



Terms and Conditions of Use of Digitised Theses from Trinity College Library Dublin

Copyright statement

All material supplied by Trinity College Library is protected by copyright (under the Copyright and Related Rights Act, 2000 as amended) and other relevant Intellectual Property Rights. By accessing and using a Digitised Thesis from Trinity College Library you acknowledge that all Intellectual Property Rights in any Works supplied are the sole and exclusive property of the copyright and/or other IPR holder. Specific copyright holders may not be explicitly identified. Use of materials from other sources within a thesis should not be construed as a claim over them.

A non-exclusive, non-transferable licence is hereby granted to those using or reproducing, in whole or in part, the material for valid purposes, providing the copyright owners are acknowledged using the normal conventions. Where specific permission to use material is required, this is identified and such permission must be sought from the copyright holder or agency cited.

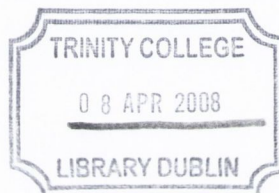
Liability statement

By using a Digitised Thesis, I accept that Trinity College Dublin bears no legal responsibility for the accuracy, legality or comprehensiveness of materials contained within the thesis, and that Trinity College Dublin accepts no liability for indirect, consequential, or incidental, damages or losses arising from use of the thesis for whatever reason. Information located in a thesis may be subject to specific use constraints, details of which may not be explicitly described. It is the responsibility of potential and actual users to be aware of such constraints and to abide by them. By making use of material from a digitised thesis, you accept these copyright and disclaimer provisions. Where it is brought to the attention of Trinity College Library that there may be a breach of copyright or other restraint, it is the policy to withdraw or take down access to a thesis while the issue is being resolved.

Access Agreement

By using a Digitised Thesis from Trinity College Library you are bound by the following Terms & Conditions. Please read them carefully.

I have read and I understand the following statement: All material supplied via a Digitised Thesis from Trinity College Library is protected by copyright and other intellectual property rights, and duplication or sale of all or part of any of a thesis is not permitted, except that material may be duplicated by you for your research use or for educational purposes in electronic or print form providing the copyright owners are acknowledged using the normal conventions. You must obtain permission for any other use. Electronic or print copies may not be offered, whether for sale or otherwise to anyone. This copy has been supplied on the understanding that it is copyright material and that no quotation from the thesis may be published without proper acknowledgement.



THESIS
8345

**Cell Signalling
and ADP Ribosylation Factor 1 (Arf1)
Effector Proteins of African
Trypanosomes**

by

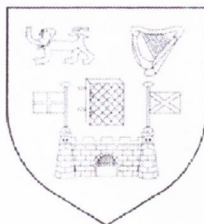
Miriam McAndrew

B.A. (Mod.)

A thesis submitted to
The University of Dublin
for the degree of

Doctor of Philosophy

Department of Genetics
Trinity College
University of Dublin



December 2007

Declaration

This thesis has not been submitted as an exercise for a degree at any other University. Except where otherwise stated, the work described herein has been carried out by the author alone. This thesis may be borrowed or copied upon request with the permission of the Librarian, University of Dublin, Trinity College. The copyright belongs jointly to the University of Dublin and Miriam McAndrew.

Signature of Author...*Miriam McAndrew*.....

Miriam McAndrew

30th May 2007

Summary

The control of population size and differentiation status is essential for the survival of all organisms. Tight regulation of these processes is of particular importance for parasites. The African trypanosome, *Trypanosoma brucei*, represents a highly successful parasite capable of surviving and indeed thriving in the harsh environment in the bloodstream of vertebrate hosts, and in the midgut and salivary glands of the tsetse fly vector. This project seeks to address issues in relation to control mechanisms of population size and differentiation status of mammalian-infective forms of the African trypanosome via the identification, characterisation and elucidation of components of the signalling process. This includes the identification of a putative receptor/ transporter for a density controlling and differentiation signalling factor.

African trypanosomes exist in two stages in mammalian hosts, a long slender stage and a short stumpy stage. In the course of this work a putative transporter for the trypanosome stumpy inducing factor (SIF), also referred to as T-GIF, was identified. Candidates for the putative receptor/transporter thought to facilitate the internalisation of the putative signalling factor were identified in the *T. brucei* database. The mostly likely candidate, an Expression Site Associated Gene, *ESAG10*, was overexpressed in the *T. brucei* cell. *ESAG10* was implicated in the long slender to short stumpy differentiation of bloodstream forms.

In addition, the transformation from long slender to short stumpy cells involves a cell density dependent signalling cascade in which trypanosomal Arf1 is implicated. Arf1 is a small GTP binding protein, highly conserved from yeasts to

humans. Arf1 is involved in signal transduction, cytoskeleton dynamics and intracellular trafficking in the cell. ArfGEF and ArfGAP proteins are positive and negative regulators of Arf1, respectively. In this study, we present data identifying, phylogenetically classifying and partially functionally elucidating Arfs, ArfGEFs and ArfGAPs in *T. brucei*. Subsequently, trypanosomal Arf1 was shown to interact with ESAG10.

Candidates for the density controlling and differentiation signalling factor were also examined in the *T. brucei* cell using *in vivo* and *in vitro* mechanisms of action. Trypanosomal Arf1 was shown to interact with cAMP as part of the differentiation process and a model of this long slender to short stumpy differentiation process is proposed.

Acknowledgements

I wish to express my appreciation and gratitude to my colleagues in the Noel Murphy Lab, Kathrin Reiber and Sinead Diviney. I wish to thank all the members of the Department of Genetics, Trinity College Dublin, for all their friendship, support and encouragement throughout my PhD. In addition, I wish thank all the members of the School of Immunology, NUI Maynooth, for their welcoming attitude in the last year of my PhD. I would like to thank my family (and my new family) for all their encouragement and support over the last couple of years. Special thanks to my supervisor Noel Murphy for his guidance during this PhD, and my unofficial supervisor, Derek Nolan.

Most importantly, I would like to thank my husband Dave for all his help, endless support and advice in the course of this PhD. Only by his constant effort and encourage am I able to complete this PhD.

Table of Contents

Title	Page Number
Chapter 1: Introduction	
1.1 Introduction to <i>Trypanosoma brucei</i>	23
1.1.1 Classification of the Trypanosome	23
1.1.2 Impact of Trypanosomal Diseases	25
1.1.3 The Life Cycle of the African Trypanosome, <i>T. brucei</i>	25
1.1.4 <i>T. brucei</i> uses Variant Surface Proteins to Evade the Host Immune Response	28
1.1.5 The <i>T. brucei</i> Genome	30
1.1.6 Endocytosis of VSGs	30
1.1.7 Population Density and Control	32
1.2 ADP-Ribosylation Factor 1 (Arf1)	37
1.2.1 Classification of ADP-Ribosylation Factors	37
1.2.2 Arf-Like Proteins	38
1.2.3 ADP-Ribosylation Factor 1	39
1.3 Arf1 Associated Proteins	40
1.3.1 GTPase Activating Proteins	41
1.3.2 Mechanism of ArfGAP Type 1	43
1.3.3 ArfGAPs in <i>Saccharomyces cerevisiae</i>	44
1.3.4 Guanine Nucleotide Exchange Factors	45
1.3.5 Mechanism of ArfGEFs	46
1.3.6 Additional Effectors of Arf1	48

1.3.7 Arf1 Involvement in Vesicle Formation	48
1.4 Thesis Aims: Elucidating the Mechanism of Cellular Differentiation in <i>T. brucei</i>	51
Chapter 2: Materials and Methods	
2.1 Identification and Analysis of Trypanosome and <i>Leishmania</i> Genes	53
2.1.1 Identification of Effectors of Arf1 and Arl Proteins in <i>T. b. brucei</i>	53
2.1.2 Identification of the TbPP69 Putative Receptor	54
Laboratory Work	
2.2 Nucleic acid preparation	55
2.2.1 Preparation of DNA	55
2.2.2 Preparation of RNA	56
2.2.3 Isolation of mRNA	57
2.2.4 Complementary DNA (cDNA) Synthesis	58
2.3 PCR Amplification	59
2.3.1 Standard PCR amplification	59
2.3.2 Qiagen Taq PCR Master Mix	60
2.3.3 Qiagen-Proof-Start PCR Kit	60
2.3.4 KOD Hot Start DNA Polymerase	61
2.4 Purification of DNA Products	63
2.4.1 Purification of PCR Products	63
2.4.2 Gel Extraction	64
2.4.3 Phenol-Chloroform Extraction	64
2.4.4 Ethanol Precipitation	65

2.4.5 Small Scale Plasmid DNA Preparation (Mini-Prep)	65
2.4.6 Large Scale Plasmid DNA Preparation (Midi-Prep)	65
2.5 Cloning of PCR Products	66
2.5.1 Restriction Enzyme Digestion of Nucleic Acids	66
2.5.2 Ligation Reactions	67
2.5.3 Ek/LIC Cloning Mechanism	68
2.5.4 Preparation of Competent Bacterial Cells	69
2.5.5 Transformation	70
2.5.6 Identification of Recombinant Clones	71
2.5.7 Glycerol Stocks	71
2.6 Electrophoresis	72
2.6.1 Conventional Agarose Gel Electrophoresis	72
2.6.2 SDS-PAGE Gels	72
2.6.3 2-D Electrophoresis	73
2.6.4 Coomassie Blue Staining of SDS-PAGE Gels	74
2.6.5 Colloidal Blue Staining of SDS-PAGE Gels	75
2.6.6 Silver Staining of Gels	75
2.7 Protein Expression	76
2.7.1 Recombinant Protein Expression (small scale)	76
2.7.2 Recombinant Protein Purification (small scale, denaturing conditions)	77
2.7.3 Recombinant Protein Purification (small scale, native conditions- phosphate buffer)	78
2.7.4 Recombinant Protein Purification (small scale, native conditions-tris buffer)	78
2.7.5 Recombinant Expression of TbArfGAP I and TbArfGAP V	78

2.7.6 Purification of recombinant TbArfGAP I and TbArfGAP V	79
2.7.7 Use of Millipore Amicon Membranes	80
2.7.8 Recombinant Co-Expression Studies	80
2.7.9 Desalting Samples using PD 10 Columns (2.5 ml Volumes)	81
2.7.10 Desalting Samples using Nap 10 Columns (1 ml Volumes)	81
2.8 Recombinant TbArf1 Protein Interaction Assay	81
2.9 Tomato-Lectin Based Interaction Assay	83
2.10 Trypanosome Culturing Techniques	83
2.10.1 HMI-9 Medium	84
2.10.2 SDM-79 Medium	84
2.10.3 Culturing Cells	84
2.11 Prostaglandin response Assays	85
2.12 Trypanosome Transfection Procedure	85
2.12.1 Preparation of Cytomix	86
2.12.2 Transfection	86
2.12.3 Assessing Effectiveness of Transfection	87
2.12.4 Selecting a Clone	87
2.12.5 Tetracycline Induction	87
2.12.6 Making Liquid Nitrogen Stocks	88
2.13 Microscopy Procedures	89
2.13.1 Slide Preparation	88
2.13.2 DAPI Staining	89
2.13.3 Tomato Lectin staining	89
2.13.4 Immuno Fluroescent Histochemistry	89
2.13.5 Microscopy	90

Chapter 3: Identifying the TbPP69 Receptor/Transporter Involved in *T. brucei*

Differentiation

3.1 Summary	91
3.2 Investigating the TbPP69 Receptor/Transporter	92
3.3 Twin Mass Spectrometry Analysis of Tomato Lectin Purified	
Surface Proteins	95
3.3.1 Matches of Peptides Derived from Purified Surface Proteins	
to the Sanger <i>T. brucei</i> Database	95
3.4 TbPP69 Receptor/Transporter Candidates	98
3.4.1 A Candidate Approach to Identifying the TbPP69	
Receptor/Transporter	98
3.4.2 Expression Site Associated Genes (ESAGs)	99
3.4.3 ESAG10 as a Candidate for the TbPP69 Receptor/Transporter	99
3.4.4 ESAG10 is Developmentally Regulated	102
3.5 Transfection Studies	104
3.5.1 The Overexpression Plasmid of Choice	104
3.5.2 The p2216 Plasmid for Transfection Studies	105
3.5.3 Cloning of ESAG10 into pLEW100 and p2216 Plasmids	106
3.5.4 Transfection with the pLEW100 ESAG10 Overexpression Plasmid	107
3.5.5 Transfection with the p2216 ESAG10 Overexpression Plasmid	108
3.6 Analysis of ESAG10 Positive Clones	108
3.6.1 Growth Curves to Assess Transfection	108
3.6.2 Confirmation that ESAG10 has Inserted in the <i>T. brucei</i> Genome	112
3.6.3 RT-PCR to Show Expression of the Inserted ESAG10 ORF	113

3.6.4 Induction of ESAG10 Protein	116
3.6.5 Immunofluorescence Analysis of ESAG10 Transfected Cells	119
3.7 Discussion - ESAG10 a Cell Surface Receptor Involved in Density Dependent Differentiation	123
Chapter 4: Identification, Phylogenetic Classification and Initial Characterisation of TbArf1, Related Proteins and Effector Proteins	
4.1 Summary	126
4.2 Identification of Arf Proteins in <i>T. b. brucei</i>	128
4.2.1 Introduction to Arf, Arls and Effector Proteins	128
4.2.2 Identification of Sequences in <i>T. b. brucei</i> Encoding Arf Proteins	129
4.2.3 Phylogenetic Classification of Sequences in <i>T. b. brucei</i> Encoding Arf and Arf-Like Proteins (Arls)	131
4.2.4 Phylogenetic Classification of Arf1	134
4.3 TbArf1 Genomic Organisation and Transcription	137
4.3.1 Analysis of the TbArf1 Contig	137
4.3.2 Differential Expression of TbArf1 ORFs in Different Life cycle Stages of the Trypanosome	140
4.4 Effectors of Arf1; ArfGAPs	142
4.4.1 Identification of ArfGAPs in the <i>T. b. brucei</i> Database	142
4.4.2 Phylogenetic Classification of TbArfGAPs	144
4.4.3 Initial Characterisation of TbArfGAPs Based on Protein Sequence	150
4.4.4 Cloning of TbArfGAPs for Protein Expression Studies	152
4.4.5 TbArf1 and TbArfGAP V Co-Expression Studies	154
4.5 Effectors of TbArf1; TbArfGEFs	156

4.5.1 Identification of ArfGEFs in the <i>T. b. brucei</i> Sanger Database	156
4.5.2 Phylogenetic Classification of TbArfGEFs	158
4.5.3 Initial Characterisation of TbArfGEFs Based on Sequence Composition	162
4.6 Other Effectors of TbArf1	163
4.6.1 Identification of Other Effectors of TbArf1	163
4.6.2 Identification of the P24 Family in <i>T. b. brucei</i>	164
4.6.3 Identification of COPI-Arf-GTP Interacting Components in <i>T. b. brucei</i>	165
4.6.4 Identification of Adaptin-Arf-GTP Interacting Components in <i>T. b. brucei</i>	165
4.6.5 GGAs	166
4.6.6 Phospholipid kinases	166
4.6.7 Other Factors that Interact with Arf-GTP	167
4.7 Discussion: Arf1 and Effectors of Arf1 in the <i>T. b. brucei</i>	168
 Chapter 5: Signalling in the African Trypanosome, <i>T. brucei</i>	
5.1 Summary	172
5.1.1 Introduction to Cell Signalling in the African Trypanosome, <i>T. b. brucei</i>	172
5.1.2 cAMP is Implicated in Differentiation in <i>T. b. brucei</i>	173
5.1.3 Programmed Cell Death in the African Trypanosome	175
5.2 Effects of Signalling Factors on the Growth of Long Slender Trypanosomes	176
5.2.1 The Effect of cAMP on the Growth of Bloodstream Form Trypanosomes	176

5.2.2 The Effect of Prostaglandins on the Growth of Bloodstream Form <i>T. b. brucei</i>	181
5.2.3 The Effect of Arf1 as an Inhibitor of Signalling Factors	187
5.3 Detection of TbArf1 Binding Proteins	189
5.3.1 An Assay to Demonstrate the Effect of Signalling Factors on TbArf1, <i>in vitro</i>	189
5.3.2 Production of Recombinant TbArf1	189
5.3.3 TbArf1 Treated with PGD2 and cAMP	190
5.3.4 2-D Analysis of Arf1-Binding Proteins	194
5.3.5 T-GIF Blocks TbArf1 Protein Binding	197
5.4 TbArf1 Interacts with TbArfGAP I and TbArfGAP V	199
5.5 Interaction between TbArf1 and ESAG10	201
5.6 Discussion: Signalling Factors Implicated in TbArf1 Mediated Differentiation	204

Chapter 6: Conclusions

6.1.1 Cell Density Dependent Differentiation of <i>T. b. brucei</i> Bloodstream Form	210
6.1.2 Molecular Characterisation of the TbPP69 Receptor/Transporter	211
6.1.3 ESAG10 as a Candidate for the TbPP69 Receptor/Transporter	211
6.1.4 Characterisation of Arfs/Arls and Effector Proteins	214
6.1.5 cAMP Signalling in Differentiation	218
6.1.6 The Role of T-GIF in Differentiation	220
6.1.7 A Model for Differentiation in <i>T. b. brucei</i>	220
6.2 Future Work	225

Chapter 7: Appendix : ILTat 1.1 VSG Versus ILTat1.4 VSG Expression

7.1 Further Investigation of the ILTat1.4 VSG Apparent in the TbPP69 kDa Protein Preparation	227
7.2 Investigation of ILTat Expression in Different Life cycle Stages of <i>T. b. brucei</i>	229
7.3 Interpretation of the ILTat VSG Results	231
References	233

List of Figures

Title	Page Number
Chapter 1	
1.1. A phylogenetic tree of kinetoplastids generated using SSUrRNA	24
1.2. The life cycle of <i>T. brucei</i>	27
1.3. A bloodstream form VSG expression site	29
1.4. A surface protein of 69 kDa that displays differential abundance in pleomorphic and monomorphic trypanosomes	34
1.5. Illustration of previously proposed model for population density control and the differentiation of trypanosomes from long slender to short stumpy stage	36
1.6. The primary structure of human Arf1	40
1.7. Protein domains of mammalian ArfGAPs	42
1.8. The formation of Clathrin-coated vesicles	49
1.9. The formation of COPI-coated vesicles	50
1.10. Localisation of Arf1 in the <i>T. b. brucei</i> pleomorphic parasite with polyclonal IgG specific for the TbArf1	52
Chapter 3	
3.1. Tomato lectin purified surface protein of pleomorphic <i>T. b. brucei</i> , ILTat 1.1	94
3.2. Schematic of mass spectrometry generated peptide fragments that align to ILTat 1.4 VSG variants	97
3.3. Protein sequence of ESAG10 showing the predicted transmembrane domains	101

3.4. ESAG10's predicted N-glycosylation sites	101
3.5. ESAG10 is specifically expressed in the short stumpy form	103
3.6. Schematic representation of the p2216 plasmid	105
3.7. Digests to confirm presence of ESAG10 inserts in the pLEW100 and p2216 vectors	106
3.8. Growth curve of induced versus uninduced positive p2216 ESAG10 clones	110
3.9. Growth curve of induced versus uninduced positive p2216 ESAG10 clones in stressful conditions	111
3.10. Designing PCR experiments to show that the p2216 ESAG10 plasmid was inserted correctly into the rDNA spacer region	113
3.11. PCR to confirm that p2216 ESAG10 is inserted into the rDNA spacer region of the ribosomal DNA	114
3.12. RT-PCR to confirm that there is expression of the ESAG10 in the transfected cells	116
3.13. Comparison of surface proteins purified from induced versus uninduced ESAG10 transfected cells	118
3.14. Results of the immunofluorescence experiments carried out on ESAG10 transfected cells	121
3.15. Replicated results of the immunofluorescence experiments carried out on multiple ESAG10 transfected cells	122

Chapter 4

4.1. T-Coffee alignment of human (Hs) and <i>T. b. brucei</i> (Tb) Arf and Arf-like proteins	132
---	-----

4.2. A neighbour-joining tree depicting T-Coffee alignment of human (Hs) and <i>T. b. brucei</i> (Tb) Arf and Arf-like proteins	133
4.3. A T-coffee alignment of Arf1 sequences from trypanosomes and other eukaryotes	136
4.4. Neighbour-joining tree of Arf1 proteins from trypanosomes and other eukaryotes	137
4.5. Schematic of the TbArf1 contig of <i>T. b. brucei</i>	139
4.6. Expression of TbArf1 ORFs in the <i>T. b. brucei</i> lifecycle stages	141
4.7. Domain structure of TbArfGAP I, II, III, IV, and V	143
4.8. T-coffee alignment of Trypanosome and <i>Leishmania</i> ArfGAP domains	146
4.9. A neighbour-joining tree produced from a T-coffee alignment of Trypanosome and <i>Leishmania</i> ArfGAP domains	147
4.10. A neighbour-joining tree produced from a T-coffee alignment of ArfGAP domains of <i>H. sapiens</i> , <i>S. cerevisiae</i> , <i>L. major</i> , <i>T. b. gambiense</i> , <i>T. b. brucei</i> , <i>T. congolense</i> , <i>T. vivax</i> and <i>T. cruzi</i>	148
4.11. Alignment of the ArfGAP domains of known and putative ArfGAP 1 proteins	149
4.12. pET51a Ek/LIC Amplified PCR products for cloning	153
4.13. Expression of TbArfGAP I	153
4.14. Expression of TbArfGAP V	154
4.15. Co-Expression of TbArf1 and TbArfGAP V	155
4.16. Characterisation of the domain structure of TbArfGEFA, TbArfGEFB and TbArfGEFC	157
4.17. A neighbour-joining tree depicting the evolutionary relationship of putative trypanosome and <i>Leishmania</i> ArfGEF proteins	160

4.18. A neighbour-joining tree depicting the evolutionary relationship of human, yeast and kinetoplastid ArfGEF proteins	161
4.19. The FRLPGE loop of the Sec7 domain of ArfGEFs	163

Chapter 5

5.1. The effect of cGMP on bloodstream form <i>T. b. brucei</i> cells	179
5.2. The effect of pCPTcAMP on the growth of bloodstream form <i>T. b. brucei</i> cells	180
5.3. The effect of PGD ₂ on bloodstream form <i>T. b. brucei</i> cells	183
5.4. The effect of 100-300 µM PGE ₂ on bloodstream form <i>T. b. brucei</i> cells	184
5.5. The effect of PGE ₂ on bloodstream form monomorphic 328.114 <i>T. b. brucei</i> cells	185
5.6. The effect of PGF ₂ on bloodstream form monomorphic 328.114 <i>T. b. brucei</i> cells	186
5.7. The effect of recombinant TbArf1 on bloodstream form <i>T. b. brucei</i> cells inhibited with 5 µM PGD ₂	188
5.8. Expression of recombinant TbArf1	190
5.9. Demonstrating extensive washing prior to elution of TbArf1-bound proteins	192
5.10. Comparison of proteins bound to TbArf1 treated with signalling factors	193
5.11. 2-D gels of treated and untreated TbArf1 elutions	195
5.12. Trypanosomal proteins binding to TbArf1 following treatment with prostaglandins	196
5.13. Effect of binding of trypanosomal proteins to TbArf1 following treatment with T-GIF and cGMP	198
5.14. TbArfGAP I and TbArfGAP V bound trypanosomal proteins	200

5.15. Comparison of TbArf1 interacting proteins using protein lysates generated from ESAG10 induced and uninduced transfected and wild type cells	202
5.16. Comparison of TbArf1 interacting proteins from protein lysates generated from ESAG10 induced and uninduced transfected and wild-type cells via 2-D gel electrophoresis	203

Chapter 6

6. 1. Schematic of long slender to short stumpy differentiation in <i>T. b. brucei</i> cells	224
---	-----

Chapter 7

7.1. Agarose gel depicting cDNA amplified with the ILTat VSG primers	230
--	-----

List of Tables

Title	Page Number
2.1. Primer Information	62
2.2. Primer Information	62
2.3. Primer Information	63
2.4. Table of Plasmids utilised	67
2.5. Strains of <i>E.coli</i> used in the study	70
4.1. TbArf1 proteins encoded in the <i>T. b. brucei</i> genome	131
4.2. Arrangement of Arf1 genes in <i>T. b. brucei</i> and related species	139
4.3. Characterisation of TbArfGAP I, II, III, IV, and V	143
4.4. Comparison of HsArf1 residues, known to be important in the Arf1/ArfGAP1 interaction, with the orthologous positions in TbArf1	151
4.5. Comparison of Human ArfGAP 1 residues, known to be important in the Arf1/ArfGAP interaction, with the orthologous positions in TbArfGAP I, II, III, IV, and V	151
4.6. COPI- Arf1 interacting proteins in <i>T. b. brucei</i>	167
4.7. Adaptin proteins in <i>T. b. brucei</i>	167
7.1. Table of the ILTat 1.1 VSG, ILTat 1.2 VSG, ILTat 1.3 VSG and ILTat 1.4 VSG primer names, PCR product names and expected sizes	230

List of Abbreviations

AA- acrylamide INJV-methylene

ABC- ATP-binding cassette

Arf- ADP ribosylation factor

ArfGAP- Arf GTP activation protein

ArfGEF- Arf guanine exchange factor

Arl- Arf-like protein

ACAP- Arf GAP with coiled-coil, ankyrin repeat and pleckstrin homology domains

AGAP- Arf GAP with GTP-binding protein-like, ankyrin repeat and pleckstrin homology domains

ARAP- ArfGAP with ankyrin repeat, pleckstrin homology domain

BES- bloodstream form expression site

CHAPS- 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate

DAPI- 4',6-diamidino-2-phenylindole

DTT- DL-Dithiothreitol

E-64- (2S,3S)-3-(N-{(S)-1-[N-(4-guanidinobutyl)carbamoyl]3-methylbutyl}carbamoyl)oxirane-2-carboxylic acid

ER- endoplasmic reticulum

ES- expression sites

ESAG- expression site associated gene

FCS- foetal calf serum

FITC- fluorescein in its reactive isothiocyanate form

IEF- isoelectric focusing

IPG- immobilized pH gradient

IPTG- isopropyl-beta-D-thiogalactopyranoside
 GGA- Golgi localised, γ -ear-containing, monomeric clathrin adaptor
 GIT- G protein-coupled receptor kinase-interacting target
 GPI- glycosylphosphatidylinositol
 MOPS- 3-N-morpholino propansulfonic acid; 4-Morpholinepropanesulfonic acid
 MRP- multidrug resistance-associated proteins
 PARP- procyclic abundant surface proteins
 PH- pleckstrin homology
 PKA- protein kinase A
 PMSF- phenylmethanesulphonylfluoride
 pNAL- poly-N-acetyllactosamine
 PNGase- *N*-linked-glycopeptide-(*N*-acetyl-beta-D-glucosaminyl)-L-asparagine
 amidohydrolase
 SA-PMP- streptavidin-coated paramagnetic particle
 SIF- stumpy inducing factor
 TEMED- N,N,N',N'-Tetramethylethylenediamine T-GIF- trypanosome growth
 inhibitor factor
 TGN- *trans*-Golgi network
 TLCK- Na-Tosyl-Lys-chloromethylketone·HCl
 VSG- variant surface glycoprotein

Chapter 1: Introduction

1.1 Introduction to *Trypanosoma brucei*

1.1.1 Classification of the Trypanosome

The trypanosome is a unicellular, protozoan parasite. Trypanosomes are extracellular parasites and are classified with *Leishmania*, a related intracellular parasite, as kinetoplastids (Donelson *et al.* 1999). Kinetoplastids are so called due to the presence of a condensed body of mitochondrial DNA, the kinetoplast. There are many species of trypanosomes, which infect a range of different hosts throughout the world. In Africa, there are three major species, *Trypanosoma brucei*, *Trypanosoma vivax*, and *Trypanosoma congolense*. *T. vivax*, *T. congolense* and *T. brucei* all infect cattle in Sub-Saharan Africa. *T. brucei* can infect both humans and cattle.

Based on the Linnean classification, *T. brucei* belongs to the genus *Trypanosoma*, and the sub-section *salivaria*, which is further sub-divided into *Duttonella*, *Nannomonas*, *Trypanozoon* and *Pycnomonas*, of which *T. brucei* belongs to the subgenus, *Trypanozoon*. There are three subspecies of *T. brucei*; *T. b. rhodesiense*, *T. b. gambiense* and *T. b. brucei*. The first two sub-species are capable of infecting humans and cause the human form of the disease, sleeping sickness (Welburn *et al.* 2001). *T. b. brucei* cannot infect humans, but can cause nagana, the cattle form of the disease (Hajduk *et al.* 1989).

A study using phylogenetic analysis of small subunit ribosomal RNA, SSUrRNA, and a cytosolic form of heat shock 90 protein, Hsp90, showed that kinetoplastids could be classified into four clades. Clades 1-3 were comprised of bodonid kinetoplastids (free living flagellates) and trypanosomes and *Leishmania* comprised the fourth clade (Figure 1.1) (Simpson *et al.* 2002). Kinetoplastids are classified as Euglenozoa. *T. brucei* and other members of the Euglenozoa have very long branch lengths indicating a high degree of divergence from other species (Cavalier-Smith 2004).

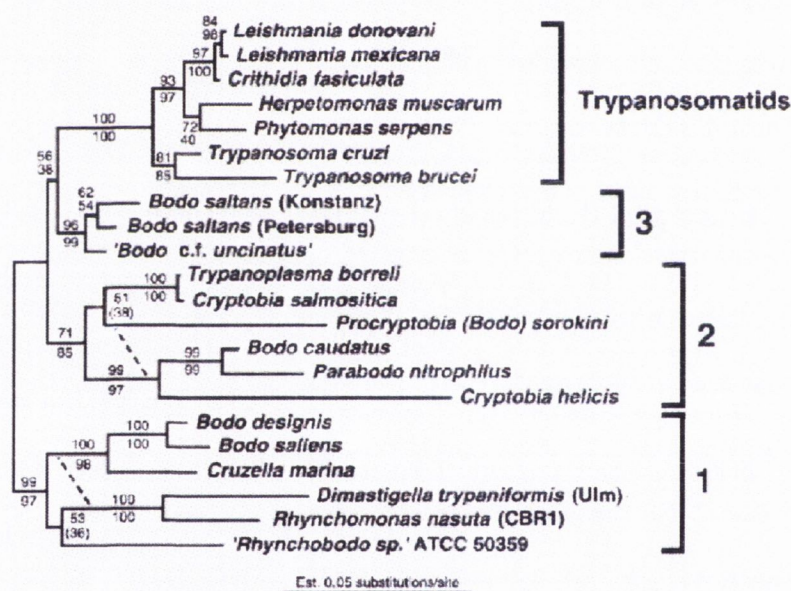


Fig. 1.1. A phylogenetic tree of kinetoplastids generated using SSUrRNA. The above figure depicts a maximum likelihood phylogenetic tree of kinetoplastids, showing four well defined clades, of which the trypanosome and *Leishmania* compose one clade (Trypanosomatids). The figure is taken from (Simpson *et al.* 2002).

1.1.2 Impact of Trypanosomal Diseases

The World Health Organisation (WHO) estimates that up to half a million people are infected by sleeping sickness in Sub-Saharan Africa, particularly in war prone regions such as Angola, Sudan, and the Democratic Republic of Congo. The Food and Agriculture Organisation of the United Nations, the FAO, suggests that nagana (the cattle form) is a major factor in rural poverty in Sub Saharan Africa, severely limiting meat and dairy production (Kabayo 2002).

Chemotherapy of sleeping sickness (human African trypanosomiasis) is difficult. There is a high frequency of severe adverse side-effects, long duration of treatment, and high costs associated with the treatments. Many of the drugs used to treat trypanosomiasis are outdated, and only one drug, melarsoprol, can treat severe cases (crossing the blood brain-barrier) but with severe side effects. In addition, there is very little pharmaceutical interest in producing new drugs to treat this disease, as it is not profitable. The African governments have set up a cross-country body, the Pan African Tsetse and Trypanosomiasis Eradication Campaign (PATTEC) in order to tackle trypanosomiasis. Using an area wide approach, they intend to eradicate tsetse infestation, creating tsetse-free zones. This step, though helpful, is unlikely to be sufficient to eradicate the disease. This disease requires integrated tsetse control, drugs, and vaccine development.

1.1.3 The Life Cycle of the African Trypanosome, *T. brucei*

The *T. brucei* parasite has both vertebrate host and tsetse insect vector stages in its life cycle (Hammarton *et al.* 2003). To cope with the extreme environmental changes in

its life cycle, the trypanosome undergoes extensive changes that allow the movement of the trypanosome from the bloodstream of the vertebrate host to the insect midgut (Vickerman *et al.* 1988). These include changes in morphology, division status, infectivity, coat-protein, gene expression, vesicular trafficking and respiration capacity. Differentiation also involves changes at the organelle level, including mitochondrial functionality, cell membrane functionality and flagellum appearance (Ziegelbauer *et al.* 1990).

The lifecycle of the trypanosome involves several differentiation steps, (Figure 1.2). Following a bite from an infected tsetse insect, trypanosomes disseminate into the bloodstream of the mammalian host via the lymphatic system. Upon infection of the mammalian host, trypanosomes divide rapidly, have high virulence, and have a long slender morphology. As peak parasitaemia approaches, cells switch to a predominantly short and fat form. These forms are infective to the tsetse vector and do not divide.

After ingestion of a bloodmeal by the tsetse fly, the parasites further transform into dividing procyclic trypomastigotes and colonise the insect midgut. These procyclic cells migrate to the proventriculus and the salivary glands of the tsetse where the cells differentiate into the epimastigote form and the trypomastigote form respectively, ready for infecting another vertebrate host.

Differentiation of the trypanosomes from long slender to short stumpy stage coincides with a partial switch in energy metabolism from glycolysis to oxidative phosphorylation in the mitochondria (Vickerman 1965, 1985). There are substantial morphological differences between the different forms of the trypanosome during differentiation. This extensive reorganisation of the cell is initiated in the first 24 hours of differentiation and appears to occur in all cells at the same time (Ziegelbauer

et al. 1990). It is believed that this non-dividing, stumpy form is preadapted to survival and establishment in the insect vector, the tsetse fly (Wijers and Willett 1960; Turner *et al.* 1988; Ziegelbauer *et al.* 1990).

These bloodstream form trypanosomes express a variant surface glycoprotein (VSG) coat that assists in evasion of the host immune system. With the uptake of the trypanosome from the vertebrate host to the tsetse vector, the VSG coat is shed. Within the salivary glands of the fly, *T. brucei* trypanosomes acquire a second new coat, called procyclin.

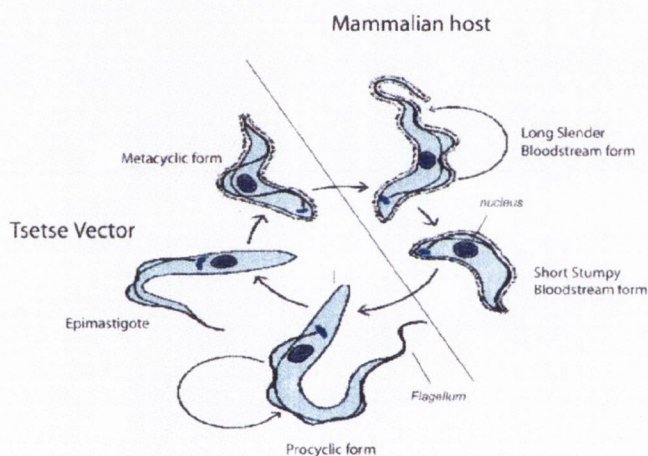


Fig 1.2. The life cycle of *T. brucei*. The trypanosomes in this figure are indicated by the single-celled structure. The cells of the metacyclic trypanosomes are inoculated into the vertebrate host in tsetse fly saliva when taking a bloodmeal. Here the extracellular parasites undergo asexual reproduction by binary fission in the bloodstream, and, later in the infection, the spinal fluid. Long slender forms transform to a form capable of infecting the tsetse fly, the short stumpy form. When taken up by the tsetse fly in its bloodmeal into the fly's midgut, the parasite's mitochondrion becomes active, and further cycles of binary fission take place. The parasites migrate to the fly's proventriculus and salivary glands, where they transform to the epimastigote forms and further cycles of binary fission take place. In the salivary glands, trypomastigote forms appear, capable of infecting the vertebrate host in the fly's blood meal. Figure is taken from <http://www.tulane.edu/wiser/-protozoology/notes/kinet.html>.

1.1.4 *T. brucei* uses Variant Surface Proteins to Evade the Host Immune Response

African trypanosomes exist freely within the bloodstream and lymphatic system of the vertebrate host, and are subject to specific and non-specific immune attack. African trypanosomes combat this immune attack by concealing their invariant surface proteins and receptors from the host immune system and covering their entire outer membrane with an ever-changing VSG coat (Barry and Turner 1991). This allows the population to avoid antibody-mediated killing throughout infection (McCulloch 2004). These changes in the coat are associated with waves of proliferation of the parasite (Barry and Turner 1991).

Within the vertebrate host, *T. brucei* uses a class of expression sites (ESs) called bloodstream ESs, (BESs) (Berriman *et al.* 2002). BESs are long polycistronic units (up to 60 kb) containing up to a dozen genes that have been described as expression site associated genes, ESAGs. BESs are comprised of expression site associated genes (ESAGs) in conjunction with VSGs. The general structure of BESs is modelled with the VSGs located proximal to the telomere, many kilobases away from the promoter, but, the number and order of the ESAGs differs between ESs (Berriman *et al.* 2002) (Figure 1.3).

Though there are multiple telomeric ESs where, VSGs are located, the trypanosome has developed a mechanism of transcription to ensure expression of only a single VSG at any one time. Three processes account for the switching of activity between different ESs. The first process involves the suppression of an active BES, and the activation of a silent BES (Navarro *et al.* 1999). The second, and more

commonly occurring switching mechanism, involves genes conversion. Here a silent VSG is copied and moved to the active BES where the resident VSG is deleted and the new copy is inserted instead (McCulloch *et al.* 1997). This is the first demonstration of gene expression being regulated at the level of transcription among the *Kinetoplastida*; all other developmentally regulated protein-coding genes examined in these organisms appear to be under posttranscriptional control (Graham and Barry 1995). Only active ES are contained within an expression site body (ESB) with Polymerase 1 (PolI), allowing only one ES to be active at any one time (Reviewed Borst 2002).

In *T. b. rhodesiense* for example, the serum-resistant associated gene (SRA) confers resistance to human APOL-I, a protein that can kill *T. b. brucei* parasites (De Greef *et al.* 1989; Xong *et al.* 1998; Vanhamme *et al.* 2003). Other important genes may be located in the VSG expression site in *T. brucei*.

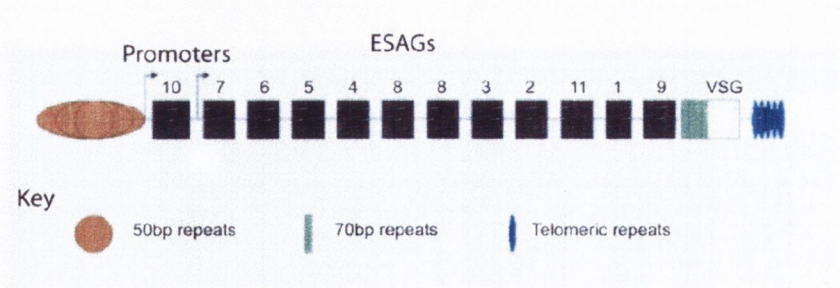


Fig 1.3. A bloodstream form VSG expression site. The expression site starts with a 50 bp repeat, and ends with a telomeric repeat. A 70 bp repeat region separates the ESAGs from the VSG gene. Not all ESAGs are present at all expression sites; ESAGs 10, 11 and 9 are often omitted.

1.1.5 The *T. brucei* Genome

The *T. brucei* genome is very gene dense, containing polycistronic transcriptional units, and only a single spliceosomal intron (Myler *et al.* 1999; Liniger *et al.* 2001). There are 11 pairs of megabase-sized chromosomes, numbered I-XI, several intermediate-sized chromosomes (0.2-0.9 Mb), and a number of mini-chromosomes (50-100 bp) (Desquesnes and Davila 2002). Most VSG genes are located in polycistronic transcriptional ESs at the telomeric ends of megabase and intermediate-sized chromosomes. VSGs found on other chromosomes are usually silent or pseudogenes.

1.1.6 Endocytosis of VSGs

In the case of *T. brucei*, the majority of transported proteins are variant surface glycoproteins (VSGs), which are transported to the cell surface where they attach via glycosylphosphatidylinositol (GPI) membrane protein anchors. VSGs make up approximately 10% of all the proteins in the *T. brucei* cell (Overath *et al.* 1994), unlike higher eukaryotes where GPI-anchored proteins are the minority. Interestingly, VSG equivalent to the area of the flagellar pocket is internalised every 2 mins (Coppens *et al.* 1987), and is quickly endocytosed and recycled (Seyfang *et al.* 1990).

The rate of endocytosis is developmentally regulated, i.e. dependent on the life cycle stage of *T. brucei* (Morgan *et al.* 2001). The long slender stage of bloodstream form cells have a surprisingly fast rate of endocytosis based on the rate of biotinylated VSG endocytosis. Endosomal turnover is even faster in the short stumpy stage (Overath and Engstler 2004). However, once differentiation into the procyclic form

has occurred, the rate of endocytosis is dramatically reduced (Langreth and Balber 1975).

T. brucei has a highly active endocytotic system which has been implicated in the uptake of nutrients (Coppens and Courtoy 1995; Steverding *et al.* 1995), sensitivity to serum lytic factors (Hager and Hajduk 1997; Vanhamme *et al.* 2003), turnover of VSG (Gruszynski *et al.* 2003), and removal of surface immunocomplexes (O'Beirne *et al.* 1998; Pal *et al.* 2003). It has a compact, highly polarised cell structure where nutrient uptake is restricted to the flagellar pocket. Endosomes are concentrated between the flagellar pocket, a deep invagination of the cell membrane at the base of the flagellum, and the nucleus of the cell, creating a continuous system of cisternal/tubulovesicular structure. In addition, *T. brucei* only contains one Golgi, making it a compact model for study.

In *Saccharomyces cerevisiae*, GPI anchoring of proteins has been studied in detail. It has been shown that following the attachment of a GPI-anchor, proteins are allowed to leave the endoplasmic reticulum (ER) (Doering and Schekman 1996). Movement of GPI-anchored proteins from the ER to the Golgi is facilitated by vesicular carriers in yeast (Muniz *et al.* 2001). Sorting of GPI-anchored proteins occurs via Rab GTPase (member of the Ras superfamily of G-proteins) that use Sec 34/35 (membrane proteins involved in translocating proteins) as a tethering factor (Morsomme and Riezman 2002).

In *T. brucei*, VSG is endocytosed from the cell surface via a clathrin-dependent mechanism (Grunfelder *et al.* 2003). This is an unusual mode of internalisation as most organisms facilitate the concentration of proteins using cytoplasmic receptors. Specifically, VSGs enter the cell at the site of invagination, the flagellar pocket, in the form of large clathrin coated vesicles (135 nm in diameter) that deliver cargo to the

endosomes. In the lumen of the cisternal endosomes the VSG is concentrated by 'default' because a distinct class of small clathrin coated vesicles budding from cisternae is depleted. TbRab11 (a trypanosome Rab GTPase) mediates VSG positive disk-like carriers which fuse to the flagellar pocket and recycle VSG back to the surface (Overath and Engstler 2004).

1.1.7 Population Density and Control

During the life cycle of *T. b. brucei* in the mammalian host, the trypanosomes undergo a developmental transition from long slender stage to short stumpy forms. Vassella and Boshart (1996) established conditions for primary *in vitro* culture of trypanosomes undergoing this event. In the course of these studies, it found that above a critical density, cultures entered a stationary phase and were morphologically differentiated into stumpy forms (Vassella and Boshart 1996). These short stumpy forms have a relatively short half-life estimated to be between 24 to 72 hours.

Wild type trypanosomes undergo this process naturally, but, several lab-derived strains do not undergo this long slender to short stumpy transition (Vickerman 1985). These lab-derived strains are called monomorphic strains. Wild type trypanosomes that can undergo this long slender to short stumpy differentiation event are called pleomorphic trypanosomes (Vickerman 1985). In situations of high culture density, long slender trypanosomes from monomorphic strains replicate continuously, without differentiating into short stumpy forms. Studies *in vivo* lead to extreme parasitaemia followed by rapid death of the host (Vassella *et al.* 1997).

Unlike monomorphic strains, the long slender stage of pleomorphic strains do differentiate into the short stumpy form upon reaching a threshold density (Vassella *et*

al. 1997). These strains undergo a programmed cell death process if not taken up by the tsetse vector. This suggests that monomorphic trypanosomes are insensitive to or cannot detect or respond to cell density threshold markers whereas the pleomorphic trypanosomes can (Vassella *et al.* 1997).

Deciphering the mechanism by which cell density translates into a differentiation signal was difficult. Vassella *et al.* (1997) replaced 30% of the culture medium in agarose plates with conditioned medium taken from bloodstream cultures at maximal density, and recorded the percentage NADH dehydrogenase activity. The depletion of nutritional components of growth factors in the culture medium was shown not to be a factor in this differentiation step. Rather, the accumulation of a differentiating inducing activity in the medium was found to be the cause. They named this factor stumpy-inducing factor (SIF).

SIF was postulated to be a pheromone-like factor (a signalling factor or a catabolite product) of *T. brucei* trypanosomes that acts as the initial differentiation signal and subsequently transduces along the cAMP pathway (Vassella *et al.* 1997). Due to its apparent small size, SIF would be expected to be accessible to receptors buried within the VSG coat. The precise structure of SIF was not deduced. Subsequent experiments showed the rapid rate of differentiation induced upon take up of the SIF factor. SIF is produced by both pleomorphic trypanosome strains capable of differentiating between long slender and short stumpy forms, and monomorphic laboratory-derived strains that have lost the ability to differentiate (Vassella *et al.* 1997). Specifically, a monomorphic strain, MITat1.2, was found to be capable of producing SIF, but could not properly receive or transmit cell density information in the culture. Vassella *et al.* (1997) suggested that there was loss of a receptor to uptake SIF in the monomorphic strain. Purified pleomorphic surface proteins of the

trypanosome were compared with those of monomorphic strains, using a recently developed tomato lectin technology that enabled purification of surface protein without VSG contamination (Murphy and Olijhoek 2001). Pleomorphic trypanosomes were shown to contain an abundant tomato lectin binding 69 kDa protein, TbPP69, which was significantly less abundant in monomorphic parasites (Figure 1.4) (Murphy and Olijhoek 2001). This TbPP69 protein was proposed to be the receptor that facilitates the recognition of SIF, and triggers the long slender to short stumpy differentiation process. The composition of this TbPP69 protein has not been deduced, and is one of the aims of this thesis.

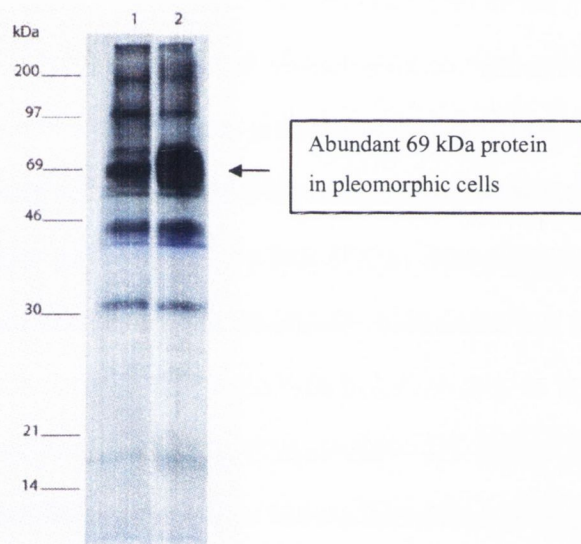


Fig 1.4. A surface protein of 69 kDa displays differential abundance in pleomorphic and monomorphic trypanosomes. The above figure depicts a SDS-10% PAGE silver stained gel. *T. brucei* proteins that bind to tomato lectin from monomorphic (lane 1) and pleomorphic (lane 2) cells are shown (Murphy and Olijhoek 2001). Trypanosomes are of the same serodeme. Note the abundant 69 kDa protein, named TbPP69, in the pleomorphic parasites (lane 2).

In conjunction with SIF, another low weight growth inhibitory and differentiation inducing factor, the trypanosome growth inhibitory factor (T-GIF) is produced by bloodstream forms of the different species of African trypanosomes and is active only in pleomorphic bloodstream forms (Murphy and Olijhoek 2001). Both SIF and T-GIF have stumpy inducing activities and have been derived from rapidly dividing long slender pleomorphic culture medium, the difference being that SIF was isolated from *T. brucei* cell culture medium and T-GIF was isolated from *T. congolense* cell culture medium. In the search for a target for this T-GIF factor, several candidates were examined, based on differential levels of mRNA expression within bloodstream form of the trypanosome. Trypanosomal ADP-ribosylation factor 1 (TbArf1) was identified as a candidate, displaying a 5 to 7 fold increase in mRNA expression between long slender and short stumpy forms of the trypanosome (Murphy and Olijhoek 2001). When conditioned medium (containing T-GIF) was passed through a column, containing recombinant TbArf1, the T-GIF factor was removed from the medium (Murphy and Olijhoek 2001). This binding of T-GIF to TbArf1 was shown to be very strong, occurring in both native and denatured conditions of the Arf1 protein, and was shown by pepscan analyses to be specific to the helix $\alpha 3$ region of this protein (Murphy and Olijhoek 2001). From these experiments it was concluded that TbArf1 was a likely target of T-GIF. An illustration of the previously proposed model for population density control and the differentiation of trypanosomes from long slender to short stumpy stage is shown (Figure 1.5).

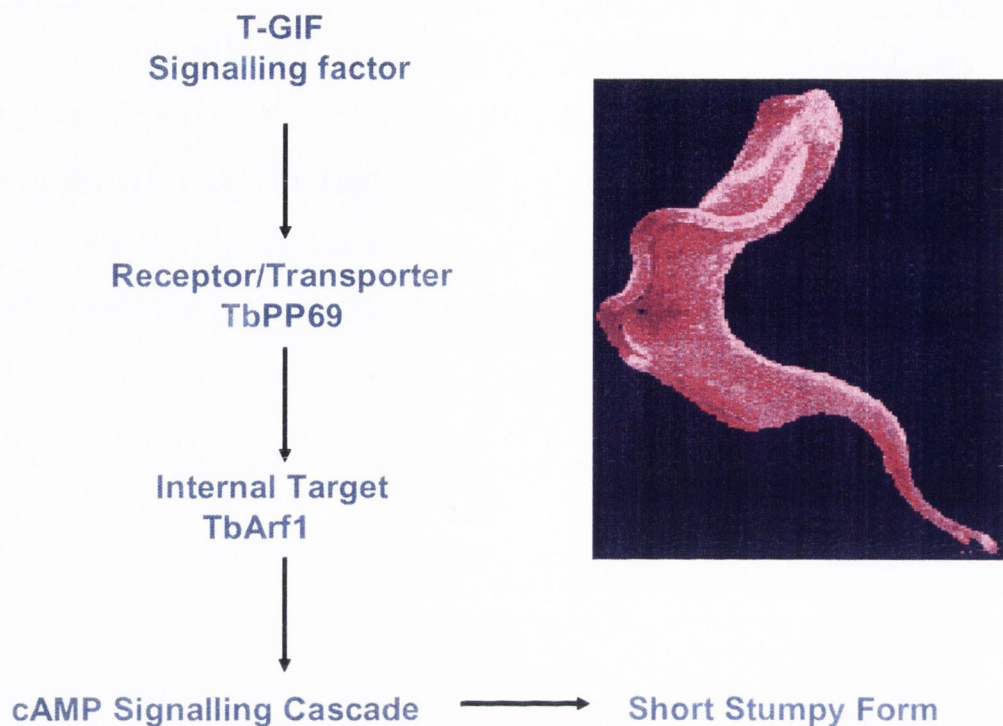


Fig 1.5. Illustration of previously proposed model for population density control and the differentiation of trypanosomes from long slender to short stumpy stage. The above figure depicts a schematic diagram of the previously proposed model for long slender to short stumpy differentiation prior to work carried out in this thesis. Pleomorphic long slender cells export and sense a T-GIF differentiation factor using a TbPP69 kDa surface protein receptor or transporter. Trypanosomal Arf1, TbArf1, is the internal target of this factor, which subsequently triggers a cAMP dependent signalling cascade triggering the differentiation event into short stumpy cells.

1.2 ADP-Ribosylation Factor 1 (Arf1)

1.2.1 Classification of ADP-Ribosylation Factors

ADP-ribosylation factor (Arf) proteins are small GTP-binding proteins of 21 kDa belonging to the Ras superfamily. Arfs function as molecular switches in fundamental cellular events such as signal transduction, cytoskeleton dynamics and intracellular trafficking (Brown *et al.* 1993). Arfs cycle between GTP bound active and GDP bound inactive forms. This cycling is mediated by specific guanine-nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs).

Arf1 was originally identified by its ability to enhance ADP-ribosylation of Guanine nucleotide binding protein stimulating gene alpha ($G_s\alpha$) by cholera toxin *in vitro* (Kahn and Gilman 1986). Subsequently, Arf1 was found to have numerous properties in addition to its ribosylation effect. These other activities include activation of phospholipase D activity and inhibition of endosome-to-endosome and nuclear vesicle fusion (Donaldson *et al.* 1991; Bornancin *et al.* 1992; Donaldson *et al.* 1992; Taylor *et al.* 1992; Brown *et al.* 1993; Cockcroft *et al.* 1994).

Six different Arfs have been found in mammals, designated Arf 1 to 6. Arfs are subdivided into three classes according to sequence identity. Class I consists of Arf1, Arf2 and Arf3, class II consists of Arf4 and Arf5, and class III contains only Arf6. Little is known about class II Arfs (Donaldson *et al.* 2005). Class I Arfs have been implicated in trafficking in the endoplasmic reticulum (ER)-Golgi and endosomal systems (Takeuchi *et al.* 2002). Arf1 was first discovered as a component of the coat found on vesicles produced from isolated Golgi membranes. Arf1 was subsequently found to be necessary to form these vesicles (COPI vesicles) *in vitro* (Donaldson *et al.*

1992). Later, Arf1 was found to be required to form clathrin-coated vesicles containing adaptor protein 1 (AP1) or adaptor protein 3 (AP3) adaptor complexes at the *trans*-Golgi network (TGN) and on endosomes (Stamnes and Rothman 1993; Ooi *et al.* 1998). This role of vesicle trafficking has been confirmed *in vivo* by expressing Arf1 mutants that were defective in either GTP binding or GTP hydrolysis (See section 1.3.7 for further details on the roles of Arf1 in vesicle formation).

Arf6, the only Class III Arf, functions exclusively in the endosomal plasma membrane system (D'Souza-Schorey *et al.* 1998). It is involved in endosomal recycling to the plasma membrane, in regulating secretion and in coordinating actin cytoskeleton changes at the plasma membrane (D'Souza-Schorey *et al.* 1998; Honda *et al.* 1999). Arf6 is present on the apical surface of Madin-Darby Canine Kidney Cells (MDCK cells) where it plays a role in modulating clathrin endocytosis (Altschuler *et al.* 1999). Arf6 has also been implicated in Fc-mediated phagocytosis in macrophages (Zhang *et al.* 1998), in insulin-regulated adiponectin secretion in 3T3-L1 adipocyte cells (Yang and Mueckler 1999) and glucose transporter (GLUT4) translocation (Lawrence and Birnbaum 2001). Arf6 induces actin rearrangement through porin voltage-dependent anion channel (VDAC) POR-1, a protein that was implicated previously in mediating actin rearrangement induced by the small GTPase, Ras (D'Souza-Schorey *et al.* 1997).

1.2.2 Arf-Like Proteins

In addition to the six Arf proteins found in mammals, there is a sub-family of highly similar homologous proteins, Arf-like proteins (Arls). These Arl proteins are structurally similar to the Arf proteins, but Arls appear to lack the cholera toxin

cofactor activity of the Arf proteins (Clark *et al.* 1993). At least 16 Arl proteins have been identified in mammals (Kahn *et al.* 2006). Arl1 has been shown to be essential for viability in *Drosophila melanogaster* (Tamkun *et al.* 1991) and appears to be closely related to Arf1. Arl1 has also been identified and studied in *T. brucei*, where Arl1 was also shown to be essential for cell viability (Price *et al.* 2005a; Price *et al.* 2005b).

1.2.3 ADP-Ribosylation Factor 1

The Arf1 protein can be divided into a number of key domains. These include an N-terminal α helix, four regions involved in GTP binding, two switch regions, switch 1 and switch 2, and a helix α 3 region (Figure 1.6). The switch 1 and 2 and α -helix 3 regions react with effectors of Arf1 (Mossessova *et al.* 1998).

Arf1 attaches to the membrane of the cell using its myristoylated N-terminus. This involves the coupling of the Arf1 protein to the membrane, and occurs upon GTP-binding (Antonny *et al.* 1997). Early models of Arf1 function suggested that the myristyl group and the helix would be retracted into the core of the protein in GDP-bound conformation, and upon GTP binding, they would be extended outward, allowing insertion of myristate into membrane lipid bilayers (Franco *et al.* 1995). This has been confirmed by recent studies using neutron diffraction and circular dichroism (Harroun *et al.* 2005). Among the several models proposed, most suggested that the myristate group is exposed and interacts with phospholipids when Arf1 is in the GDP-bound conformation. This weak membrane association is completely abolished if the myristate is removed. The Arf1-membrane interaction is stabilised in the GDP-bound form by a conformational change in the N-terminal helix that exposes several

hydrophobic residues, including Leu 8, Phe 9 and Phe 12 (which are buried inside the Arf1 protein in its GDP-bound form), and allows their insertion into the membrane (Antonny *et al.* 1997).

GNIFANLFKGLFGKKEMRIIMVGLDAAGKTITILYKLKLGEIVTTIPTIGFNVETVEYKNI
SFTVWDVGGQDKIRPLWRHYFQNTQGLIFVVDSDNRERVNEAREELMRMLAEDELDAVL
LVFANKQDLPNAMNAAEITDKLGLHSLRHRNWIQATCATSGDGLYEGLDWLSNQLRNQK

Fig 1.6. The primary structure of human Arf1. This figure depicts the amino acid sequence of human Arf1 (HsArf1). The amino-terminal α helix (underlined), the conserved Ras family GTP-binding proteins consensus sequence (black boxes), switch 1 (highlighted in red), switch 2 (highlighted in blue) and helix $\alpha 3$ (highlighted in green) are shown.

1.3 Arf1 Associated Proteins

As with all GTPase type proteins, Arf1 undergoes a nucleotide-dependent conformational change between a GTP-bound (on state) and GDP-bound (off state) form. GTPase-activating proteins (ArfGAPs) are negative regulators of this process. ArfGAPs recognise Arf-GTP and induce hydrolysis of GTP (Szafer *et al.* 2001). Specifically, ArfGAPs hydrolyse GTP-bound Arf via a highly conserved zinc finger domain that mediates this catalysis. ArfGAPs are structurally diverse proteins

containing catalytic, protein-protein and lipid interaction domains, in addition to the ArfGAP domain.

Guanine nucleotide exchange factors (ArfGEFs) are positive regulators of Arf-GDP. All ArfGEFs identified to date possess a Sec 7 domain, a module of approximately 200 amino acids in length, that mediates the interaction with Arf1 and catalyses the exchange of GDP for GTP (Klarlund *et al.* 1997; Abergel *et al.* 1998). Some ArfGEFs are involved in G-protein signalling pathways in response to extra- or intracellular signals, which can induce translocation of ArfGEFs to specific membrane compartments in the cell where they then activate G proteins. Certain ArfGEFs also mediate cross-talk between different receptor-mediated signalling pathways, thereby acting as signal integrators (Geiger *et al.* 2000). ArfGEFs also serve as an initial point of control for most G protein regulated pathways. Mammalian ArfGEFs comprise a family of 14 proteins divided into two subfamilies, all containing a conserved Sec7 domain (Jackson and Casanova 2000).

1.3.1 GTPase Activating Proteins

ArfGAPs are structurally diverse proteins containing catalytic, protein-protein and lipid interaction domains, in addition to the ArfGAP domain. ArfGAPs interact directly with the switch 2 and helix $\alpha 3$ of the Arf1 protein, although there is some evidence to suggest that the N-terminus is also important (Bernards 2003; Randazzo and Hirsch 2004). There is also speculation that different members of the ArfGAP families interact with different parts of the Arf1 protein.

The human genome encodes 15 ArfGAPs, all possessing functional ArfGAP domains (Figure 1.7). There are two main classes, based on the positioning of the

ArfGAP domain. These are further sub-divided based on accessory protein structure. The first major class consists of proteins with the ArfGAP domain at the N-terminus (ArfGAP1 type and G protein-coupled receptor kinase-interacting target, GITs). Those that contain a pleckstrin homology (PH) domain immediately N-terminal to the ArfGAP domain belong to the second group, ArfGAP with ankyrin repeat, pleckstrin homology domain (ARAP) (Nie *et al.* 2003a).

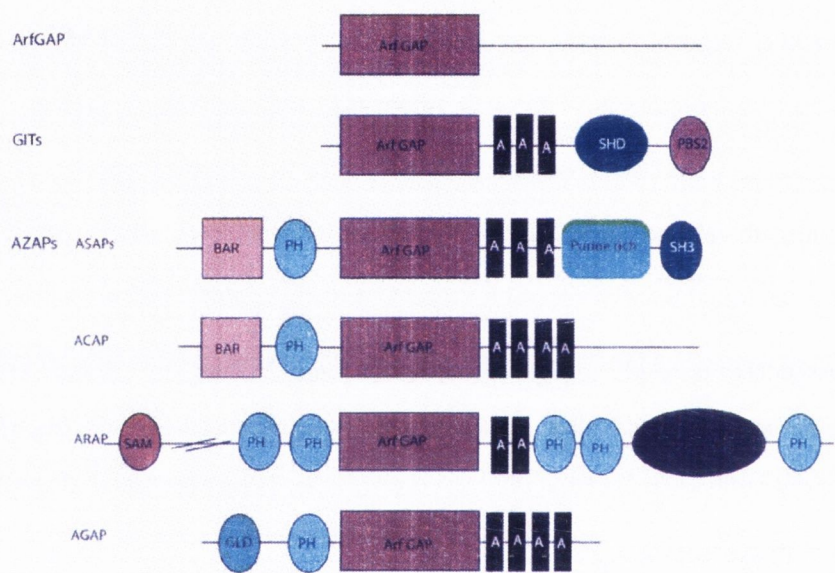


Fig 1.7. Protein domains of mammalian ArfGAPs. The above figure depicts the classification of mammalian ArfGAPs, three major groups are presented: ArfGAPs, GITs, and AZAPs, as defined in surrounding text. ArfGAP1 type proteins contain only an ArfGAP domain, lacking extra domains. Other ArfGAPs contain additional domains: the GITs have additional domains only to the C-terminus of the ArfGAP domain and AZAPs: have additional domains on both sides of the ArfGAP domain, as shown in the diagram above. AZAPs are further divided into ASAPs, ACAPs, ARAPs and AGAPs, which are defined based on presence of additional domains. The above figure depicts the following abbreviated domains: (A)-ankyrin repeat; (BAR)-Bin, amphiphysin and Rvs 167 and Rvs 161; (GLD)-GTP-binding protein-like domain; (PBS)-paxillin-binding sequence; (PH)-pleckstrin homology; (SAM)-sterile α motif; (SH3)-src homology 3 and (SHD)-spa-homology domain.

ArfGAPs appear to bind preferentially to one particular Arf protein over the other Arfs. Arf GAP with GTP-binding protein-like, ankyrin repeat and pleckstrin homology domains 1 (AGAP1), for example, preferentially hydrolyses Arf1 (Ooi *et al.* 1998), while Arf GAP with coiled-coil, ankyrin repeat and pleckstrin homology domains 1 (ACAP1) hydrolyses Arf6 (Jackson *et al.* 2000). ArfGAPs localise to specific areas of the cell, contributing to the site-specificity of Arf, enabling efficient functioning and regulation of Arf at each site independent of other sites. For instance, overexpression studies of ArfGAP1 show that it binds to the cargo receptor at the Golgi apparatus (Aoe *et al.* 1997; Majoul *et al.* 2001).

1.3.2 Mechanism of ArfGAP Type 1

ArfGAPs can stimulate Arf1-bound GTP hydrolysis, returning Arf1 to the inactive GDP-bound state. All ArfGAPs share a common ArfGAP domain of approximately 70 amino acids, that includes a zinc finger motif of CXXCX(16-17)CXXC, which is critical for ArfGAP activity (Cukierman *et al.* 1995). In addition to this zinc finger, all ArfGAPs have a conserved arginine within the GAP domain, which is implicated in GTP hydrolysis. This arginine is positioned on the surface of the molecule, close to several hydrophobic residues. When it or adjacent hydrophobic residues are substituted there is a reduction in GAP activity (Mandiyan *et al.* 1999). A 100,000 fold decrease of GTPase activity was observed in the case of a mutation of the arginine (Randazzo *et al.* 2000).

As not all ArfGAPs function in the same manner, the mechanism for type 1 ArfGAPs, the most fundamental ArfGAP, is described. All other ArfGAPs apart from

type 1 ArfGAPs have additional domains, which contribute additional functions to the ArfGAPs. Goldberg (1999) demonstrated that the interface between Arf1 and ArfGAP1 occurs in the switch 2 and helix $\alpha 3$ domains of Arf1 and in the strand $\beta 5$ -adjoining loop and helix αF regions of ArfGAP1. In this crystallization study, the switch 1 region was free, and the surface of the zinc-finger motif including an arginine critical for activity was orientated away from Arf1. In support of this model, the vesicle coat protein, coatamer, which binds to switch 1 and switch 2 of Arf1 was found to stimulate GAP activity (Zhao *et al.* 1997). This is consistent with the idea that Arf1 is hydrolysed within a three-part complex consisting of Arf1, coatamer and ArfGAP1. However, this theory has been contested (Jacques *et al.* 2002) and there is mounting evidence against this proposed tripartite complex. Even the region of interaction is in question, the N-terminal α -helix has been implicated along with switch 1 in the interaction between Arf1 and the ArfGAP (Jacques *et al.* 2002).

1.3.3 ArfGAPs in *Saccharomyces cerevisiae*

Six yeast genes that encode proteins containing cysteine rich ArfGAP-like domains have been identified in the *Saccharomyces cerevisiae* genome (Zhang *et al.* 1998). Four of these are functional: GCS1 (1,3-beta-D-glucan synthase), GLO3 (Gcs1 like ORF), AGE1, and AGE2 (ArfGAP Effector protein 1 and 2) (Cukierman *et al.* 1995; Poon *et al.* 1996). GCS1 and GLO3 have been implicated in vesicular transport between the Golgi and the ER (Ireland *et al.* 1994), while GSC1 and AGE2 have been implicated in trans Golgi network (TGN) endosomal trafficking (Dogic *et al.* 1999; Poon *et al.* 1999; Poon *et al.* 2001). Deletion studies of *S. cerevisiae* ArfGAPs revealed that none was essential (Ireland *et al.* 1994; Zhang *et al.* 1998; Poon *et al.*

1999). Double deletion mutants have shown there is redundancy in the yeast ArfGAPs (Poon *et al.* 1999; Poon *et al.* 2001). Triple mutants were also generated, showing that the gene encoding the GCS1 protein was more important than the other ArfGAPs. The reason for this effect appears to be related to the expression level of the ArfGAP genes. GCS1 was expressed at higher level than the other ArfGAPs. Expression of the ArfGAPs, AGE1 and AGE2 was undetectable (Zhang *et al.* 2003).

1.3.4 Guanine Nucleotide Exchange Factors

ArfGEFs have been subdivided into two classes based on molecular weight. Those with a higher molecular weight, greater than 100 kDa, and those less than 100 kDa. The higher molecular weight ArfGEFs function in the ER-Golgi system, and include guanine nucleotide exchange factor for ADP ribosylation factors (Gef)/ membrane-associated guanine-nucleotide exchange factor GNOM (*S. cerevisiae*) and Sec7/ Brefeldin A inhibited guanine nucleotide exchange factor (BIG) (human) subfamilies. To further aid classification, larger ArfGEFs can be inhibited through the use of Brefeldin A (BFA), an antiviral agent that inhibits an early step in secretion (Tamura *et al.* 1968), while the lower molecular weight class cannot.

The lower molecular weight ArfGEFs include the well-known human ADP-ribosylation nucleotide opener site (ARNO) subfamily and function primarily in the endosomal-plasma membrane system (Santy and Casanova 2001). They are involved in signalling pathways downstream of phosphoinositide-3 (PI₃) kinase, a lipid kinase, in actin cytoskeleton remodeling, and integrin signalling (Frank *et al.* 1998). All of the low molecular weight family members contain a PH domain, that mediates membrane localisation via interaction with specific polyphosphoinositides, and an

adjacent carboxy-terminal polybasic domain that cooperates with the PH domain to enhance membrane-binding (Frank *et al.* 1998).

There are four ArfGEFs in *S. cerevisiae*, Sec7 protein (Sec7p), synaptotagmin 1 (Syt1), and guanine nucleotide exchange factors for Arf 1 and 2 (Gea1, Gea2) (Peyroche *et al.* 1996; Peyroche *et al.* 1999; Sprang 2001). Gea1 and Gea2 can functionally replace one another (Peyroche *et al.* 2001), and have been assigned a role in the organisation of the actin cytoskeleton (Zakrzewska *et al.* 2003). Gea1 and Gea2 have been shown to recognize an integral membrane protein, Golgi transmembrane-domain partner of any of the high-molecular-weight ARF-GEFs (Gmh1), which is located in the cis-Golgi in *S. cerevisiae*, acting as a positive regulator of Gea 1 and 2 (Chantalat *et al.* 2003).

1.3.5 Mechanism of ArfGEFs

The ArfGEF Sec7 domain engages both the switch 1 and switch 2 regions of the Arf1 molecule during nucleotide exchange. These regions of the Arf1 molecule undergo substantial conformational changes that facilitate the exchange reaction (Chardin *et al.* 1996). Arf1 can interact weakly with membrane phospholipids when bound to GDP, whereas its GTP-bound form is tightly membrane bound due to the interaction of the N-terminal helix of Arf1 with the lipid bilayer. The presence of the membrane phospholipids alone can stimulate exchange of GDP for GTP on Arf1 in the absence of a ArfGEF (Franco *et al.* 1995) but this reaction is extremely slow compared with that catalysed by the Sec7-domain of ArfGEF. It appears that these lipids are required first to interact with Arf1 to free the sites of interaction, so that ArfGEF can engage

with Arf1. ArfGEF accelerates nucleotide exchange on Arf1 by 4-5 fold (Beraud-Dufour *et al.* 1998).

The crystal structure of the human ARNO Sec7 domain has been solved at an approximately 2Å resolution (Cherfils *et al.* 1998; Mossessova *et al.* 1998). It consists of 10 alpha-helices (A-J) arranged in a right-handed superhelix, similar to the armadillo-repeat, 40 amino acid long tandemly repeated sequence motif first identified in *Drosophila melanogaster* Hatzfeld *et al.* (1999). The C-terminus of the Sec7 domain mediates the interaction with Arf1 through hydrophobic and polar residues. Specifically α helices F, G and H form a deep hydrophobic groove. A hydrophilic loop between helices F and G acts as the binding site for nucleotide free Arf1. A conserved FRLPGE sequence makes up most of α helix H. This loop contains a large number of solvent exposed hydrophobic residues, and contributes most of the exchange activity. Point mutations in either of these sequences dramatically reduces the ability to exchange nucleotides, demonstrating that this is the area of Sec7 activity (Betz *et al.* 1998; Cherfils *et al.* 1998; Mossessova *et al.* 1998).

The nucleotide exchange activity of ArfGEF molecules is dependent on a conserved glutamate residue located in the loop between helices F and G (Cherfils *et al.* 1998; Mossessova *et al.* 1998). It has been proposed that this invariant glutamate at the C-terminus of the F-G loop (E156 in ARNO) forms a “glutamatic finger” that inserts into the nucleotide-binding fold of Arf1, displacing the coordinating Mg^{2+} ion and possibly the β -phosphate of the bound GDP (Beraud-Dufour *et al.* 1998). The crystal structure of a complex between the Sec7 domain of Gea2p and nucleotide-free Arf1 supports this model, indicating that the glutamate side chain comes within 3Å of the β -phosphate site (bound to GDP) and is likely to exert steric and electrostatic repulsive forces on the bound nucleotide (Goldberg 1998).

1.3.6 Additional Effectors of Arf1

Apart from ArfGEFs and ArfGAPs, Arf1 interacts with several other proteins. In its GDP-form, Arf1 interacts with members of the p24 family of type I integral-membrane proteins, which are localised in the ER and the Golgi apparatus along with ArfGEFs (Donaldson and Lippincott-Schwartz 2000). These p24 proteins are thought to function as receptors for cargo exit from the ER and in transport vesicle formation. They contain diphenylalanine and dilysine sorting signals that are involved in coat-protein recruitment and anterograde and retrograde transport of the secretory pathway (Schimmoller *et al.* 1995; Stamnes *et al.* 1995; Belden and Barlowe 1996; Sohn *et al.* 1996; Rojo *et al.* 1997; Bremser *et al.* 1999).

In its GTP bound form Arf1 interacts with several vesicle coat components (COPI, Adaptin Protein 1/3/4, and GGA1/2/3), phospholipids (e.g. phosphatidylinositol) and other factors such as Arfaptin and phospholipase D (Nie *et al.* 2003a).

1.3.7 Arf1 Involvement in Vesicle Formation

Arf1 plays a key role in the composition of vesicles used in vesicular trafficking (Balch *et al.* 1992). Arf1 is involved in the formation of vesicles composed of COPI and clathrin coatomer, but not in COPII vesicles. Formation of clathrin vesicles has not been fully elucidated yet, and involves many more proteins than COPI vesicle formation. Figure 1.8 depicts assembly of clathrin coated vesicles.

COPI vesicles are primarily involved in retrograde transport from the Golgi to the endoplasmic reticulum (ER) and also in forward transport from Golgi to the cisternae. Clathrin vesicles are involved in transport between the plasma membrane to early endosomes and from Golgi to the endosome. COPI vesicles are composed of the COPI coatomer: α , β , β' , γ , δ , ϵ , and ζ subunits. COPI vesicles capture cargo with cytoplasmic carboxy-terminal domain sorting signals in the form of KKXX, dilysine motifs (Kirchhausen 2000). Coat assembly of COPI is depicted in Figure 1.9. Arf1 is activated by ArfGEF, and in its myristolated state Arf1 binds to the Golgi membrane. Coat assembly is activated and the COPI vesicle forms.

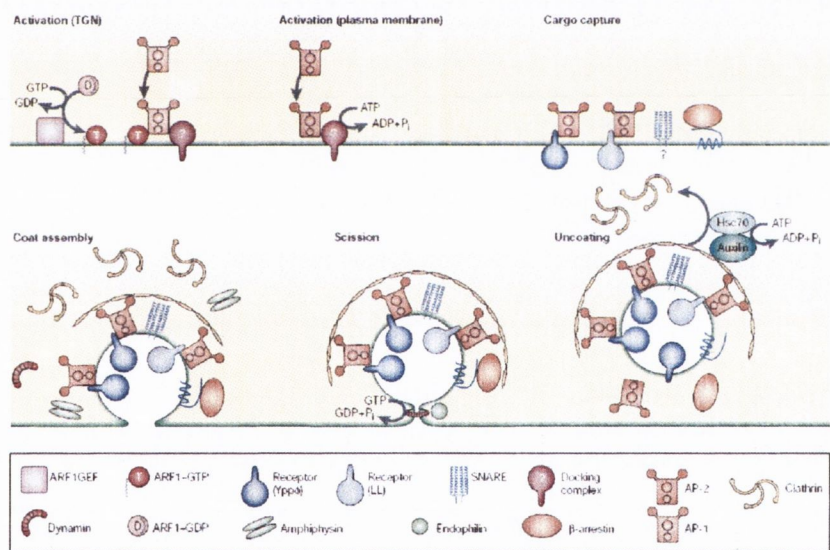


Fig 1.8. The formation of Clathrin-coated vesicles. Activation of Arf1 occurs at the trans-Golgi network (TGN) triggering coat assembly. Coat assembly is activated at the plasma membrane. Adaptor proteins bind to cargo molecules and other coat components, including clathrin. Clathrin forms a cage around the cargo using clathrin triskellons that polymerise into hexagons and pentagons, leading to membrane deformation. When the coat is almost complete, dynamin and accessory proteins pinch off the vesicle. Uncoating of the vesicle is facilitated by ATP hydrolysis by Hsc 70 (cognate of Hsp 70) and auxillin. Figure is taken from Kirchhausen (2000).

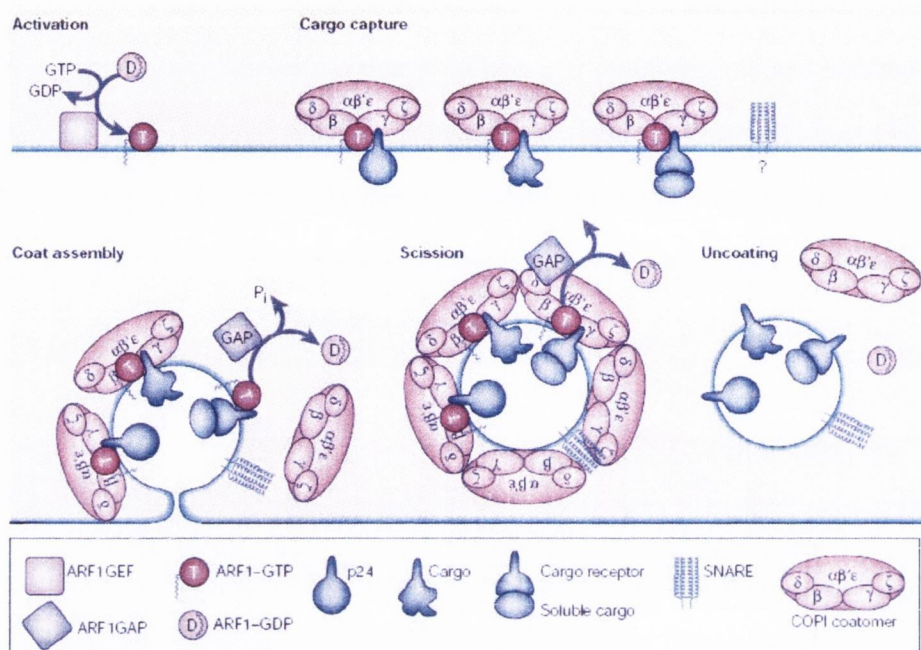


Fig 1.9. The formation of COPI-coated vesicles. Coat assembly is activated by recruitment of Arf1-GTP to the membrane, following activation of Arf1 by ArfGEF. COPI is captured, cargo (material for transporting) is recruited, and membrane deformation occurs. When the coat has been completed, the vesicle buds. Arf1GAP enhances the GTPase activity of Arf1, and leads to the inactivation of Arf1 and uncoating. Figure is taken from Kirchhausen (2000).

1.4 Thesis Aims: Elucidating the Mechanism of Cellular Differentiation in *T. brucei*

Bloodstream forms of *T. b. brucei* parasites undergo a long slender to short stumpy differentiation process. This process is cell density dependent (Vassella *et al.* 1997). Upon reaching a specified density, long slender cells release a T-GIF signal which is internalised by other long slender cells initiating differentiation (Vassella *et al.* 1997). A receptor or transporter protein is required to take up this signal. A surface protein, TbPP69, is upregulated in differentiating pleomorphic strains and has been implicated in binding of T-GIF (Murphy and Olijhoek 2001). One of the objectives of this thesis was the identification and characterisation of the TbPP69 receptor/transporter and its involvement in differentiation.

Arf1 in *T. b. brucei* has also been implicated in differentiation as the internal target of T-GIF (Murphy and Olijhoek 2001). TbArf1 is one of several proteins that are specifically upregulated in the long slender cells, where differentiation occurs (Murphy and Olijhoek 2001). Furthermore, T-GIF has been shown to bind TbArf1 in the helix $\alpha 3$ region (Noel Murphy, unpublished). Antisera raised against *T. b. brucei* Arf1 (TbArf1) recognised the trypanosome endosomal system, suggesting a similar role for TbArf1 in vesicle formation, as in its mammalian and yeast counterparts (Murphy and Olijhoek 2001, see Figure 1.10). Unexpectedly, TbArf1 has also been localised to the flagellar region of the trypanosome. This suggests a novel function in this flagellar organelle, that TbArf1 is involved in signalling. The second objective of this project was the identification and characterisation of Arf1 effector proteins, in *T. b. brucei*, to examine their role in differentiation.

The signal of differentiation must be propagated throughout the cell via a signal transduction pathway. cAMP has been implicated in the differentiation of long slender cells of *T. b. brucei*, as shown by the addition of a membrane permeable derivative of cAMP (pCPTcAMP) to monomorphic long slender cells (Breidbach *et al.* 2002). cAMP was sufficient to induce differentiation, independent of T-GIF (SIF). The third objective of this thesis was the elucidation of the role of cAMP pathways in Arf1-mediated *T. b. brucei* differentiation.

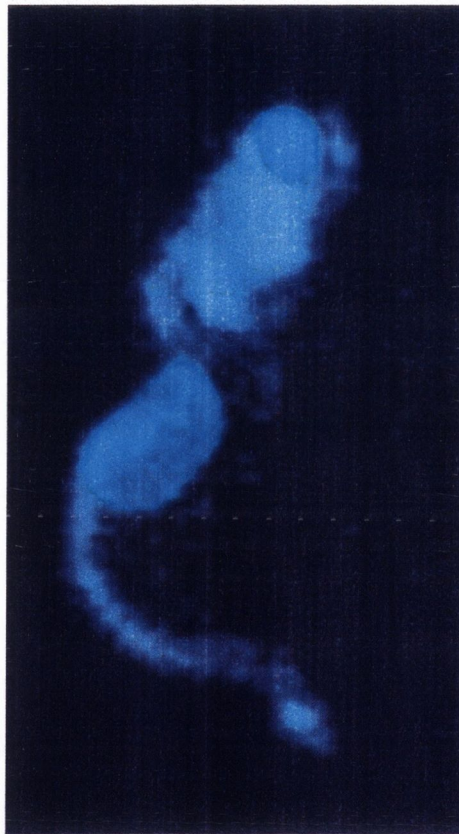


Fig 1.10. Localisation of Arf1 in the *T. b. brucei* pleomorphic parasite with polyclonal IgG specific for the TbArf1. Nuclear and kinetoplast DNA are stained with DAPI (blue) and antibodies are stained with FITC (green). Note there is concentrated staining between the nucleus and kinetoplast where the endosomal and Golgi regions occur and where TbArf1 is expected. However, there is also staining of TbArf1 along the flagellum. This figure is taken from Murphy & Olijhoek (2001).

Chapter 2: Materials and Methods

Bioinformatic Analysis

2.1 Identification and Analysis of Trypanosome and *Leishmania* Genes

2.1.1 Identification of Effectors of Arf1 and Ar1 Proteins in *T. b. brucei*

ADP-ribosylation factor (Arf) genes, and their effectors related to vesicular signalling, that have been characterised in other species were identified in the literature. Protein sequences of Arf1, Arf-like proteins (Arfs) and effectors of Arf1 (GTPase-Activating Proteins of Arf1 (ArfGAPs) and Guanine Nucleotide Exchange Factors of Arf1 (ArfGEFs)) from *Homo sapiens*, *Mus musculus*, *Rattus norvegicus*, *Drosophila melanogaster*, *Xenopus laevis*, *Caenorhabditis elegans*, *Dictyostelium discoideum* and *Saccharomyces cerevisiae* were downloaded from the NCBI database (<http://www.ncbi.nlm.nih.gov/>). Each of these protein sequences was compared to the *T. brucei* gene database (GeneDB) (<http://www.genedb.org/>), using the OmniBLAST program (<http://www.ncbi.nlm.nih.gov>). A protein motif scan program, ScanProsite (<http://www.expasy.-org/tools/scanprosite/>), was used to confirm the presence of conserved domains in the newly identified homologues. Similarly, homologues were also identified in *T. b. gambiense*, *T. cruzi*, *T. vivax*, *T. congolense*, and *L. major*.

T-coffee alignments of the Arf1, Arf, Arl, ArfGAP and ArfGEF protein families were produced (T-coffee, Notredame *et al.* 2000). The Genedoc program was used to identify and extract conserved domains (Genedoc, Nicholas *et al.* 1997). Protein alignments were used to construct neighbour-joining phylogenetic trees (Mega 3.1, Kumar *et al.* 2004). T-coffee alignments of the Arf1, Arf, Arl, ArfGAP and ArfGEF protein families were produced (T-coffee Notredame *et al.* 2000). The Genedoc program was used to identify and extract conserved domains (Genedoc, Nicholas *et al.* 1997). Protein alignments were used to construct neighbour-joining phylogenetic trees, using Mega 3.1. 1000 bootstrap replicates were carried out to assess the statistical significance of each node (Mega 3.1, Kumar *et al.* 2004).

2.1.2 Identification of the TbPP69 Putative Receptor

To identify candidates for the TbPP69 receptor/transporter, partially purified protein (tomato-lectin purified pleomorphic and monomorphic surface proteins, prepared from monomorphic strain *T. brucei* 328.114 and pleomorphic strain *T. brucei* ILTat1.1 according to section 2.9) containing the putative TbPP69 factor was run on a SDS-12% PAGE gel and 69 kDa bands were extracted and subjected to tandem-tandem mass spectrometry analysis (as outlined section 3. 3, materials and methods are as described in Untalan *et al.* 2005).

Analysis produced strings of peptide sequences which were compared with the *T. b. brucei* GeneDB database (<http://www.genedb.org/>), using the OmniBLAST server program (Hertz-Fowler *et al.* 2004) to produce best possible matches. The GeneDB OmniBLAST program performs a full database search using the Blosum 62 matrix for scoring the protein alignments. The open reading frame finder (ORF finder,

NCBI, <http://www.ncbi.nlm.nih.gov/>, Wheeler *et al.* 2007) was used to identify open reading frames of the best matches that were then translated into protein sequences using protein translator program, Translate (Expasy <http://www.expasy.org/tools/dna.html>). The TbPP69 receptor/transporter was known to be in the order of 40-70 kDa. Proteins in this range were selected using a range exclusion function of GeneDB. Selected candidates were assessed using a transmembrane program to search for multiple domains as the TbPP69 protein was thought to be a multi-pass transmembrane protein. Candidates were tested with the TMHMM program (Krogh *et al.* 2001) for identification of transmembrane helices in proteins (CBS; Denmark, <http://www.cbs.dtu.dk/services/TMHMM-2.0/>). Subsequently, candidates were assessed using a program to identify signal cleavage sites, as the TbPP69 receptor/transporter was known to possess an N-terminal signal peptide (SignalP 3.0, (Bendtsen *et al.* 2004)). A program to predict GPI-anchors was run on candidate sequences, to ensure that they did not have GPI-anchoring domains, DGPI (http://129.194.185.165/dgpi/index_en.html, Kronegg and Buloz, 1999).

Laboratory Work

2.2 Preparation of Nucleic Acids

2.2.1 Preparation of DNA

Bloodstream form *T. brucei* 328.114 trypanosomes grown according to conditions (section 2.11.3) were resuspended at a concentration of 1×10^9 cells/ml in NET buffer [150 mM NaCl, 100 mM ethylenediamine tetraacetic acid (EDTA), 10 mM Tris-HCl

pH 7.4] which was brought to a final concentration of 1% SDS with 100 µg of boiled RNase A added per ml. The trypanosomes were incubated at 37°C for 3 h to lyse the cells and digest the RNA. Proteinase K (10 mg/ml) was added to a final concentration of 50 µg/ml and the digestion reaction was allowed to continue overnight at 55°C. The lysate was then gently but thoroughly extracted with an equal volume of phenol pre-equilibrated with TE [10 mM Tris-HCl, pH 8.0, 1 mM EDTA]. Any residual phenol was removed by 2-3 extractions with ether. The DNA was precipitated by the addition of 2.5 volumes of cold absolute ethanol pre-chilled at -20°C and pelleted by centrifugation at 14,000 rpm for 20 min at 4°C in an Eppendorf minifuge or mikro 200R centrifuge. The DNA precipitate was washed twice with 70% ethanol then dried under a vacuum (Heto, spedivac). The pellet was re-dissolved in an appropriate volume of sterile water or TE depending on the yield of the DNA. The DNA concentration was estimated by measuring the absorbance at 260 nm in an Eppendorf photometer. The DNA was stored at 4°C for later use. For longer storage, the DNA was kept as an ethanol precipitate at -70°C.

2.2.2 Preparation of RNA

Isolation of RNA followed the procedure described by Chomczynski and Sacchi (1987). This procedure involved lysis of trypanosomes in the presence of a strong denaturant and RNase inhibitor. To a pellet of approximately 1×10^9 trypanosomes, 2 ml of Solution D [4 M guanidinium thiocyanate, 25 mM sodium citrate pH 7.0, 0.5% (w/v) sarcosyl, 0.1 M 2-mercaptoethanol], 200 µl of 2 M sodium acetate, pH 4.0, 2 ml of water-saturated phenol and 400 µl of chloroform were added. The contents were mixed vigorously by vortexing repeatedly.

The resulting suspension was placed on ice for 10 min and then aliquoted into sterile 1.5 ml Eppendorf tubes, then centrifuged at 14,000 rpm for 10 min (Eppendorf minifuge or mikro 200R centrifuge). The RNA was completely dissolved in Solution D and precipitated using 2.5 volumes of absolute ethanol for 10 min on dry ice. The RNA was recovered by centrifugation at 14,000 rpm for 10 min at 4°C (Eppendorf minifuge or mikro 200R centrifuge) and the resulting RNA pellet washed with 70% ethanol and dried under vacuum (Heto, speedivac). The RNA pellet was dissolved in 100 µl of sterile RNase-free water.

The concentration of RNA was estimated by measuring the absorbance at 260 nm (Eppendorf photometer). A 2–5 µg aliquot of the total RNA was electrophoresed on a 1.5% agarose gel to monitor the physical integrity of the nucleic acid. Aliquots were made and stored at –70°C for later use.

2.2.3 Isolation of mRNA

Magnetic beads were used to further isolate mRNA from an RNA preparation according to manufacturer's instructions (Promega Magnet Sphere Magnetic Separation Kit®, Madison, WI, USA). Promega Streptavidin Paramagnetic Particles (SA-PMP) beads were resuspended in solution by gently flicking the tube. 40 µl of suspended bead solution were removed and transferred into a 0.5 ml Eppendorf tube, and placed in the supplied magnetic stand. Beads were washed 3x in 300 µl of 0.5 x SSC [SSC containing 175.3 g/l NaCl, 88.2 g/l sodium citrate], and were resuspended in 20 µl of 0.5 x SSC.

0.1–1.0 mg of total RNA and 10 pmol/µl biotinylated oligo dT primer (Primers listed in Table 2.1, supplied by MWG Biotech, Ebersberg, Germany) were placed in a

0.5 ml Eppendorf tube, and heated on a PCR block (M.J. Research Inc., PTC-100 thermo cycler) at 65°C for 10 min. 20 µl of 20x SSC solution was added. The RNA solution was added to the SA-PMP beads and incubated at room temperature for 10 min. Tubes were mixed by inversion every 2-3 min. The SA-PMP beads were captured in the magnetic stand and the supernatant was removed. Beads were washed 5 times in 300 µl of 0.1x SSC. Supernatant was removed, and the RNA remained attached to the SA-PMP beads.

2.2.4 Complementary DNA (cDNA) Synthesis

The synthesis of cDNA was performed as described in the manufacturer's protocol (Sensiscript® Reverse Transcription, Qiagen, West Sussex, UK). For first strand cDNA synthesis, the following reaction was set up in a 0.5 ml microfuge tube: 5 µl of mRNA (1-2 µg/µl, attached to the SA-PMP beads) was heated at 75°C for 5 min and cooled on ice. To this, 2 µl of 10x RT buffer (provided by manufacturers), 2 µl of 5 mM dNTPs, 2 µl of 100 ng/µl oligo (dT) primer (primers listed Table 2.1, supplied by MWG Biotech), and 13 µl RNase-free water were added. The reaction was initiated by addition of 1 µl of Sensiscript® Reverse Transcriptase (4 U/µl) and incubated at 37°C for 1 h.

The second strand cDNA was PCR amplified using mini-exon and oligo (dT) primers. The PCR amplification reactions were set up in a final volume of 20 µl as described in the following section, using 2 µl of 100 ng/µl of oligo (dT) primer. The reaction mixtures were spun briefly in a microfuge. The PCR parameters: denature at 94°C for 1 min, anneal at 50°C for 1 min, synthesise at 72°C for 2 min for 40 cycles with a final synthesise at 72°C for 5 min (M.J. Research Inc., PTC-100 thermo

cycler). The thermal cycler was programmed to cool to 4°C at the end of the PCR. The double-stranded cDNA (ds-cDNA) was analysed by agarose gel electrophoresis, to assess its quality and concentration and then stored at -20°C for later use.

2.3 PCR Amplification

A number of PCR amplification protocols were followed depending on the *Taq* polymerase used. It was found that certain *Taq* enzymes were optimal at amplifying different fragments of DNA.

2.3.1 Standard PCR amplification

Standard PCR amplification reactions were performed in final reaction volume of 20 or 50 µl containing 1-10 ng/µl of DNA template, 2 mM MgCl₂ buffer, 0.2 mM dNTPs, 0.4 mM forward and reverse primers [outlined in Table 2.1 (primers for chapter 3), Table 2.2 (primers for chapter 5), on Table 2.3 (primers used in Appendix)] and 1 unit *Taq* polymerase (Qiagen). Reactions were placed in a programmable heating block (PTC-100 thermo cycler, M.J. Research Inc.) and incubated at 94°C for 1 min, 50°C for 1 min and 72°C for 2 min, followed by 30–40 cycles at 94°C for 30 s, 50°C for 1 min (the annealing temperature was optimised for the reaction) and 72°C for 2 min. A final elongation step of 10 min at 72°C, and a 4°C cooling step followed.

2.3.2 Qiagen *Taq* PCR Master Mix

For standard PCR amplification a PCR Master Mix for *Taq* polymerase (Qiagen) was used, which gave effective results with little effort. The PCR amplification reactions were performed in final reaction volume of 20 or 50 μ l containing 1-10 ng/ μ l of DNA template, 2 x Qiagen *Taq* PCR Master Mix (2.5 units *Taq* polymerase, 1 x Qiagen buffer [TrisHCl, KCl, $(\text{NH}_4)_2\text{SO}_4$, 15 mM MgCl_2 , pH 8], 200 μ M of each dNTP) and 2 ng/ μ l final concentration of each primer (primer details see Tables 2.1, 2.2, and 2.3; supplied by MWG Biotech, Germany and Sigma Genosys, West Sussex, UK). The PCR reactions were placed in a programmable heating block (PTC-100 thermo cycler, M.J. Research Inc.) and incubated at 94°C for 1 min, 55°C for 1 min and 72°C for 2 min, followed by 30–40 cycles at 94°C for 30 s, 55°C for 1 min (the annealing temperature was optimised for the reaction) and 72°C for 2 min. A final elongation period of 10 min at 72°C, and a 4°C cooling period followed. The annealing temperatures varied depending on the melting temperatures of the individual primers.

2.3.3 Qiagen Proof Start ® PCR Kit

For long DNA fragments a high fidelity PCR kit was used, Proof-start® PCR Kit (Qiagen). The procedure was followed according to manufacturer's guidelines. The kit proved difficult to optimise, so other similar kits were tested. PCR amplification reactions were performed in a final reaction volume of 20 or 50 μ l containing 1-10 ng/ μ l of DNA template, 10x Qiagen Proofstart® PCR buffer [Tris.Cl, KCl, $(\text{NH}_4)_2\text{SO}_4$, 15 mM MgSO_4 , bovine serum albumin, Triton X-100, pH 8.7], variable quantities of MgSO_4 , 10 mM dNTPs, 2.5 U/ μ l Proof Start® DNA polymerase and 2

μ M final concentration of each primer. The PCR reactions were placed in a programmable heating block (PTC-100 thermo cycler, M.J. Research Inc.) and incubated at 95°C for 5 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 1 min (adding 1 min per kb).

2.3.4 KOD Hot Start® DNA Polymerase

A second high fidelity PCR kit was used for important products that were difficult to obtain; this kit worked very well. The PCR amplification reactions were also performed in final reaction volume of 20 or 50 μ l using KOD hot start® DNA polymerase (Novagen, Nottingham, UK). Reactions were carried out according to the manufacturer's guidelines, containing 1-10 ng/ μ l of DNA template, 1 unit KOD hot start® DNA polymerase, 0.3 μ M 5' primer, 0.3 μ M 3' primer, 1 mM MgSO₄, 0.2 mM dNTPs, 10x PCR KOD hot start® DNA polymerase buffer to a final concentration of 1x DNA polymerase buffer. The PCR reactions were placed in a programmable heating block (PTC-100 thermo cycler, M.J. Research Inc.) and incubated at 94°C for 2 min, followed by 35 cycles 94°C for 15 s, 60°C for 30 s, and 72°C for 20 s (with the addition of 20 s per kb). A final elongation period of 10 min at 72°C, and a 4°C cooling period followed. The annealing temperatures varied depending on the melting temperatures of the individual primers.

5 μ l PCR products were mixed with 6x loading dye (1 x TAE [40 mM Tris-acetate, 1 mM EDTA, pH 8.0], 50% glycerol, 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol) and resolved on 1% agarose gel made with 1x TAE buffer stained with ethidium bromide (0.5 μ g/ml) and photographed under UV illumination (BioRad gel documentation system).

Table 2.1. Primer Information. Names, sequences and restriction sites incorporated into the primers used in chapter 3 are shown below.

Primer Name	DNA Sequence	Restriction Site Incorporated
T7-SL-universal	TAATACGACTCACTATAGGGAGTAGAACAGTTTCTGTACTATA	
T7-Oligo -dT	TAATACGACTCACTATAGGGAGATTTTTTTTTTTTTTTTTTTTTT	
pLEW 100 fwd (p2216 F)	GAGTTTAACATGTTCTCGTCC	
pLEW 100 Rev (P2216 R)	GTGCCATCAGATTACTCC	
pLEW 100 ESAG 10 Fwd	GCTCTAGATAGCCATGCGATTTCTTTCCTTCATGTGC	<i>Xba I</i>
pLEW 100 ESAG 10 Rev	GCGGATCCGTTTCAGATATTACTTATGGGAGG	<i>Bam HI</i>
p2216 ESAG 10 Fwd (E10 F)	CATAAGCTTATGCGATTTCTTT	<i>Xba I</i>
p2216 ESAG 10 Rev (E10 R)	GTAGGATCCCTAATTGGCTGT	<i>Bam HI</i>
ESAG10 internal reverse (E10 TR)	TCATTGCGTAACCCAACGAT	
Splice leader Fwd	TAGAACAGTTTC TGTACTATATTG	
α-Tubulin Rev	CCAGTGCAGTTGTCAGCGAGC	
β-Tubulin Rev	GACCGTATCATGATG	
rDNA spacer Fwd (rDNA F)	GAGGAAGGAGGGAAG	
rDNA spacer Rev (rDNA R)	ACAACCGAGCAATGA	

Table 2.2. Primer Information. Names, sequence and restriction enzymes incorporated into the primers used in chapter 4 are shown below.

Primer Name	DNA Sequence	Restriction Site Incorporated
TbArfI ORF 12R	GGGCACATCACCAGAAC	
TbArfI ORF 3R	CAAGCTCACAATTTTCGC	
TbArfI ORF 4R	GGTATTAACAATAACCTACTTC	
pET21 ArfGAP I Fwd	CATGGATAATCACATACTGCTGAGGATGG	<i>BamHI</i>
pET21 ArfGAP I Rev	GCGTGTGCGACGATTGGAAGTCCCATCG	
pET21 ArfGAP V Fwd	CATGGATCCCAAGTCGTAATAGGGATGAAG	<i>BamHI</i>
pET21 ArfGAP V Rev	GTCAAGCTTGTGCTTCTTACCAAAGG	<i>HindIII</i>
pET21 ArfGAP V Trun Rev	GGCAAGCTTGTTAGTATTCTGCTGTGCG	<i>HindIII</i>
P51 ek/lic ArfGAP I Fwd	GACGACGACAAGATGTCACATACTGCTGAG	
P51 ek/lic ArfGAP I Rev	GAGGAGAAGCCCGGTGATTGGAAGTCC	
P51 ek/lic ArfGAP V Fwd	GACGACGACAAGATGAGTCGTAATAGGGAT	
P51 ek/lic ArfGAP V Trun Rev	GAGGAGAGGCCCGGAGTATTCTGCTGTGCG	
T7 Promoter Fwd	TAATACGACTCACTATAGGG	
T7 Rev	CTAGTTATTGCTCAGCGGT	

Table 2.3. Primer Information. Names and sequences of the primers used in the Appendix are shown below.

Primer Name	DNA sequence
ILTat 1.1 Fwd	GACAGGCGCCGTCGGTATCAC
ILTat 1.1 Rev	TGGGAGTCCATCGTGCTGGCC
ILTat 1.2 Fwd	CGGCGTAGCAGACTCTCACCA
ILTat 1.2 Rev	GCCTGCTGTGCCTCCGTTAGT
ILTat 1.3 Fwd	CTTTCAGACCTCGGCAAGTGG
ILTat 1.3 Rev	GTTTGCGCTGCTGCGCGAAGT
ILTat 1.4 Fwd	AAAGACGCCGAGCTGACAGCC
ILTat 1.4 Rev	GTCCCGACACCAGTTTAGCGT
Biotin SL-brucei	AACTAACGCTATTATTAGAACAGTTTCTG
SR-T23	GTTAACTCGAGAATTCTTTTTTTTTTTTTTTTTTTTTT

2.4 Purification of DNA Products

2.4.1 Purification of PCR Products

Purification of PCR products was performed using the JET® quick PCR purification spin kit (Genomed, Limerick, Ireland) to remove any contaminants that would interfere with further reactions. For each 100 µl of PCR reaction sample, the aqueous phase was transferred to a 1.5 ml microfuge tube. To this, 400 µl of binding H1 buffer [concentrated guanidine hydrochloride, EDTA, Tris/ HCl isopropanol] was added. The contents in each tube were inverted briefly. The DNA/H1 mix was then pipetted into the assembled spin column and 2 ml receiver tube and spun at 13,000 rpm (Thermo IEC micromax centrifuge or mikro 200R centrifuge) for 1 min, the flow-through was discarded. 500 µl of a second H2 solution [ethanol, NaCl, EDTA and Tris/HCl] was added as a washing step. The column was spun again at 13,000 rpm for 1 min, and the flow-through was discarded. The column required a second centrifugation step to remove residual ethanol. The used receiver tube was removed

from the spin column and a second sterile receiver tube was put in its place. 50 µl of, pre-heated (65°C) TE buffer was added into the spin column and allowed to stand on the bench for at least 1 min. To elute DNA the spin column was then spun at 13,000 rpm for 1 min, and purified PCR products were removed from the receiver tube, and stored at 4°C or -20°C for later use.

A second similar PCR purification kit and protocol was also used to purify PCR products according to manufacturer's instructions (QIAquick® PCR purification kit, Qiagen).

2.4.2 Gel Extraction

Two gel extraction procedures were carried out according to the manufacturer's guidelines with the following changes: samples were eluted with 20 µl warmed TE. Kits were obtained from JET® quick gel extraction spin kit (Genomed) and QIAquick® gel extraction kit (Qiagen).

2.4.3 Phenol-Chloroform Extraction

Equal volumes of phenol-chloroform [phenol/chloroform/isoamyl alcohol (25:24:1)] and the sample for cleaning were placed into 1.5 ml eppendorf tubes. The tubes were spun in a centrifuge for 5 min at 13,000 rpm (Thermo IEC micromax centrifuge or mikro 200R centrifuge). Two phases were produced; the aqueous layer was removed and transferred into a new 1.5 ml eppendorf tube. Two volumes of ice-cold 100% ethanol were added, and the eppendorf tube was stored in the freezer for 20 min. On removal, the eppendorf tube was spun in a centrifuge for 20 min at 13,000 rpm at 4°C,

supernatant was removed, and 400 µl of 70% ethanol were added. The tube was spun for a further 10 min at 13,000 rpm, the supernatant was removed and the pellet was air-dried. The pellet was resuspended in an appropriate amount of TE.

2.4.4 Ethanol Precipitation

Ethanol precipitations used 1 µl of 100% glycogen, a tenth of the total volume of 3 M sodium acetate, pH 7, 2x volume of ice-cold 100% ethanol and sample. The reaction was cooled at -20°C for 20 min, and sedimented at 13,000 rpm for 20 min at 4°C (Thermo IEC micromax centrifuge or mikro 200R centrifuge). The supernatant was removed and the sample was washed with 70% ethanol, and sedimented for 10 min at 13,000 rpm, the supernatant was removed and the pellet was air-dried. The pellet was resuspended in an appropriate amount of TE.

2.4.5 Small Scale Plasmid DNA Preparation (Mini-Prep)

Two small scale plasmid preparation kits were used to prepare the DNA. Procedures were carried out in accordance with the manufacturer's instructions. A Nucleospin® Plasmid Kit (Macherey-Nagel, Düren, Germany) and a QIAprep® Spin Miniprep Kit (Qiagen) were used.

2.4.6 Large Scale Plasmid DNA Preparation (Midi-Prep)

Larger scale preps were prepared according to manufacturer's instructions (Qiagen QIAprep® plasmid midi kit), with the following changes. DNA was precipitated with

1x cold isopropanol, and cooled at -20°C before spinning in a centrifuge. On resuspending the pellet following isopropanol extraction, 200 µl of TE was used and pellet was briefly spun again to remove residual DNA.

2.5 Cloning of PCR Products

Primers were designed for the *T. brucei* putative gene sequences (Sigma Genosys and MWG). Primers contained 12-15 bp flanking the 5' and 3' regions of interest, a restriction enzyme site (if required), and GC clamp. PCR products were acquired as outlined above (section 2.3 PCR amplification) using *T. brucei* strain 328.114 DNA.

2.5.1 Restriction Enzyme Digestion of Nucleic Acids

All restriction enzymes were used under optimal conditions as recommended by the manufacturer (New England Biolabs, Boston, USA and Sigma). Double digests of DNA were carried out with two enzymes simultaneously, using a single appropriate buffer as indicated by the supplier (New England Biolabs and Sigma). All buffers used were obtained as 10x stocks. Where indicated, one-hundredth of the reaction volume of 100x bovine serum albumin (provided by the supplier) was also used in the reactions. All digests of DNA were carried out at 37°C for 2 or 3 hrs. All the DNA digests were separated by electrophoresis as described (section 2.6 Electrophoresis) and photographed.

Purification of PCR products and plasmid DNA (the plasmids used are listed in Table 2.4) were described in section 2.4. Genomic DNA prepared from trypanosomes and other organisms was digested in a total reaction volume of 10 or 50 µl. Each

reaction contained 0.5 to 2 µg of DNA, 2 to 10 units of appropriate restriction enzyme, 0.1 or 0.5 µl of 100x BSA (if required), 1 or 5 µl of appropriate 10x buffer and sterile water added to make the final volume. The restriction enzymes used for digestions included *Bam* HI (Sigma), *Hind* III (Sigma), *Eag* I, *Pac* I, *Xba* I and *Xho* I (New England Biolabs).

Digested PCR products and plasmid preps were cleaned using phenol-chloroform extraction procedure as described (section 2.4.3).

Table 2.4. Table of plasmids utilised. Plasmid name, antibiotic resistance gene and references are given.

Plasmid Name	Resistance genes	Reference/Source
plew100	Ampicillin, Phleomycin	Wirtz <i>et al.</i> 1999
pQE30	Ampicillin	Qiagen
pET21a	Ampicillin	Novagen
pET15b	Ampicillin	Novagen
pET28b	Kanamycin	Novagen
pET51a Ek/LIC	Ampicillin	Novagen

2.5.2 Ligation Reactions

The ligation reactions of the PCR products and plasmid vectors were performed at a molar concentration of 2:1, respectively. Using the NEB Quick ligation® kit (New England Biolabs), each ligation reaction was set as follows; 3 µl of the vector (25 ng/µl), 6 µl of appropriately diluted target DNA (25 ng/µl), 1 µl provided NEB ligase® enzyme (10 U/µl), 10 µl of 2x ligation buffer [100 mM Tris-HCl, pH 7.8, 20 mM MgCl₂, 20 mM dithiothreitol (DDT)]. The ligation reaction tubes were incubated at 25°C for 5 min (PTC-100 thermo cycler, M.J. Research Inc.). Up to 10 µl of each ligation mix were used to transform 200 µl competent *Escherichia coli* JM109 bacterial cells.

2.5.3 Ek/LIC Cloning Mechanism, Novagen

Primers were designed and manufactured containing the integration sequence as given by the manufacturers, (5' forward sequence GAC GAC GAC AAG ATX, 5' reverse sequence GAG GAG AAG CCC GGT) with 12 bp flanking the gene sequence of interest (Sigma Genosys). PCR products were amplified according to the KOD hot start polymerase directions (Novagen), as described (section 2.3.3, KOD hot start DNA Polymerase). PCR products were purified using PCR purification kits. Purified PCR products were treated with T4 DNA polymerase (Novagen) according to the manufacturer's guidelines with 0.2 pmol purified PCR product in up to 14.6 µl TE buffer, 10x T4 polymerase buffer, 25 mM dATP, 100 mM DTT, 1 unit T4 DNA polymerase. The reaction was incubated at 22°C for 30 min, and inactivated at 75°C for 20 min (PTC-100 thermo cycler, M.J. Research Inc.).

Following T4 polymerase treatment, the pET Ek/LIC 51a vector was annealed to the treated PCR product of interest. 1 μ l of Ek/LIC vector (50 ng/ μ l) and 2 μ l of T4 polymerase treated Ek/LIC insert (0.02 pmol), were incubated for 5 min at 22°C. 1 μ l of 25 mM EDTA was added to the reaction, and was further incubated for 5 min at 22°C. Up to 1 μ l of annealed reaction was used in a transformation procedure.

2.5.4 Preparation of Competent Bacterial Cells

Preparation of competent cells was based on the protocol of Sambrook *et al.*, (1989). The *E. coli* strains (Table 2.5) for transformation were streaked from a frozen stock onto agar plates and a single colony was used to grow a 10 ml overnight LB broth. 8 ml of the overnight culture were inoculated into 200 ml LB broth in a 500 ml conical flask. The culture was incubated at 37°C in a thermal shaker (C25 incubator shaker, New Brunswick Scientific or Multitron® H7 Infors AG CH-4103, Bottmingen, Switzerland) at 220 rpm until the cell density reached $OD_{600} = 0.6$ as measured on a spectrophotometer (Biochrom). The culture was chilled on ice for 10 min. The cells were pelleted by centrifugation at 2,500 rpm at 4°C for 8 min (Centra CL3 centrifuge, Thermo IEC). The medium was decanted and the cells were resuspended in 25 ml of 50 mM $CaCl_2$ and centrifuged at 2,500 rpm for 8 min, before the supernatant was removed. Cells were resuspended in 25 ml of 10 mM NaCl, spun at 2,500 rpm for 8 min, and the supernatant was removed. Cells were incubated in 25 ml of $CaCl_2$ for 30 min on ice to make them competent. Cells were spun for a further 8 min at 2,500 rpm, and resuspended in 50 mM $CaCl_2$ and 15% (w/v) glycerol.

The cells were transferred in aliquots of 250 µl into pre-chilled sterile Eppendorf tubes and frozen immediately. The tubes containing the cells were stored at -70°C for later use.

Table 2.5. Strains of *E.coli* used in the study.

<i>E. coli</i> strain	Genotype	Reference
JM109	F ⁺ , <i>TraD36</i> , <i>proA</i> ⁺ , <i>proB</i> ⁺ , <i>lacI</i> ^q , <i>lacZ/recA1</i> , <i>endA1</i> , <i>gyrA96</i> , <i>Nal</i> ^r , <i>thi</i> , <i>hsdR17</i> , <i>supE44</i> , <i>relA1</i>	Sambrook <i>et al.</i> 1989
BL 21 DE3	<i>hsd S gal (λcIts857 ind1 Sam7 nin5 lac UV5-T7 gene1)</i>	Studier and Moffatt 1986

2.5.5 Transformation

The procedure described by Sambrook *et al.* (1989) for transformation of bacteria was used. The competent cells were thawed on ice and were gently mixed by inversion. 200 µl aliquots were transferred into pre-chilled 1.5 ml Eppendorf tubes. 10 µl of the ligation reaction mixture (see ligation reaction) were added to the competent cells, mixed gently and then incubated on ice for 5 min. The tubes were then placed in a water bath at 42°C for 45 s, to heat-shock the cells and then immediately chilled on ice for 3 min. To this, 0.9 ml of LB broth (Sigma-Aldrich), preheated at 37°C, was added and then incubated in a thermal shaker at 37°C, 220 rpm for 45 min (C25 incubator shaker, New Brunswick Scientific or Multitron® H7 Infors AG CH-4103 Bottmingen, Switzerland). Dilute and 10x concentrated reaction volumes were plated

onto agar plates containing 100 (pLEW100 and pET21a vectors) or 50 (Ek/LIC vector) µg/ml ampicillin or 30 µg/ml kanamycin (pET28b vector). Plates were incubated inverted at 37°C overnight.

2.5.6 Identification of Recombinant Clones

Clones were picked and spotted on agar plates and emulsified in 20 µl of deionised water, and denatured at 95°C for 5 min (M.J. Research Inc., PTC-100 thermo cycler). 1 µl of the denatured DNA clone was amplified with selected primers (T7 Fwd and T7 Rev, and pLEW100 Fwd and Rev primers, table 2.1, 2.2) to show presence of insert. Positive clones were prepared and restriction digests were performed. Results were also confirmed by sequencing (MWG Biotech, GENE Crust, Poitiers cedex, France using the T7 forward or pLEW forward primer to check the reading frame, <http://www.mwgbiotech.com/html/all-/index.php>).

2.5.7 Glycerol Stocks

Glycerol stocks of the positive recombinant clones were made using a 5 ml over night culture, which was centrifuged at 13,000 rpm for 15 min. The supernatant was removed and the pellet was resuspended in 30% glycerol-broth [30% (v/v) glycerol in L.B. broth]. Glycerol stocks were fast frozen, dipped in dry ice and ethanol, and stored at -70°C for future use.

2.6 Electrophoresis

2.6.1 Conventional Agarose Gel Electrophoresis

Conventional agarose gel electrophoresis was used to fractionate various nucleic acid fragments that included restriction enzyme digests of genomic DNA, recombinant plasmid DNA and PCR products. The agarose gels were prepared with 1x TAE [40 mM Tris-acetate, 1 mM EDTA, pH 8.0]. The gels were prepared by boiling agarose (Pronadisa, Conda laboratories) in 1x TAE containing 0.5 µg/ml ethidium bromide. Gels were placed in an appropriate horizontal electrophoresis tank (Thermo EC) containing sufficient 1x TAE buffer. The samples were mixed with 6x gel loading buffer [1 x TAE, 50% glycerol, 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol] before loading into the wells. Gels were run at a constant voltage (usually 120 V) using a Thermo EC105 power supply source. The location of nucleic acid fragments within the gels was determined by UV illumination (BioRad gel documentation system) and bands were compared with size markers (1 kb DNA ladder, Promega) to determine fragment size.

2.6.2 SDS PAGE Gels

SDS-12% Polyacrylamide gels were prepared. The resolving gel consisted of 2.5 ml 4x resolving gel buffer for protein separation (1.5 M TrisHCl, pH 8.8, 0.4% SDS), 3.5 ml dd H₂O, 4.0 ml AA/bis acrylamide (Protogel, National diagnostics), 50 µl 10% ammonium persulfate and 5 µl N,N,N',N'-Tetramethylethylenediamine (TEMED). The stacking gel consisted of 1.25 ml 6x stacking gel buffer (0.5 M TrisHCl, pH 6.8,

0.4% SDS), 3.2 ml dd H₂O, 0.5 ml AA/bis acrylamide, 100 µl 10% ammonium persulfate, 10 µl TEMED.

20 µl of eluted purified protein fractions were mixed with 5x loading dye [56% volume/volume (v/v) glycerol, 11% weight/volume (w/v) of 10% SDS, 2.8% (v/v) β-mercaptoethanol, 1.4% of 0.5% (w/v) bromophenol, 7% (v/v) 0.5 M TrisHCl, pH 6.8]. Samples were denatured, boiled at 95°C for 5 min, cooled to 4°C for 5 min, and centrifuged quickly on the minifuge.

Prepared samples were loaded onto SDS-12% PAGE gels. Samples were compared with a 220 kDa bench mark ladder (PEQ lab, bench mark protein ladder) and run at 150 V on the electrophoresis apparatus (Consort E815) in a running buffer [1x running buffer: 14.4 % (w/v) glycine, 3% (w/v) Tris base, 1% (w/v) SDS] for ~75 minutes.

Polyacrylamide gels (Invitrogen, Dublin, Ireland, NuPAGE® 12% Bis-Tris gel, 1.0 mm x 12 well) were also used at 200 V on the electrophoresis apparatus in a running buffer [10% SDS, 5% 20x 3-N-morpholino propanesulfonic acid; 4-Morpholinepropanesulfonic acid (MOPS)] for ~45 minutes.

2.6.3 2-D Electrophoresis

Two-dimensional electrophoresis was carried out according to Reiber *et al.* (2005), with the following changes. Protein samples were precipitated with acetone, as follows: 4x volume of ice cold 100% acetone was added to the sample. Sample was treated vigorously on a vortex, before incubation in freezer for 2 hours. Samples were sedimented on the centrifuge at 4°C at 14,000 rpm for 15 min (Mikro 200R). A white

pellet was produced, and the supernatant was discarded. The pellet was dried in a Speedivac® for 5 min (Heto).

Pellets were resuspended in 250 µl IEF buffer [30°C in 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate (CHAPS) buffer (7 M urea, 2 M thiourea, 2% CHAPS, 2% ampholytes 3–10 (Amersham, Bucks, UK), 65 mM DL-Dithiothreitol (DTT), 0.1% bromophenol blue)] and loaded onto immobilized pH gradient (IPG) strips (Amersham, 7 cm, pH range 3–10) for isoelectric focusing (IEF). Following the initial conditioning step, voltage ramping was set at 2,000 volts for 2 hours, 3,500 volts for 2 hours and a further 3,500 volts for 1 hour. A final step of 200 volts for 10 hours was used to keep the sample intact.

Treated IPG strips were incubated with solution 1 for 10 min [IPQ buffer (6 M urea, 375 mM Tris/HCl pH 7.4, 2% SDS, 2% glycerol), 2% (w/v) DTT], followed by solution 2 [IPQ buffer, 2.5% (w/v) iodoacetamide, bromophenol blue crystals] for 10 min.

Second dimension SDS-PAGE gels were prepared using double the quantities that are previously described, as 2 mm thick bioRad gel plates were used. Gels were prepared only from resolving gel components. Each strip was placed onto the gel, the protein ladder (PEQ labs) was loaded, and strip was overlaid with 1.5% agarose (Pronadisa, Conda laboratories) containing bromophenol blue. Gels were run as previously described (section 2.6.2) a longer running time of 1 hour 30 min was used.

2.6.4 Coomassie Blue Staining of SDS-PAGE Gels

Gels were stained in Coomassie blue stain [1% (w/v) coomassie blue, 45% (v/v) methanol, 10% (v/v) glacial acetic acid; filtered] for 2 h or overnight while agitating

on a rocker (Bellco technology), and destained in 25% (v/v) methanol, 2% (v/v) glacial acetic acid.

2.6.5 Colloidal Blue Staining of SDS-PAGE Gels

Gels were stained according to Neuhoﬀ *et al.* (1988). Gels were fixed for 1-4 days in 50% (v/v) ethanol, 3% (v/v) orthophosphoric acid. Gels were washed three times in ddH₂O, and equilibrated for 1 hour in Neuhoﬀ's solution [16% (w/v) ammonium sulphate, 25% (v/v) methanol, 5% (v/v) orthophosphoric acid]. 1 g of Coomassie blue (CBB) G-250 was sprinkled into solution, and the gel was stained for 2-3 days.

2.6.6 Silver Staining of Gels

SDS-PAGE gels were silver stained based on the Amersham protocol (wolfson.huji.ac.il/purification/PDF/Stains/PHARMACIA_SilverStainKit.pdf) with the following deviations. Gels were incubated in silver stain destain [100 mM sodium thiosulfate, 13 µM Potassium ferricyanide] for 5 min, and washed vigourously in ddH₂O. Gels were then fixed in 40% (v/v) ethanol, 10% (v/v) acetic acid for 30 min or overnight. Gels were then washed in 50% (v/v) methanol for 10 min, followed by ddH₂O for 10 min. Gels were then sensitised for 25 min in 30% (v/v) ethanol, 6.8% (w/v) sodium acetate, 0.2% (w/v) sodium thiosulfate, 0.125% (v/v) glutaldehyde. Gels were subsequently rinsed in ddH₂O, washed for 5 min with ddH₂O, rinsed ddH₂O again and washed for 3 min in ddH₂O. Gels were stained for 30 min in 0.25% (w/v) silver nitrate. Gels were then rinsed ddH₂O, washed for 5 min with ddH₂O, rinsed ddH₂O again and washed for 3 min ddH₂O. Gels were developed in 2.5% (w/v)

sodium carbonate, 0.015% (v/v) formaldehyde. Developing was stopped by addition of 5% acetic acid. Gels were preserved in 30% (v/v) ethanol, 4% glycerol.

Gels were also silver stained according to manufacturer's guidelines (Invitrogen, silver staining kit), with the following changes, volumes of 50 ml were used for gel staining instead of 100 ml volumes.

SDS-PAGE gels were gel-dried, following incubation in preserving solution for 1 hour or more, gels by placing onto pre-wetted cellophane and drying according to the manufacturer's guidelines (Heto master jet and gel drier).

2.7 Protein Expression

Protein expression and purification was based on manufacturer's guidelines (Novagen and Qiagen) with the following deviations, to produce an optimised method.

2.7.1 Recombinant Protein Expression (Small Scale)

The protein expression plasmid of interest (Novagen and Qiagen) was transformed into BL21 DE3 competent cells (Novagen) and the bacteria were plated onto appropriate antibiotic plates. A single colony from these plates was picked and emulsified in 5 ml of 2 x YT media (16 g/l tryptone, 5 g/l NaCl, 10g/l yeast extract) and 5 µl of ampicillin (100 or 50 µg/ml) or kanamycin (30 µg/ml) and grown in a thermal shaker (C25 incubator shaker, New Brunswick Scientific) overnight. The starter culture was diluted 1 in 500 to the desired volume and was grown at 37°C until it reached an OD₆₀₀ of 0.6, when isopropyl-beta-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM. The induced culture was grown for another

3-5 h, the OD₆₀₀ readings were taken and compared to noninduced culture. Cultures were centrifuged at 3,500 rpm for 15 min (Thermo IEC, Centra CL3 centrifuge).

2.7.2 Recombinant Protein Purification (Small Scale, Denaturing Conditions)

Small scale purification conditions were based on the manufacturer's guidelines (Qiagen). The pellet was resuspended in lysis buffer [100 mM NaH₂PO₄, 10 mM TrisHCl, 8 M urea, pH 8]. The material was incubated on ice for 30 minutes, and sonicated 6x for 10 seconds at 15-20% intensity (Bandelin Sonoplus sonicator, probe UW 2200). The lysed solution was transferred in the 1.5 ml Eppendorf tubes and was centrifuged at 4°C at 13,000 rpm for 30 minutes (Thermo IEC micromax centrifuge or Mikro 200R). The supernatant was kept and placed in Qiagen 5 ml polypropylene columns, and incubated with 1 ml Ni₂₊ resin (Qiagen) for 45 minutes. The Ni₂₊ prepared column was allowed to stand, and the flow-through was discarded. The column was washed 2 times with lysis buffer, and then washed 5 times in wash buffer [100 mM NaH₂PO₄, 10 mM TrisHCl, 8 M urea, pH 6.3]. The His₆-tagged protein was eluted in three fractions of elution buffer [100 mM NaH₂PO₄, 10 mM TrisHCl, 8 M urea, pH 4.5]. The pellet was incubated in 1 ml lysis buffer, for solubility studies. Samples from all stages of the process were taken to assess the solubility of the protein.

2.7.3 Recombinant Protein Purification (Small Scale, Native Condition-Phosphate Buffer)

Small scale purification conditions were based on manufacturer's guidelines (Qiagen). The procedure was followed as described above, but the buffers differed. The lysis buffer consisted of 50 mM NaH_2PO_4 , 300 mM NaCl, 10 mM imidazole, pH 8, the Wash buffer consisted of 50 mM NaH_2PO_4 , 300 mM NaCl, 20 mM imidazole, pH 8, 1 mM ATP and the Elution buffer of 50 mM NaH_2PO_4 , 300 mM NaCl, 250 mM imidazole, pH 8.

2.7.4 Recombinant Protein Purification (Small Scale, Native Conditions-Tris Buffer)

The procedure was followed as above, but the buffers differed. Lysis buffer and wash buffer consisted of 10 mM TrisHCl, 150 mM NaCl, 10 mM MgCl_2 , 10 mM β -mercaptoethanol, and the Elution buffer of 10 mM TrisHCl, 150 mM NaCl, 10 mM MgCl_2 , 10 mM β -mercaptoethanol, 250 mM imadazole, pH 7.7.

2.7.5 Recombinant Expression of TbArfGAP I and TbArfGAP V (Large Scale)

Novagen plasmids p51 Ek/LIC containing putative genes of interest were transformed into BL21 DE3 competent cells (Novagen) and the bacteria were plated onto ampicillin (50 $\mu\text{g/ml}$) antibiotic plates. A single colony was emulsified in 20 ml of 2 x YT medium [16 g/l tryptone, 5 g/l NaCl, 10g/l yeast extract] and 10 μl of ampicillin (100 mg/ml) and grown in a thermal shaker overnight (C25 incubator shaker, New

Brunswick Scientific). The starter culture was diluted 1 in 500 to the desired volume and was grown at 37°C until it reached an OD₆₀₀ of 0.6, when IPTG (Sigma) was added to a final concentration of 0.5 mM. The induced culture was grown for another 3-5 hours at 37°C (TbArfGAP V) and 28°C (TbArfGAP I). The OD₆₀₀ readings were taken and compared to uninduced culture. Cultures were placed into pre-cooled centrifuge tubes for the 3500 Sorvall. Cultures were centrifuged at 6,500 rpm for 30 min at 4°C (RC 5C Plus, Sorvall). Pellets were stored at -20°C overnight, and purified the following day.

2.7.6 Purification of Recombinant TbArfGAP I and TbArfGAP V (Large Scale)

The pellet, as described in section 2.7.5, was thawed on ice, and resuspended in 10-30 ml (300 ml culture is lysed in 10 ml lysis buffer) of native Tris lysis buffer (section 2.7.4). The culture was lysed on ice for 30 minutes, and sonicated at 15-20% (Bandelin Sonoplus sonicator, probe UW 2200) 6 times for 20 seconds, until viscosity was reduced. The lysed solution was transferred to SS34 tubes and centrifuged at 4°C at 15,000 rpm for 30 minutes (RC 5C Plus, Sorvall). The supernatant was reserved for further purification. The pellet was preserved to test protein solubility, and the pellet was lysed in a 5 ml of lysis buffer for analyses.

10 ml of supernatant were placed in Qiagen 5 ml polypropylene columns, and incubated with 0.5 ml Ni₂₊ agarose resin (Qiagen) for 30 min to 4 hours. The column was allowed to stand, and the flow-through was discarded. The column was washed 3 times with 10 ml native Tris wash buffer, and then washed once in 10 ml native Tris wash buffer. The bound protein was eluted with one 2 ml elution, and two 1 ml elutions with native Tris elution buffer. A solution of 0.5 M NaOH was used to clear

remaining proteins from the Ni₂₊ resin before the column was washed in 10 ml H₂O, and stored in 30% (v/v) ethanol. Samples from all stages of the process were taken to optimise the purification procedure. The His₆-tag purification step was repeated, to further purify the product.

2.7.7 Use of Millipore Amicon Membranes

Following the initial His₆-tag purification step, it was found that the recombinant protein was not as pure as required, therefore another procedure was undertaken to increase the purity of the fraction. An Amicon ultra-4® centrifugal filter device (Millipore) was utilised to exclude all proteins above 50 kDa in molecular weight. The centrifugal device was washed with Tris wash buffer. 4 ml of the desired fraction was loaded onto the filter and it was spun in 4,000 rpm at 4°C (Thermo IEC, Centra CL3 centrifuge). Protein <50 kDa flowed through the filter, >50 kDa proteins remained in the retentate. This enabled the elution of a purer recombinant fraction, and removal of unwanted larger proteins.

2.7.8 Recombinant Co-Expression Studies

Based on the method used in Mossessova *et al.* (1998), co-expression of two plasmids with different antibiotic resistance markers was carried out. The plasmids were transformed into competent BL21 DE3 cells, and plated onto ampicillin (100 µg/ml) and kanamycin (30 µg/ml) plates. Resistance colonies were picked, and inoculated into larger cultures as described in section 2.7.1.

2.7.9 Desalting Samples using PD 10 Columns (2.5 ml Volumes)

Following purification procedures, the eluted proteins contained 250 mM imadazole, had to be desalted for future use. Elutants were rebuffered in native Tris buffer (detailed in section 2.7.4) using PD10 columns according to manufacturer's guidelines (GE Healthcare).

2.7.10 Desalting Samples using Nap 10 Columns (1 ml Volumes)

Elutants were rebuffered in native Tris buffer using Nap10 columns according to manufacturer's guidelines (GE Healthcare).

2.8 Recombinant TbArf1 Protein Interaction Assay

Ni₂⁺-NTA® His₆ bind resin (Novagen) was packed into a 1 ml Qiagen column. The beads were equilibrated with 10 ml native Tris buffer [10 mM Tris, 20 mM NaCl, 10 mM MgCl₂, 10 mM β -mercaptoethanol, pH 7.8]. The stopper was placed into position, and 300 µg of recombinant TbArf1 (wild type or mutant, depending on the experiment) was loaded onto the column. The cap was placed onto the column, and the column was incubated on a rocker for 4-24 hours at 4°C.

Potential signalling factors were added to the column several hours after loading with recombinant TbArf1. Prostaglandins D₂, E₂, F₂, cAMP, cGMP, were all added to column in 10x molar excess quantities of recombinant TbArf1. Signalling factors and Arf1 were incubated at 4°C on a rocker overnight.

Trypanosome protein lysates were produced using 4×10^8 trypanosomes per 300 μg recombinant TbArf1. Trypanosome pellets were produced from bloodstream form 328.114 *T. brucei* cells, and frozen at -70°C for future use. Pellets were thawed, and lysed in protein lysis buffer on ice for 1 hour [25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 50 mM NaCl, 1% (v/v) CHAPS, 30 $\mu\text{g}/\text{ml}$ leupeptin, 0.2 mM phenylmethanesulphonylfluoride (PMSF), 20 μM (2S,3S)-3-(N-{{(S)-1-[N-(4-guanidinobutyl)carbamoyl]3-methylbutyl}carbamoyl}oxirane-2-carboxylic acid (E-64), 50 μM Na-Tosyl-Lys-chloromethylketone·HCl (TLCK), 1 mM EDTA]. Lysed protein pellets were spun at 15,000 rpm for 30 min at 4°C (Mikro 200R) and the supernatant was used in the following steps.

Arf1 was allowed to flow through column, and was washed in two 2 ml volumes of low salt buffer [10 mM TrisHCl, 10 mM MgCl_2 , 20 mM NaCl, pH 7.5]. Trypanosome protein lysates were added to the columns and incubated on a rocker for 1 hour at room temperature.

The lysate was allowed to flow through, and the column was washed twice with 2 ml low salt buffer. The column was then washed with 4x 2 ml volumes of high salt buffer [10 mM TrisHCl, 500 mM NaCl, 10 mM MgCl_2 , pH 7.5]. Proteins were eluted in 3x 1 ml 250 mM imidazole buffer [10 mM TrisHCl, 150 mM NaCl_2 , 10 mM MgCl_2 , 10 mM β -mercaptoethanol, 250 mM imidazole, pH 7.7]. The resin was treated with 0.5 M NaOH on a rocker at room temperature, before the columns were treated with 10 ml H_2O , and stored in 30% (v/v) ethanol.

2.9 Tomato Lectin Based Interaction Assay

T. brucei 328.114 bloodstream form cells, and cells transfected with ESAG10 pLEW100 overexpression plasmid (both induced and uninduced cells) were lysed, and protein was extracted as described below. Surface protein was isolated using agarose tomato-lectin containing beads. As described below protein extracts were run on an SDS-10%PAGE gels, and expression of surface proteins was compared.

4×10^8 cells were lysed as in section 2.8 for 1 hour on ice. The lysate was spun in a centrifuge at 15,000 rpm for 30 min at 4°C (Mikro 200R); the supernatant was removed and incubated with 100 µl of tomato-lectin agarose beads (Vector labs, Peterborough, UK) for 1 h at room temperature. The supernatant was then spun in a centrifuge at 15,000 rpm for 10 min at 4°C, and the supernatant was again removed. The pellet was washed in 500 µl of Tris buffer [10 mM TrisHCl, 10 mM MgCl₂, 20 mM NaCl, pH 7.5] (to remove non-tomato lectin binding proteins), and spun again in a centrifuge at 15,000 rpm for 10 min at 4°C. The washing step was repeated twice more. The resulting pellet was resuspended in 40 µl of Tris buffer, and run on a SDS-10% PAGE gel as described previously.

2.10 Trypanosome Culturing Techniques

Culturing media were prepared based on the TrypanoFAN protocols (www.trypanofan.org).

2.10.1 HMI-9 Medium

Bloodstream form trypanosomes require a rich medium such as HMI-9 for growth. One portion of HMI-9 medium powder [Sigma, 90.8 g containing 0.005 mM bathocuproine, 1.50 mM L-cysteine, 1.00 mM hypoxanthine, 1.00 mM sodium pyruvate, 0.16 mM thymidine] makes up 5 litres of medium. To complete the medium 15.12 g NaHCO₃, and 71.15 µl 2-mercaptoethanol were added to 4.5 l ddH₂O. The medium was mixed vigorously and the pH was adjusted to 7.5.

50 mg of Pen/Strep (Gibco, Dublin, Ireland), and G-418 (Sigma, 25 µg/ml) and 100 ml of thawed foetal bovine serum (BioSera, heat inactivated at 56°C for 30 min) were added to 1 l of medium before filter sterilisation (0.22 µm filters, Sarstedt, Nümbrecht, Germany). The medium was stored at 4°C until use.

2.10.2 SDM-79 Medium

SDM-79 powdered medium was acquired from Sigma. To complete the medium, 2 g/l sodium hydrogen carbonate and 10 % foetal bovine serum (Jenna Biosciences) were added, and the pH was adjusted to 7.3.

2.10.3 Culturing Cells

T. b. brucei bloodstream form 328.114 cells (acquired from Derek Nolan, Dept. of Biochemistry, Trinity College Dublin) were cultured in T25, T40 and T400 flasks at 37°C, 5% CO₂, with HMI-9 medium until they reached 2 x 10⁶ cells/ml (as counted by a haemocytometer). (G-418 at 2.5 µg/ml concentration was required to keep the

strain from reverring back to wild type strain, losing the plasmid that allowed for the necessary machinery for the transfection to work).

T. b. brucei procyclic form 2930 cells (acquired from Derek Nolan, Dept. of Biochemistry, TCD) were cultured in T25, T40 and T400 flasks at 27°C, with no additional CO₂, in SDM-79 medium, keeping growth between 5 x10⁵ and 1 x10⁷ cells/ml. (Hygromycin [25 µg/ml], and G-418 [7 µg/ml] were required to keep the strain from reverring back to wild type strain, losing the plasmid that allowed for transfection).

2.11 Prostaglandin Response Assays

The response of bloodstream form *T. b. brucei* 328.114 cells to prostaglandins was measured as described by Figarella *et al.* (2005). Prostaglandins were resuspended in sterile HMI-9 medium and added to the trypanosome cells. Prostaglandins D₂, E₂, and F₂ (PGD₂, PGE₂, PGF₂) were added to the cells in 2.5, 5.0 and 7.5 µM concentrations; PGE₂, PGF₂ were also added in 100, 200 and 300 µM concentrations. Based on assays by Vassella *et al.* (1997), cAMP and a control cGMP were added in 1, 2, and 4 mM concentrations. The effects of the prostaglandins, cAMP and cGMP on the cells were noted using daily cell counts of the cells with a haemocytometer.

2.12 Trypanosome Transfection Procedure

Transfection of trypanosomes was carried out based on the method of Wirtz *et al.* (1999).

2.12.1 Preparation of Cytomix

Cytomix is required by trypanosome cells to buffer the electroporation process. A stock cytomix solution was made, containing 2 mM Ethylene-bis(oxyethylenenitrilo)tetraacetic acid (EGTA) (pH 7.6), 120 mM KCl, 0.15 mM CaCl_2 , 10 mM $\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$ (pH 7.6). Subsequently, less stable components: 25 mM HEPES, 5 mM MgCl, 0.5% (w/v) glucose, 100 $\mu\text{g/ml}$ BSA (defatted), 1 mM hypoxanthine, 2 mM ATP, 5 mM glutathione were added to the stock solution upon use. The cytomix solution was filter sterilised (Millipore 0.22 μm) before use.

2.12.2 Transfection

Bloodstream form 328.114 cells were cultured to a density of $2\text{-}3 \times 10^7/\text{ml}$. The culture was centrifuged at 1500 rpm for 5 min (Thermo IEC, Centra CL3 centrifuge), and supernatant was siphoned off. Cells were washed once in 10 ml volume of cytomix. The cells were counted, and cell density was adjusted with cytomix to give $3 \times 10^7/\text{ml}$. 10 μg of *NotI* digested plasmid DNA of interest was placed in an electroporation cuvette, and 0.4 ml of cells resuspended in cytomix were added. The settings for electroporation were 1.4 kV and 25 μF . Cells were removed from the cuvette and added to 36 ml of complete HMI-9 medium and incubated at 37°C in the incubator overnight (Nuaire DH Autoflow CO_2 air-jacketed incubator).

10-14 hours later, 36 μl of 1000x phleomycin (2.5 $\mu\text{g/ml}$) were added and the cells were aliquoted into a 24 well plates and incubated for 7-14 days. As the overexpression plasmid encoded phleomycin, clones which had taken up the overexpression vector were able to grow in the presence of phleomycin.

2.12.3 Assessing Effectiveness of Transfection

The effectiveness of the electroporation was assessed by comparing pre- and post-electroporated cells. The percentage death of the trypanosome cells was calculated. 70% - 90% of the cells must die in the electroporation process for the procedure to be successful.

2.12.4 Selecting a Clone

Phleomycin resistant growth was expected in 1-5 of the 24 wells, within 7 days, for successful transfection. In the presence of trypanosome growth (live, motile cells), clones were monitored and subsequently subcultured in complete HMI-9 medium with continued selection with phleomycin. Once successful growth had been achieved, aliquots of cells were frozen down, and tetracycline induction (section 2.13.5) was carried out.

2.12.5 Tetracycline Induction

Tetracycline induction allowed for expression of the cloned pLEW100 plasmids in the *T. b. brucei* 328.114 cells. Growth of trypanosome cultures was followed over a period of 6-7 days to monitor the effect of induced versus uninduced cultures, based on cell density. Effective expression of the deleterious overproduced protein was expected to reduce cell growth as compared to cells not expressing protein.

2.12.6 Making Liquid Nitrogen Stocks

Bloodstream forms were grown to a cell density of 1×10^6 cells/ml, spun at 1,500 rpm for 5 min (Thermo IEC, Centra CL3 centrifuge) before the supernatant was siphoned off. Cells were resuspended in HMI-9 freezing buffer (20% FCS (foetal calf serum), 30% glycerol in HMI-9 medium). Cells were aliquoted in 1.5 ml cryotubes, and wrapped up in gloves and tissue for slow freezing at -70°C before being transferred into liquid nitrogen 8-12 hours later, for long term storage. For procyclic cells, SDM-79 medium was used instead of HMI-9 medium.

2.13 Microscopy Procedures

2.13.1 Slide Preparation

Premium microscope slides (Ultima, 76 x 26 mm) were coated with poly-L-lysine (10 μl) and air-dried overnight. Bloodstream form cells were grown to a density of 10^6 cells/ml, they were spun at 1,500 rpm for 5 min (Thermo IEC, Centra CL3 centrifuge), and the supernatant was siphoned off. Cells were resuspended in sterile PBS (Sigma-Alrich), spun at 1,500 rpm for 5 min, and supernatant was siphoned off. Cells were resuspended in PBS so that 100 μl of PBS contained 10^4 cells. Wax circles were drawn on the slides, and 100 μl of resuspended cells were placed on the slides. Slides were air-dried for 10 min to allow adherence of the poly-L-lysine, excess PBS was removed and the slides were air-dried. Slides were incubated in 100% ice-cold methanol at -20°C for 5 min and subsequently air-dried. Slides were wrapped in tin foil and stored at -20°C until required.

2.13.2 DAPI Staining

A 20 mg/ml solution of 4',6-diamidino-2-phenylindole (DAPI Sigma) was prepared, and diluted 1 in 200 in 1x TBS [10 mM TrisHCl, 15 mM NaCl, pH 7.5]. Prepared slides were placed at room temperature and incubated with a 50 µl drop of DAPI in the dark for 5 min. The slides were rinsed with PBS and tapped dry. 5 µl of Vectashield® (Vector Labs) mounting medium was used, and overlaid with a coverslip.

2.13.3 Tomato Lectin staining

A 1 mg/ml solution of Texas Red *Lycopersicon esculentum* tomato-lectin (Vector Labs) was diluted 1 in 200 in 1x TBS. Prepared slides were thawed and incubated with 50 µl drop of tomato-lectin in the dark for 30 min. The slide was then rinsed with PBS and tapped dry. 5 µl of Vectashield® (Vector Labs) mounting medium was used, and overlaid with a coverslip.

2.13.4 Immuno Fluroescent Histochemistry

Slides were thawed at room temperature, equilibrated with 1x TBS and the excess removed. Slides were blocked in a 1 in 5 dilution of blocking serum [20% goat serum, Dako, Dublin, Ireland in 1x TBS] for 30 min at room temperature. Slides were incubated in a 1 in 50 dilution with the primary antibody [Arf1 antibody, BT164 (not commercially manufactured, produced by Dr. Noel Murphy, referenced: Murphy and

Olijhoek 2001)) in 1x TBS overnight at 4 °C. Slides were washed with 1x TBS-T [1x TBS + 0.1% Tween] and rocked for 3 min. This step was repeated twice more. The slides were incubated with a 1 in 50 dilution of the secondary antibody (fluorescein-labelled affinity purified antibody to bovine IgG) in 20% goat serum for 30 min in at room temperature in the dark. The previous washing step was repeated. Slides were mounted with 5 µl of Vectashield®, and overlaid with a coverslip.

2.13.5 Microscopy

Slides were viewed under 40x power using an Olympus BioSystems illumination system MT20. Cell and Cell software 2.1 (Olympus) was used to visualise and record the image. Images were superimposed on one another using this programme.

Chapter 3: Identifying the TbPP69

Receptor/Transporter Involved in *T. brucei* Differentiation

3.1 Summary

T-GIF is an external signalling factor that is released by long slender bloodstream form *T. b. brucei* cells which causes differentiation into short stumpy cells. Though both pleomorphic (cells capable of differentiation from long slender to short stumpy bloodstream forms) and monomorphic cells (cells incapable of this differentiation process) are able to produce T-GIF, it is thought that a loss or deficiency of a receptor or transporter renders the monomorphic cells unable to sense T-GIF (Vassella *et al.* 1997). Examination of this theory in more detail via a comparison of tomato lectin binding proteins of pleomorphic and monomorphic cells showed that there was an abundant protein of 69 kDa in pleomorphic cells that was significantly less abundant in monomorphic cells (Murphy and Olijhoek 2001). This 69 kDa protein referred to as TbPP69, was proposed to be the T-GIF receptor/transporter.

An expression site associated gene, *ESAG10*, was investigated as a candidate for TbPP69 transporter protein. *ESAG10* was chosen as a candidate for TbPP69 as it conformed to the criteria for TbPP69 as it is predicted to be a transmembrane protein, with a predicted glycosylation site and a predicted N-signal cleavage site. *ESAG10*

also had sequence similarity to a transporter, BT1, which has been implicated in differentiation in *Leishmania donovani* (Lemley *et al.* 1999). This ESAG10 protein was overexpressed and a number of assays were carried out to assess what impact this had on the cell. Overexpression of ESAG10 resulted in greater expression of a protein that migrated by SDS-PAGE similarly to the TbPP69 protein, and also altered the location of a protein with suggested involvement in differentiation, TbArf1. This suggested that ESAG10 was likely to be the TbPP69 receptor that facilitated the differentiation event.

3.2 Investigating the TbPP69 Receptor/Transporter

Differentiation of long slender, bloodstream forms of *T. b. brucei* occurs via a cell density dependent mechanism (Vassella *et al.* 1997). A signalling factor, T-GIF is released and is subsequently taken up by a receptor or transporter protein of long slender cells, initiating differentiation. A surface protein, TbPP69, was previously identified as being upregulated in pleomorphic cells (which are capable of differentiating from long slender to short stumpy forms) and not in monomorphic cells (lab derived cells, which are unable to differentiate) (section 1.1.7, Figure 1.4; Murphy and Olijhoek 2001). T-GIF, an external differentiation factor, was implicated in binding to and internalisation by the TbPP69 surface protein (Murphy and Olijhoek 2001). The uptake of T-GIF was postulated to trigger the differentiation of actively dividing long slender cells to non-dividing short stumpy bloodstream forms (Noel Murphy, personal communication). The identity of the TbPP69 receptor or transporter is currently unknown. In this chapter, I present a series of experiments that were carried out to identify and characterise the TbPP69 receptor/transporter.

The TbPP69 receptor/transporter was proposed to be located on the trypanosome cell surface, to facilitate interaction with the external T-GIF factor. A variant surface glycoprotein (VSG) coat covers the surface of the trypanosome, helping the organism to evade immune attack. Other surface proteins are located in the flagellar pocket, hidden from the immune system (Coppens *et al.* 1988). The TbPP69 receptor/transporter is likely one of these flagellar pocket surface proteins. The abundance of VSGs, however, makes the identification of any other surface receptors or transporters very difficult. Fortunately, a procedure has been developed that allows the purification of surface proteins other than VSGs (Nolan *et al.* 1999).

Surface proteins localised in the flagellar pocket are associated with glycans (linear chains of poly-N-acetyllactosamine, pNAL) (Lee *et al.* 1990), which aid the sorting of membrane proteins in polarised cells of other organisms (Fielder *et al.* 1995; Scheiffele *et al.* 1995; Gut *et al.* 1998; Alfalah *et al.* 1999). Tomato lectin, a very stable glycoprotein isolated from the tomato, *Lycopersicon esculentum*, has been found to bind with high affinity to flagellar pocket surface proteins which contain three or more linear disaccharide repeats of N-acetyllactosamine (Merkle and Cummings 1988). This characteristic of tomato lectin is frequently used to extract surface proteins of interest from the trypanosome (Nolan *et al.* 1999).

The tomato lectin isolation method was used to purify surface proteins of the *T. b. brucei* strain ILTat 1.1 (a pleomorphic strain of *T. b. brucei*). These tomato lectin isolated proteins were subsequently electrophoresed on a SDS 12% polyacrylamide gel and stained with colloidal blue. A dominant protein band of approximately 69 kDa was evident in all preparations (Figure 3.1). This band was implicated in differentiation as it is more abundant in pleomorphic cells, which can undergo differentiation from actively-dividing long slender to non-dividing short stumpy

bloodstream forms, than in monomorphic cells, which cannot (see Introduction section 1.1.7). This evidence correlates well with the hypothesis that a defect in monomorphic strains of *T. brucei*, thought to be due to an alteration in a surface receptor or a transporter, prevents cells from differentiation (Vassella *et al.* 1997). Specifically, both monomorphic and pleomorphic cells are capable of producing the stumpy inducing factor (SIF or T-GIF), but monomorphic strains cannot transmit or interpret cell density information (Vassella *et al.* 1997).

The 69 kDa band was cut from the gel, and sent away for mass spectrometry analysis at the proteomics unit of the University of Victoria, Canada, in an attempt to determine the identity of the TbPP69 receptor/transporter.

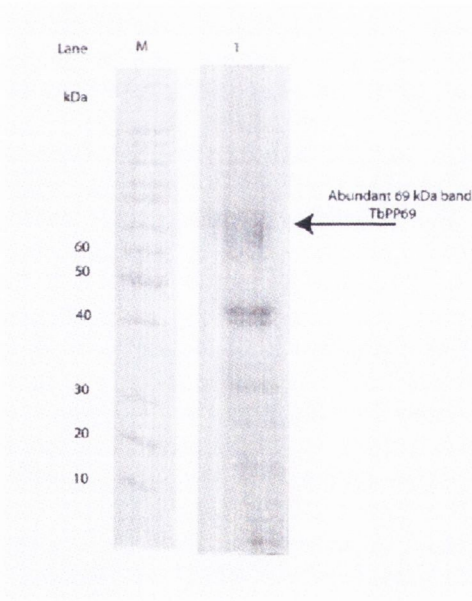


Fig 3.1. Tomato lectin purified surface protein of pleomorphic *T. b. brucei*, ILTat 1.1. Tomato lectin purified proteins from the pleomorphic strain ILTat 1.1 were run on an SDS-12% PAGE gel and stained with colloidal blue. Lane M: protein marker, Lane 1: Tomato lectin protein preparation. There was an abundant protein band located at 69 kDa, as indicated. This was the proposed TbPP69 receptor/transporter protein.

3.3 Twin Mass Spectrometry Analysis of Tomato Lectin

Purified Surface Proteins

A liquid chromatography mass spectrometry-mass spectrometry (LC-MS-MS) approach was used to identify the TbPP69 receptor/transporter protein. This allowed the determination of the amino acid sequence from the starting peptide, which could be subsequently searched against a protein database to identify the protein in question. A high performance liquid chromatography (HPLC) machine is firstly used to separate the digested protein fragments into identical peptides. These peptides were subsequently ionised via an electrospray ionisation step (ESI). The first mass spectrometry stage uses a quadrupole mass filter to select a peptide to a single mass, other peptides were discarded. A collision cell fragmented the peptide into sequential amino acids, and a second time of flight mass filter separates and detects the amino acid composition of the peptide (details of materials and methods: as described Untalan *et al.* 2005, technique reviewed McLafferty and Turecek. 1993, Roepstorff. 1997).

3.3.1 Matches of Peptides Derived from Purified Surface Proteins to the Sanger *T. brucei* Database

Approximately twenty peptide sequences ranging in length from six to twelve amino acids were generated. The derived peptide sequences were individually compared to the *T. b. brucei* Sanger database (<http://www.genedb.org/>) using the OmniBLAST

program. The Sanger OmniBLAST program performed a full database search using the Blosum62 matrix for scoring the protein alignments. In addition, sequences were compared to the NCBI non-redundant database using the BLASTP program (<http://www.ncbi.nlm.nih.gov/BLAST/>) to allow comparison to sequences from other trypanosome isolates. A number of different 'hit' proteins were identified with significant identity to the query sequences.

The 69 kDa band that was excised from the gel and sent away for mass spectrometry analysis appears to have contained several different proteins. Several of the peptide sequence fragments matched to one of the four variants of ILTat 1.4 VSG, a variant surface glycoprotein (Donelson *et al.* 1982; Rice-Ficht *et al.* 1982) (Figure 3.2). There was sufficient coverage to conclude that the ILTat 1.4 VSG protein was one of the proteins present in the 69 kDa band.

It would not be surprising to have had minor VSG contamination in the proteins sent for mass spectrometry analysis given their abundance on the cell surface. In this case, however, there seem to be significant levels of contamination since the VSG appears to be the major protein in the preparation. Why this occurred is not known; it is possible that ILTat 1.4 VSG is sufficiently different from the ILTat 1.1 VSG or is processed differently causing it to bind to the tomato lectin resin.

The tomato lectin technique was designed specifically to exclude isolation of VSG (Nolan *et al.* 1999). VSGs are constantly recycled in *T. b. brucei* cells and therefore VSGs are processed in a different manner to other proteins along the Rab11 pathway (as outlined in the introduction, section 1.1.6). This tomato lectin purification protocol has been optimised to exclude the ILTat 1.1 VSG protein (Nolan *et al.* 1999). ILTat 1.1, VSG appears to have been successfully excluded in this case but there is contamination with ILTat 1.4 VSG. To confirm that *T. brucei* pleomorphic strain

proteins, however, were from a different batch from those used in the RT-PCR results. Therefore, it is possible that the same phenomenon is occurring again in this case: some cells were expressing ILTat 1.4 VSG in a major population of ILTat 1.1 VSG expressing cells and through the tomato lectin purification procedure these ILTat 1.4 VSG expressing cells were being enriched.

3.4 TbPP69 Receptor/Transporter Candidates

3.4.1 A Candidate Approach to Identifying the TbPP69 Receptor/Transporter

Due to contamination with VSG surface proteins the mass spectrometry analysis failed to identify any suitable candidate for the TbPP69 receptor/transporter. As an alternative, a candidate based approach was implemented. Candidates were identified based on several likely characteristics of the TbPP69 receptor/transporter.

The TbPP69 receptor/transporter was originally proposed to be a 69 kDa protein. Following deglycosylation of the protein with the *N*-linked-glycopeptide-(*N*-acetyl-beta-D-glucosaminy)-L-asparagine amidohydrolase (PNGaseF) enzyme, the calculated size of the transporter was 55 kDa (N. Murphy, unpublished). (The TbPP69 preparation was treated with PNGaseF enzyme, and subsequently untreated and treated preparations were run on an SDS-PAGE gel). Enzymatic treatment, however, may not have removed all the sugar groups as some may be buried and inaccessible. Thus, the molecular weight of the mature deglycosylated protein may be less than 55 kDa. Furthermore, as a membrane protein, the TbPP69 receptor/transporter was

expected to contain an N-terminal signal peptide and at least one transmembrane domain.

3.4.2 Expression Site Associated Genes (ESAGs)

Previous studies have shown that *T. brucei* parasite strains are capable of switching from a pleomorphic phenotype to a monomorphic phenotype (Barry and Turner 1991), a process which can involve switching of VSG expression site (see introduction, chapter 1). For instance, ILTat 1.1 is a pleomorphic clone that expresses the VSG 1.1 gene (Williams *et al.* 1980), whereas monomorphic strains express alternative VSGs from a different expression site (Matthews *et al.* 1990). Similar observations have been made for the AnTar1 serodeme (E. Pays, personal communication). Furthermore, the serum resistance associated (SRA) gene, which confers human infectivity to *T. b. rhodesiense* (Xong *et al.* 1998), is activated via a switch in expression site. This suggested the possibility that pleomorphism could also be determined by an expression site associated gene (ESAG). Several ESAG genes have been documented in the last two decades (see introduction, section 1.14). Of these, ESAG10 best fits the criteria of the TbPP69 putative receptor/transporter.

3.4.3 ESAG10 as a Candidate for the TbPP69 Receptor/Transporter

ESAG10 is located in the promoter region of an expression site (clone 13J) in *T. brucei* strain 427 variant VSG 221a (Zeng *et al.* 2001; Berriman *et al.* 2002), on chromosome 10 sequenced from *T. brucei* strain 118 (Gottesdiener 1994) and also present on an intermediate-sized chromosome in the *T. brucei* TREU 927 strain

(Berriman *et al.* 2005), where it may be differentially expressed. There are three other sequences with high similarity (92-96% identity) to *ESAG10* found in the Sanger *T. brucei* database (<http://www.genedb.org>). These genes are not associated with expression sites, but are arranged in tandem on chromosome VIII. These genes encode truncated versions of the *ESAG10* sequence, containing a stop codon within the first 60 amino acids of the sequence. These genes appear to encode folate transporters. In addition, there are several more distantly related pteridine transporters within the *T. b. brucei* genome.

ESAG10 possesses many of the requirements of a potential candidate for the TbPP69 receptor/transporter, containing an N-terminal signal peptide, 12 transmembrane domains (Figure 3.3), no GPI-anchorage, and possible surface exposed N-glycosylation sites (to allow tomato lectin to bind to it) (Figure 3.4). The *ESAG10* protein is, however, outside the molecular weight range of the receptor/transporter molecule. *ESAG10* has a predicted molecular weight of 76 kDa (73 kDa when the N-terminal signal peptide is cleaved). Furthermore, glycosylation would be expected to considerably increase the molecular weight. *ESAG10*, as mentioned earlier, has been shown to be related to a biopterin transporter (BT1) in *Leishmania donovani*. BT1 displays anomalous migration in SDS-polyacrylamide gels. BT1 has a predicted molecular weight of 69.7 kDa, but runs on a polyacrylamide gel at 45 kDa (Lemley *et al.* 1999). This anomalous migration of integral membrane proteins on SDS-PAGE gels is not uncommon (Mueckler *et al.* 1985). Since *ESAG10* is closely related to BT1, it is likely that it also displays aberrant migration on SDS-PAGE gels, displaying a smaller molecular weight than predicted. Under those circumstances, *ESAG10* would fulfill all the requirements specified for the TbPP69 transporter of *T. b. brucei*.

NNKVALSFCAEYKVEPKVTVVKTFILVGIYLVMTTSPNACQDQPPQHSAPQAHEAECTTHKLSAEKTM
 DARPVHPDARALFQQLPCVWRIPVFGTAVEAFGPRCVLALGLSELFGKGIAHDMIRTTLFPMFTYTFGA
 DAKLYQRMGGMSSSLGYAMKPFAMFSDLFALFGYTKRWYLALSCVVGSTVAIVYGSLPGELSYVPVAGI
 LVFVMAFTKGILDILTQGHYSRLIRRVPLAGPSLVSWVWVCVLTGSLIASSIVGPLTDKKLQRVAVFIS
 AGIQFVPTIFFIMNWyGERRNREERAYDLTVLRKEQLEREADAAGLQSEETSGSLDNPSDAEEVAEAG
 TRILTCCCGAFEVNREVFARNKKVVFYCMLLTLGAMGMVLVTVLGTQLLITSVLASFTLCGLGFVAL
 PLVIAKANMFTFISRVAYIQLPGAIDNVFMATPDCFPGGPNFSYFYYSTVGNMIGAVGGVIGVTLFRYV
 FSKRSYRLTFIVTTLIEIVSSIFDIIIVERWNRPYVSDHVVFVLGDQIIHQVCYMMHFMPVILISRLC
 PSGYESAVYSVLGCAHFGRSVSNTLGWLLMEYVWNVQSDIKVGPCDFSNVKWLILLVGHFATPLISIPL
 VFLLIPAARLCDVLDENGKAITKEADVRAFSNDSPRMREPTAN

Fig 3.3. Protein sequence of ESAG10 showing the predicted transmembrane domains. The figure above depicts the protein sequence of ESAG10 less the predicted signal sequence, which is cleaved during processing of the protein. The predicted transmembrane domains, indicated with a grey background, were predicted using the THMM program (<http://www.cbs.dtu.dk/services/TMHMM/>, Krogh *et al.* 2001). The possible N-glycosylation sites are underlined.

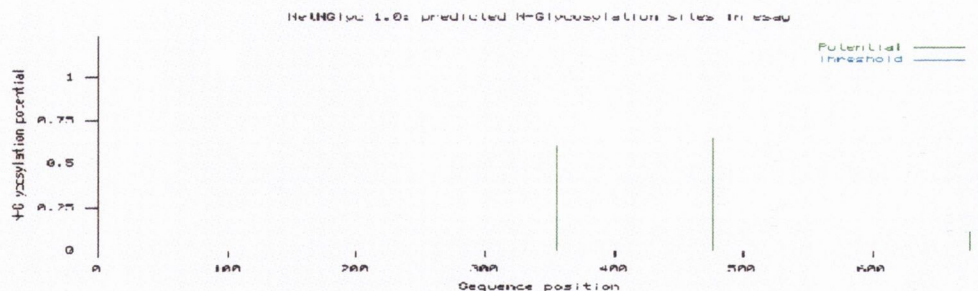


Fig 3.4. ESAG10's predicted N-glycosylation sites. The figure above depicts the predicted ESAG10 glycosylation site at position 355 and 476. Glycosylation sites are shown by vertical green lines, which exceed the threshold level (blue horizontal line). This analysis was performed using the glycosylation prediction program (<http://www.cbs.dtu.dk/services/YinOYang/>) (Gupta *et al.* 1999).

3.4.4 *ESAG10* is Developmentally Regulated

To determine whether the expression of *ESAG10* was developmentally regulated, i.e. differentially regulated depending on the life cycle stage of *T. b. brucei* cDNA was generated from the long slender and short stumpy stages of the bloodstream form, and from the procyclic stage, of *T. b. brucei*. Oligonucleotides were designed to amplify sequences specific to the *ESAG10* gene (and not the *ESAG10*-like genes found on chromosome VIII). To avoid amplification of genomic DNA, a spliced leader forward primer, that only amplifies cDNA, was used. As controls, the cytoskeletal protein genes, α - and β - tubulin, were also amplified. *ESAG10* appears to be developmentally regulated, as its expression was only detected in the short stumpy form (Figure 3.5). Although, the reference genes, alpha and beta tublin, are both more highly expressed in the short stumpy developmental stage are suggesting that *ESAG10* could be present in both of the other developmental stages, long slender and procyclic, but is not visible.

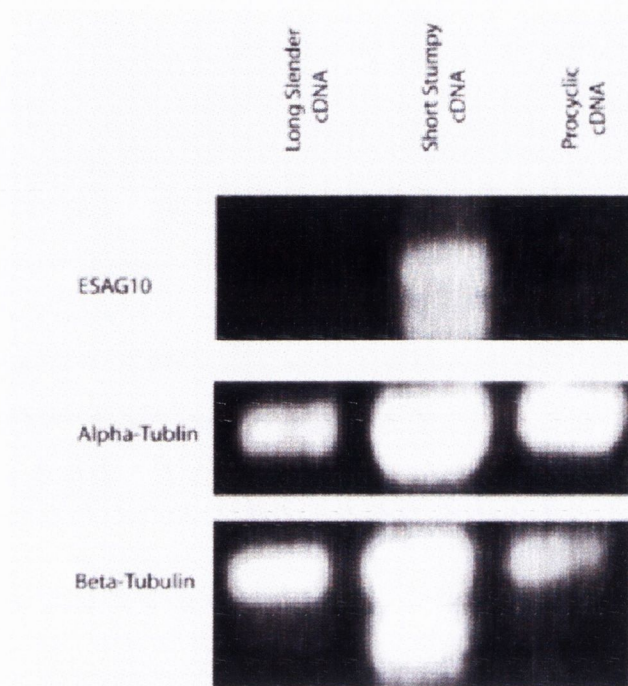


Fig 3.5. *ESAG10* is specifically expressed in the short stumpy form. The above figure depicts a 1% agarose gel displaying RT-PCR results of amplified α -tubulin, β -tubulin and *ESAG10* in long slender, short stumpy and procyclic cDNA. There are two bands apparent in the short stumpy β -tubulin amplified fragment and in the short stumpy *ESAG10* amplified fragment, these may be splice variants.

3.5 Transfection Studies

3.5.1 The Overexpression Plasmid of Choice

The aim of this part of the project was to overexpress *ESAG10*, the candidate TbPP69 transporter, in bloodstream form trypanosomes, to determine its role in *T. brucei* differentiation. Overexpression of the TbPP69 protein should enable the pleomorphic cells to detect the signalling density factor, T-GIF, and differentiate into their short stumpy forms, and eventually die. An overexpression plasmid, pLEW100, has been developed by George Cross and colleagues to allow protein overexpression in the trypanosome (similar to Figure 3.6 without the addition yellow fluorescent protein, Wirtz *et al.* 1999).

The pLEW100 plasmid is based on the T7 promoter and integrates into the ribosomal DNA (rDNA) spacer region of *T. b. brucei* genome. The plasmid contains two antibiotic resistance genes for ampicillin and phleomycin. The ampicillin resistance allows for selection of the plasmid for manipulation and growth. Phleomycin resistance ensures selection of the trypanosomes that have integrated the pLEW100 plasmid into their rDNA spacer region. Addition of tetracycline to the medium induces the expression of the tetracycline-responsive procyclic acidic repetitive protein (procyclic abundant surface proteins, PARP) promoters to drive expression of the selectable marker and gene of interest (Wirtz *et al.* 1999). The plasmid also contains a luciferase open reading frame (ORF), which can be cut out of the plasmid using *HindIII* and *BamHI* restriction enzymes, facilitating the insertion of a gene of choice. The gene encoding *ESAG10*, the TbPP69 candidate, was cloned into this vector for overexpression studies.

3.5.2 The p2216 Plasmid for Transfection Studies

Due to difficulties with the pLEW100 system, mainly the inability to achieve a positive transfection, a second plasmid, p2216, was acquired for use in overexpression studies (donated by Derek Nolan, TCD, constructed by Mark Carrington). This p2216 plasmid is derived from the original pLEW100 plasmid, but possesses an additional open reading frame adjacent to the luciferase ORF. This 740 bp ORF encodes a yellow fluorescent protein (EYFP), which enables the plasmid-bearing cells to be detected using a fluorescent microscope, and aids in the identification of transfected cells. The *ESAG10* gene also was amplified and cloned in this plasmid (Figure 3.6).

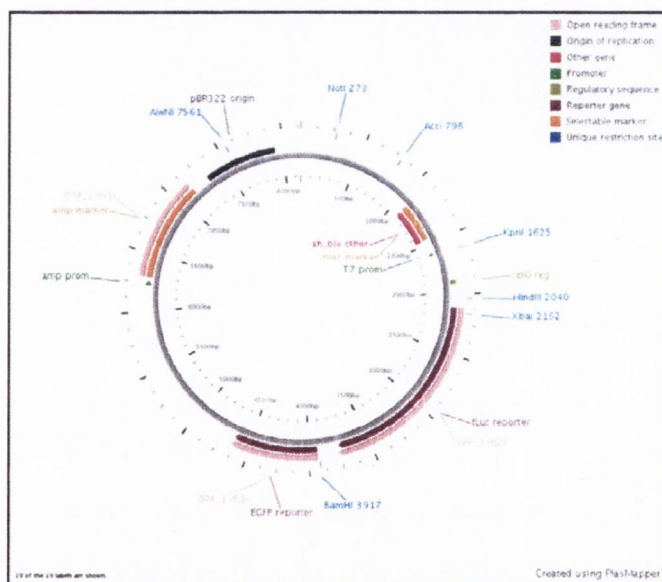


Fig 3.6. Schematic representation of the p2216 plasmid. The yellow fluorescence reporter ORF is depicted by the shorter pink/purple arrows. See figure 3.6 for other details. The figure was generated using ‘PlasMapper’ (Dong *et al.* 2004).

3.5.3 Cloning of the *ESAG10* gene into pLEW100 and p2216 Plasmids

Oligonucleotides were generated for *ESAG10* (Table 2.1). The *ESAG10* gene was successfully amplified and cloned into the pLEW100 overexpression plasmid for transfection studies. The *ESAG10* gene was also cloned into the p2216 vector. PCR products were produced and a restriction digest was carried out to confirm the presence of the *ESAG10* insert (Figure 3.7). As anticipated, the *ESAG10* insert was recovered from *XbaI* and *BamHI* double digests only. Clones were also sequenced by MWG Biotech (Ebersberg, Germany) to ensure that the gene of interest had been inserted into the pLEW100 and p2216 plasmids correctly.

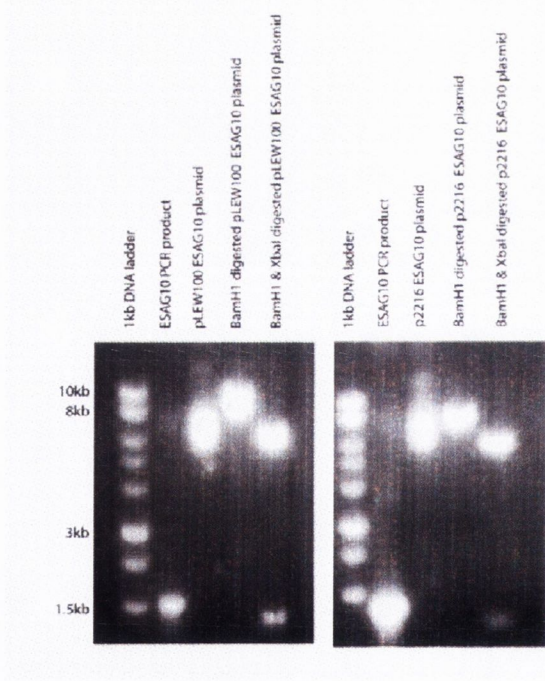


Fig 3.7. Digests to confirm presence of *ESAG10* inserts in the pLEW100 and p2216 vectors. The above figure depicts five lanes of a 1% agarose gel. Note the presence of the *ESAG10* insert at the expected size (1.5 kb) in the *ESAG10* PCR product and in the double digest of the *ESAG10*-bearing pLEW100 and p2216 plasmids.

3.5.4 Transfection with the pLEW100 ESAG10 Overexpression Plasmid

Transfections were carried out using 2×10^7 cells, with the addition of phleomycin to select for positive clones, as described in Materials and Methods. If transfectants were not produced, cells failed to proliferate in the presence of phleomycin and died within the first seven days. It proved difficult to achieve positive results so the protocol was altered in an attempt to optimise the experiment. A series of modifications were made to the protocol to achieve this. First, the cell number was altered. Experiments were carried out with 2×10^6 , 1×10^7 , 2×10^7 , 5×10^7 , 1×10^8 and 2×10^8 cells. It was found that with less than 2×10^7 cells, insufficient numbers survived the transfection procedure, and thus no clones were obtained. With greater than 2×10^7 cells, cells survived the electroporation step in greater numbers, and continued to survive long into the phleomycin selection, such that the possibility of obtaining false positives clones increased.

A second approach was taken to optimise the transfection by altering the quantity of DNA used. Experiments were carried out with 100 ng, 500 ng, 1 μ g, 5 μ g, 10 μ g and 20 μ g of *NotI* digested DNA. Experiments using less than 5 μ g of DNA produced no clones. When greater than 10 μ g of DNA was used, clones were produced.

As a further step, electroporation of the cells was optimised for killing. Cells were pulsed with a single 1.4 kV pulse, a double 1.4 kV pulse and triple 1.4 kV pulse. It was found that the pulse required to produce optimal killing of greater than 90% depended on the number of cells being used. A single 1.4 kV pulse was sufficient to kill greater than 90% of the cells when the recommended 2×10^7 cells were used. In

total, more than forty pLEW100 *ESAG10* transfections were carried out. I was unable to generate a true positive *ESAG10*-transfected clone using the pLEW100 system.

3.5.5 Transfection with the p2216 *ESAG10* Overexpression Plasmid

Transfections were also carried out with *ESAG10* gene inserted into the p2216 plasmid, a variant of the pLEW100 plasmid, as described in the materials and methods section 2.13.2. The transfection procedure was carried out three times using this p2216 *ESAG10* plasmid. A null p2216 plasmid was used as a control. Following the second transfection procedure, cells began to proliferate in a single well, and a positive transfection result was achieved. The transfected cells were subsequently assessed as described in the following section.

It is unclear why the transfection was unsuccessful using the pLEW100 plasmid but was successful with the p2216 plasmid. Other groups have also reported that the pLEW100 plasmid expression system is problematic (D. Nolan, personal communication).

3.6 Analysis of *ESAG10* Positive Clones

3.6.1 Growth Curves to Assess Transfection

To assess whether transfection of the *ESAG10* containing p2216 plasmid was successful, growth curves of the positive clones were produced. Induced and uninduced cells were monitored over five consecutive days and counts were taken on each day, as shown in the growth curves below (Figure 3.8). The induced cells were

expected to grow at a slower rate than uninduced cells, as overexpression of protein would require large amounts of the cell's energy. Figure 3.8 shows, however, equal rates of growth in the induced and uninduced cells. Over production of this protein did not appear to have a deleterious effect on the tetracycline induced cells.

Under stressful conditions, the effect of over-producing a protein in *T. brucei* is more obvious than when cells are not stressed (D. Nolan, personal communication). A second growth curve was undertaken to elucidate whether stressful conditions impacted the growth of induced cells, as compared to uninduced cells (Figure 3.9). To create stressful conditions the amount of serum required in the standard growth medium HMI-9 was lowered from 10% to 5%. Little difference, however, was again observed between the induced and uninduced cells in these conditions.

There were two likely explanations for this. Either the induced and uninduced cells did not produce the ESAG10 protein, or the overexpression system was leaky in uninduced cells. p2216 is known to have a leaky promoter (G. Cross, Laboratory Webpage, <http://tryps.rockefeller.edu/>) and it is possible that the induced cells also expressed the product of the transfected gene. Further investigation was required to ensure that the transfection was successful.

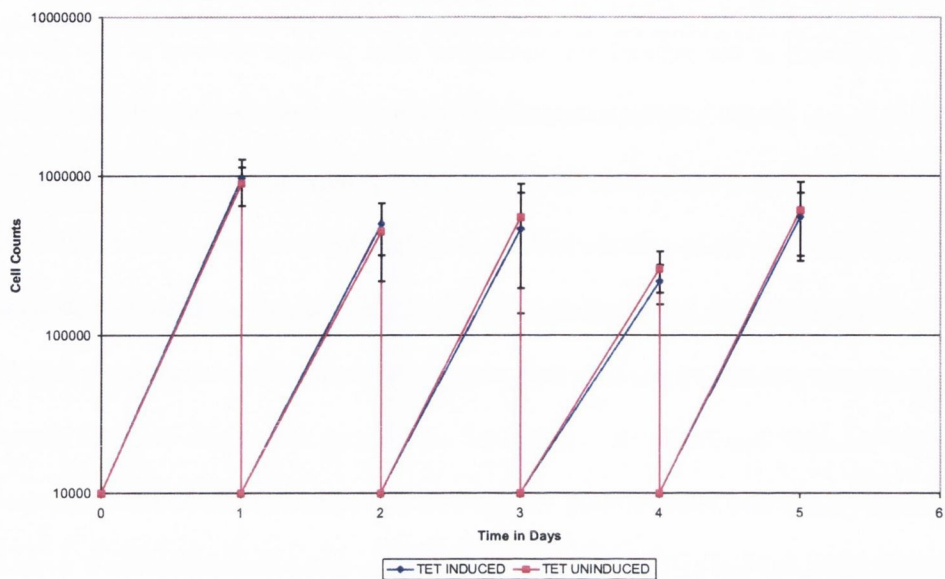


Fig 3.8. Growth curve of induced versus uninduced positive p2216 *ESAG10* clones. The above graph depicts a log growth curve of the tetracycline induced cells (2 $\mu\text{g}/\mu\text{l}$ tetracycline) as indicated by the blue line, versus the cells without addition of tetracycline, as indicated by the pink line. Cell counts were performed daily in triplicate using a haemocytometer. Cells were initially seeded at 10^4 per ml and were subsequently daily re-seeded at 10^4 . Standard error bars are shown.

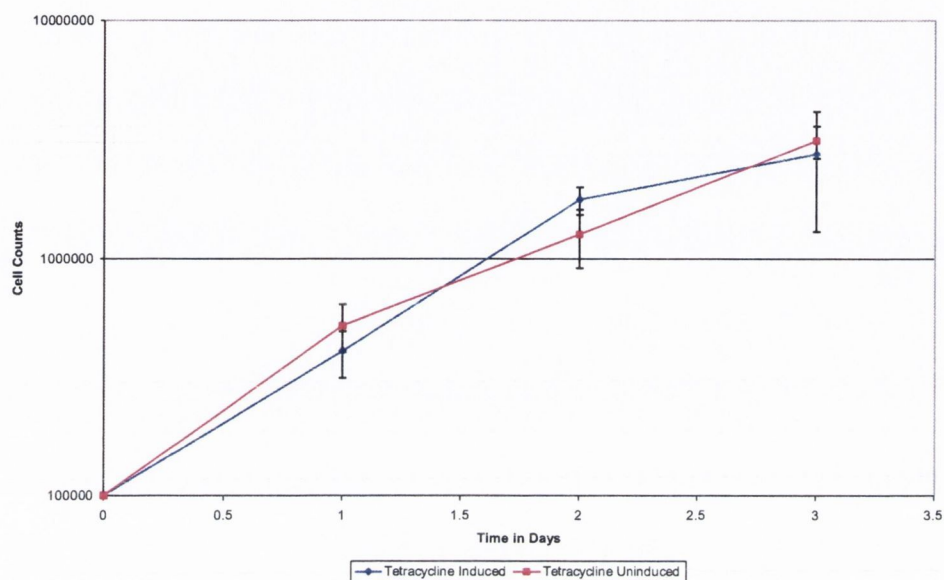


Fig 3.9. Growth curve of induced versus uninduced positive p2216 ESAG10 clones in stressful conditions. The above log graph depicts a growth curve of the tetracycline induced cells as indicated by the blue line, versus the cells without addition of tetracycline, as indicated by the pink line. Cell counts were performed daily in triplicate using a haemocytometer. Cells were initially seeded at 10^5 . Standard error bars are shown.

3.6.2 Confirmation that ESAG10 has Inserted in the *T. brucei* Genome

To show that the *ESAG10* p2216 vector had been correctly inserted into the ribosomal DNA (rDNA) spacer region of the *T. brucei* genome, a series of PCR experiments were carried out as detailed below. The primers were designed as indicated in the figure below (Figure 3.10). The PCR experiment showed that the p2216 *ESAG10* plasmid had been successfully inserted into the rDNA spacer region of the *T. brucei* genome (Figure 3.11).

The gel results show that the pLEW100 plasmid has inserted into the transfected DNA, and is not present in the untransfected genomic DNA (pLEW F + R primers). Also, that *ESAG10* is in the genomic DNA, plasmid construct and in the transfected DNA but not in the empty p2216 plasmid (E10 F + R primers). Finally, that *ESAG10* has inserted correctly into the transfected genome such that a product is formed from *ESAG10* forward primer and a reverse rDNA primer (E10 F + rDNA R primers), and likewise the reverse *ESAG10* primer with a forward rDNA primer (rDNA F + E10 R primers) only in the transfected genome, such that it is concluded that *ESAG10* has inserted correctly into the transfected trypanosome genome.

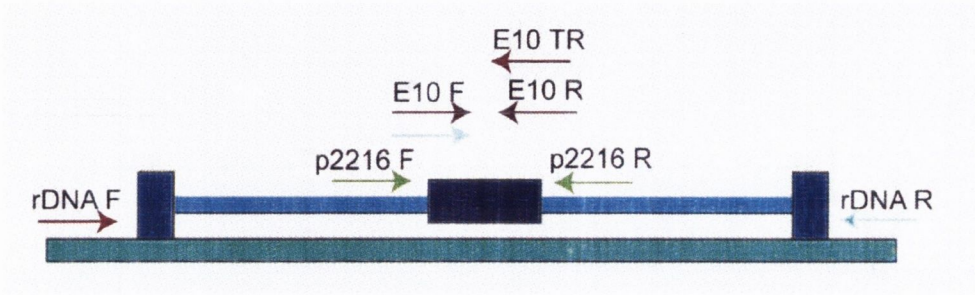


Fig 3.10. Designing PCR experiments to show that the p2216 *ESAG10* plasmid was inserted correctly into the rDNA spacer region. The p2216 F and p2216 R primers were designed to confirm the presence of the plasmid in the transfected cells. The rDNA F and E10T R and the rDNA R and E10 F primers were designed to test for the presence of the *ESAG10* insert in the rDNA spacer of the *T. brucei* genome. The E10 F and E10 R primers were designed to confirm that the insert was indeed *ESAG10*.

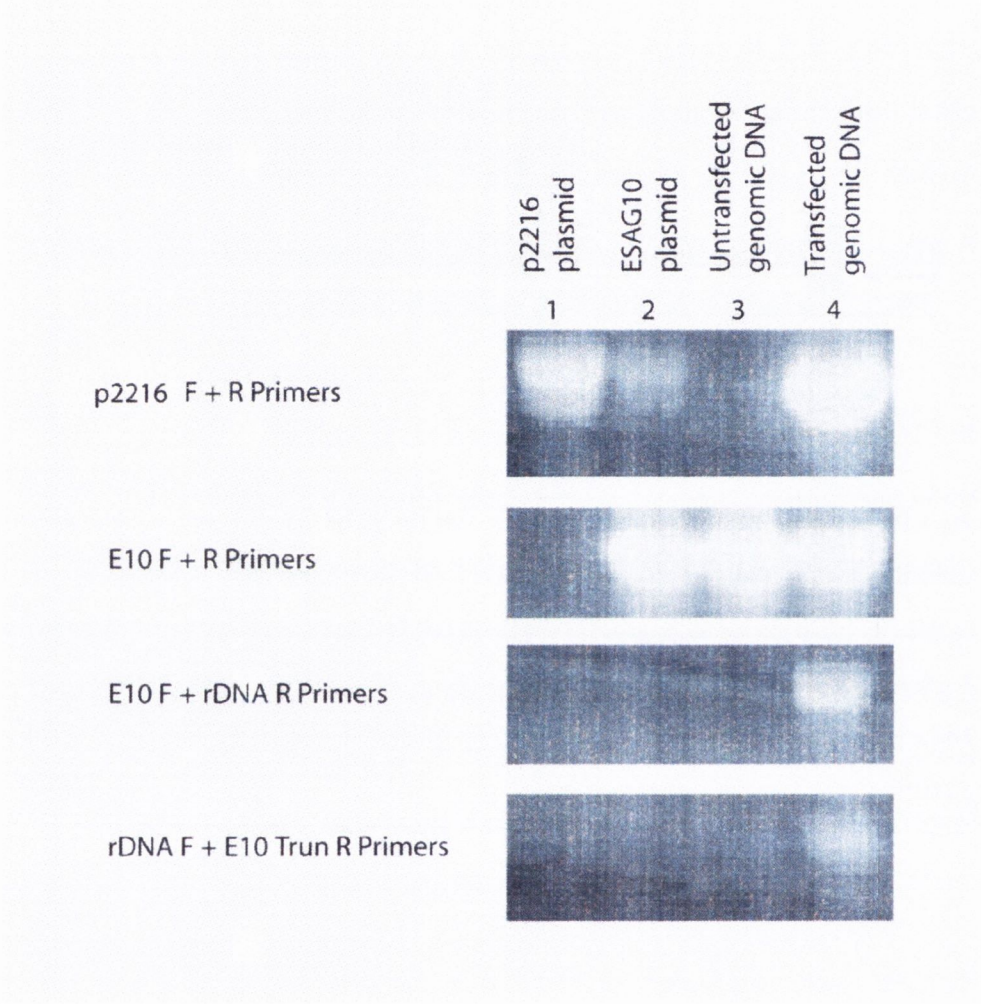


Fig 3.11. PCR to confirm that p2216 *ESAG10* was inserted into the rDNA spacer region of the ribosomal DNA. Lane 4 shows the *ESAG10* insert in transfected *T. b. brucei* genomic DNA.

3.6.3 RT-PCR to Show Expression of the Inserted *ESAG10* ORF

To demonstrate the expression of *ESAG10* at the mRNA level, mRNA was generated from both induced and uninduced *ESAG10* transfected cells, and untransfected cells. cDNA was generated from the mRNA using a reverse transcriptase enzyme, and a PCR reaction was carried out to show that there was expression of the *ESAG10* gene (Figure 3.12). A control reaction, showing that the mRNA was present, used the spliced leader forward primer with the α -tubulin house-keeping gene. A truncated version of the *ESAG10* gene was used with the spliced leader forward primer, and the amplified products showed that there was expression of *ESAG10*.

The results of this RT-PCR reaction showed that *ESAG10* was expressed in both the *ESAG10*-transfected, induced and uninduced cells. This expression of the *ESAG10* in both the induced and uninduced *ESAG10* transfected cells confirms the speculation that the overexpression plasmid is leaky, and that expression is achieved in both the induced and uninduced cells. As expected, expression of *ESAG10* was not detected in the untransfected parental cells. This result complements that result of the above section Figure 3.8, where *ESAG10* expression was not detected in the long slender cDNA, but its expression was detected in the short stumpy forms.

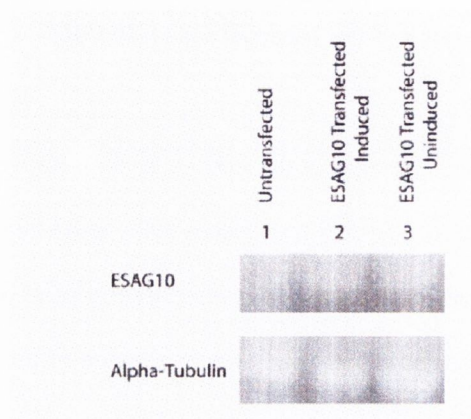


Fig 3.12. RT-PCR to confirm that there was expression of the *ESAG10* in the transfected cells.

The above figure depicts the RT-PCR results showing that the *ESAG10* gene was expressed in transfected cells. Upper panel; Lane 1: untransfected *T. b. brucei* cDNA, Lane 2: cDNA from *T. b. brucei* transfected with the *ESAG10*-p2216 plasmid and induced with tetracycline, Lane 3: cDNA from *T. b. brucei* transfected with the *ESAG10*-p2216 plasmid, but not induced. The RT-PCR products of the *ESAG10* truncated product, which gives a product in the case of both the *ESAG10* transfected induced and uninduced cDNA, showing that mRNA from *ESAG10* is being expressed. There appears to be a smudge present in the case of untransfected RNA (Lane 1, *ESAG10*) which may be due to non-specific amplification. Lower panel; Lanes 1-3: RT-PCR products of the α -tubulin.

3.6.4 Induction of *ESAG10* Protein

To test whether the induced transfected cells, which overexpressed *ESAG10*, also overexpressed the TbPP69 protein, protein extracts were generated from untransfected cells, and cells transfected with *ESAG10*, both induced and uninduced. These protein extracts were incubated with tomato lectin agarose beads, washed and subsequently run on an SDS-10% PAGE gel as described in the materials and methods section 2.9. The gel was then silver stained. The gel depicts three lanes, the untransfected cells, the induced *ESAG10* transfected cells and the uninduced *ESAG10* transfected cells

(Figure 3.13). A large band in the region of 70 kDa can be seen in each of the three lanes. This band is much more intense in the case of the induced *ESAG10* cells compared to the uninduced *ESAG10* transfectants, or the untransfected cells' protein extracts.

This result mirrors the original gel on which the project was based which showed the upregulation of a 69 kDa putative receptor/transporter in pleomorphic cells as compared to monomorphic cells, (Figure 1.4). The induced *ESAG10* transfected surface protein extract shows up-regulated expression of a band in the 69/70 kDa region, which is the same position as the proposed TbPP69 receptor/transporter, giving further support for *ESAG10* as the receptor/transporter in question.

Although RT-PCR suggested that *ESAG10* was expressed at equal levels in induced and uninduced cells, it is apparent that *ESAG10* displays higher levels of expression at the protein level in induced cells. It should be noted that RT-PCR is not a very reliable quantitative measure of expression.

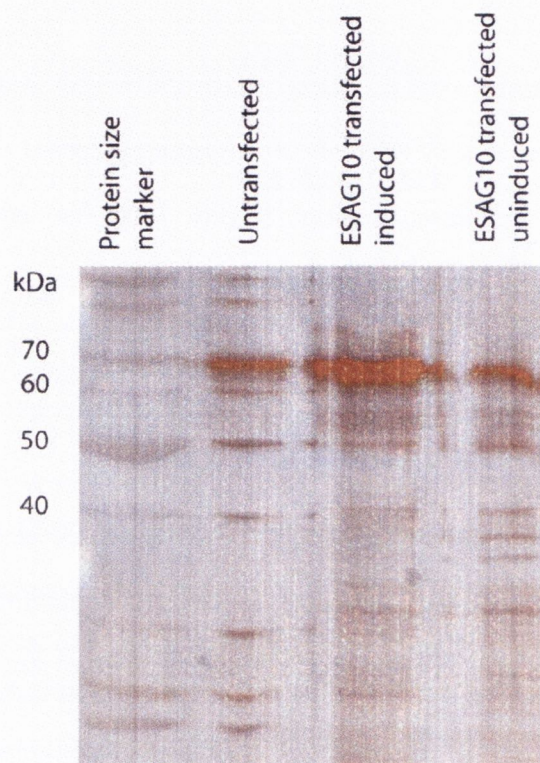


Fig 3.13. Comparison of surface proteins purified from induced versus uninduced *ESAG10* transfected cells. The above figure depicts an SDS-10% PAGE gel of surface proteins purified from induced and uninduced *ESAG10* transfected cells, and untransfected cells. Lane M: the 220 kDa ladder (Peq labs). Lane 1: untransfected *T. b. brucei* tomato lectin binding surface proteins, Lane 2: induced *ESAG10* transfected *T. b. brucei* tomato-lectin binding surface proteins. Lane 3: uninduced *ESAG10* transfected *T. b. brucei* tomato lectin binding surface proteins. There is an abundant protein visible at the 70 kDa region; this is proposed to be the overexpressed *ESAG10* protein, and also the TbPP69 receptor/transporter.

3.6.5 Immunofluorescence Analysis of *ESAG10* Transfected Cells

TbArf1, a *T. b. brucei* vesicular trafficking protein, has been previously implicated in differentiation of long slender to short stumpy forms of *T. b. brucei* (Murphy and Olijhoek 2001). TbArf1 has been shown to bind specifically to T-GIF, the trypanosome differentiation inducing factor, and likely plays a key role in transducing the differentiation signal throughout the cell. To investigate the potential interaction of ESAG10 and TbArf1, a series of co-localisation studies was carried out. Bloodstream form trypanosome cells were adhered to poly-L-lysine coated slides and were fixed in methanol before blocking and incubation overnight with a primary antibody specific to TbArf1.

A tomato lectin Texas red conjugated stain, which binds to glycosylated cell surface proteins, was used to monitor ESAG10 protein location in non-transfected and transfected induced and uninduced *T. b. brucei* cells (Figure 3.14). Overexpression of ESAG10 (Texas red) in transfected induced and uninduced cells resulted in upregulation of cell surface proteins mainly along the flagellum. (Though tomato lectin detects other tomato lectin-binding proteins apart from ESAG10, upregulation of the Texas red staining in transfected versus untransfected cells is most likely due to increased ESAG10). In comparison, the untransfected cells showed Texas red staining only in the area around the Golgi, endosome and flagellar pocket. This is typical of *T. b. brucei* cells, where staining of tomato lectin binding proteins occurs in the vicinity of the flagellar pocket (Nolan *et al.* 1999). In addition, a specific stain for Golgi and nucleus, such as TbRab 31 which localises to the trypanosome Golgi complex (Field *et al.* 2001), could also be used to confirm the location of the Golgi and ER.

TbArf1 localisation and expression was monitored using a primary Arf1-specific antibody, which was detected using a secondary antibody conjugated to fluorescein in its reactive isothiocyanate form (FITC). A secondary antibody control was carried out using buffer, instead of the primary antibody, confirming that the secondary antibody was specific. ESAG10 overexpression in transfected *T. b. brucei* cells altered the pattern of TbArf1 expression and its cellular localisation such that TbArf1, which was previously localised around the Golgi and endosomal system, was now apparent throughout the cell. In particular, intense staining of TbArf1 was noted in regions close to the cell surface and in the flagellar region. Furthermore, ESAG10 and TbArf1 were co-localised together suggestive of a direct interaction between the two proteins.

Fluorescently stained cells were viewed under an Olympus BioSystems fluorescent microscope, 40x power and photographs were taken of the different stains. A blue fluorescent stain, DAPI, which specifically binds nuclear and mitochondrial DNA in the nucleus and the kinetoplast, assisted in the identification of the Golgi, flagellar pocket, and in the orientation of the cell. The results of Figure 3.14 were replicated in several other *T. b. brucei* cells in a separate series of experiments to confirm the observed results (Figure 3.15).

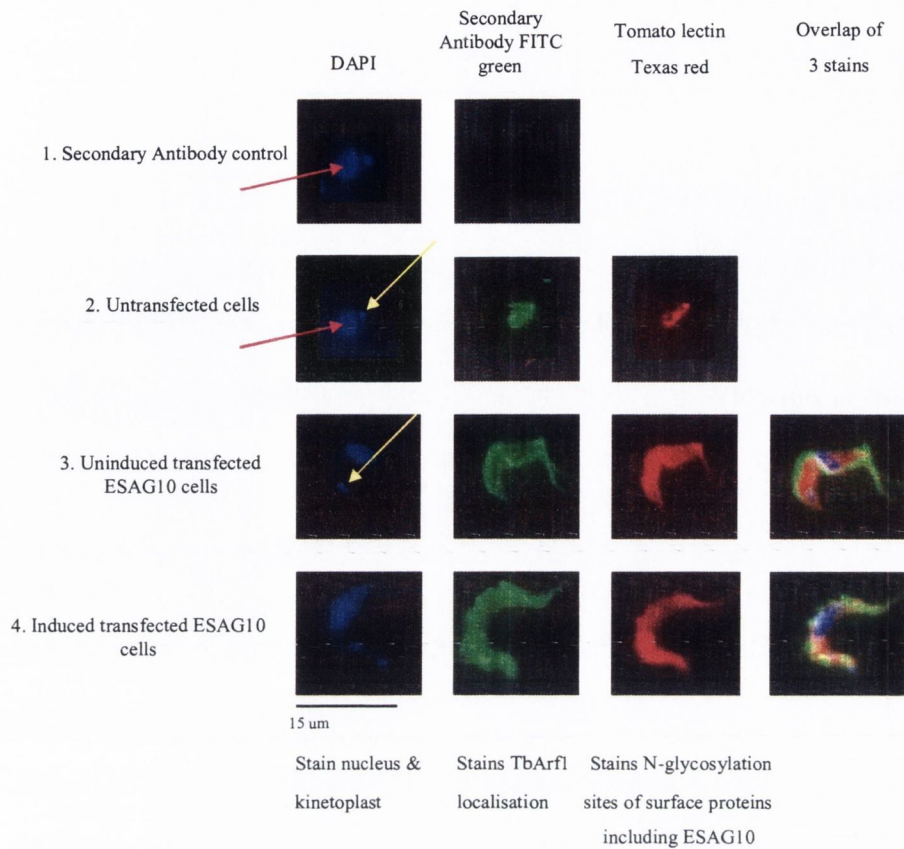


Fig 3.14. Results of the immunofluorescence experiments carried out on *ESAG10* transfected cells. Pink arrows indicate location of the nucleus, yellow arrows indicate location of the kinetoplast. (1) The secondary antibody control. No primary antibody was used and as expected no specific FITC staining is evident. (2) Untransfected *T. b. brucei* cells. Texas red stained tomato lectin binding proteins (proteins containing linear chains of poly N-acetyl glucosamine) and FITC stained TbArf1 was evident only in the vicinity of the Golgi and flagellar pocket. (3) Uninduced ESAG10 transfected cells. Cells show the same pattern as induced cells, possibly due to a leaky expression system. (4) Tet-induced *ESAG10* transfected cells. ESAG10 expression as monitored by upregulated Texas red staining, is now evident throughout the cell, mainly along the flagellum. In association with this FITC stained TbArf1 is also evident throughout the parasite, particularly along the flagellum.

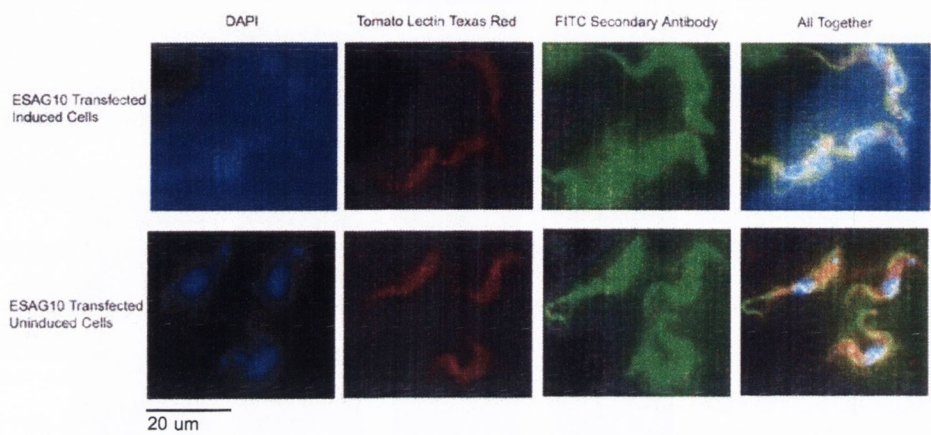


Fig 3.15. Replicated results of the immunofluorescence experiments carried out on multiple *ESAG10* transfected cells. Fluorescence stained induced and uninduced *ESAG10* transfected cells. Cells were stained with DAPI, tomato lectin Texas red, and FITC indicating the results of Fig 3.15 are replicated in multiple cells, as previously discussed. This image shows several more trypanosomes in a single field of view.

3.7 Discussion- ESAG10, a Cell Surface Receptor Possible Involved in Density Dependent Differentiation

A surface protein, TbPP69, has previously been implicated in cell density dependent differentiation of long slender *T. b. brucei* cells (Murphy and Olijhoek 2001). The molecular identity of this receptor has not been previously deduced. An aim of this thesis was to identify and characterise the TbPP69 receptor/transporter, and to elucidate its role in differentiation. The TbPP69 surface protein was previously shown to be upregulated in pleomorphic cells (Murphy and Olijhoek 2001). Purified surface proteins from pleomorphic cells were run on an SDS-12% PAGE gel, and the 69 kDa band of interest was extracted from the gel and sent away for mass spectrometry analysis. The analysis showed that there was contamination of the 69 kDa band with ILTat 1.4 VSG. No putative receptor/transporter candidates were retrieved when peptides, generated from the mass spectrometry analysis, were compared to the Sanger *T. brucei* GeneDB database. Subsequently, a candidate based approach was used to identify possible TbPP69 candidates based on the predicted characteristics of the TbPP69 receptor/transporter. An expression site associated gene, *ESAG10* was chosen as a likely candidate for the TbPP69 transporter as it fit several of the expected characteristics of TbPP69.

ESAG10 was first identified in 1994 as an expression site associated gene found in the promoter region of a 1.5 Mb chromosome of *T. brucei* bloodstream variant 118 clone 1, which encoded a protein with 10 (or 12) predicted transmembrane domains (Gottesdiener 1994). In this study, *ESAG10* was located in the 13 kb region between the two promoters of the expression site. More recent studies investigating expression

site composition has shown that *ESAG10* is often absent from expression sites, and when present it is located in the promoter region of the expression site, with a designated promoter for its expression (Berriman *et al.* 2002). The presence of *ESAG10*'s own promoter may be significant, as none of the other expression site associated genes have their own promoters. *ESAG10*'s promoter may allow differential expression of *ESAG10* with respect to the other ESAGs, to facilitate greater expression of *ESAG10* during the long slender life cycle stage to enable uptake of T-GIF, the differentiation inducing factor.

ESAG10 was shown to have high sequence similarity to a related parasite *Leishmania donovani* gene, OrfG, located at the LD1 locus on chromosome 35 (Myler *et al.* 1994). The expression of OrfG in *Leishmania* was shown to have a direct relationship with the active transport of biopterin into the cell. OrfG encodes a biopterin transporter and was renamed BT1 (Lemley *et al.* 1999). BT1 has 34% amino acid sequence identity, 64% similarity with *ESAG10* (Myler *et al.* 1994).

Biopterin is converted into tetrahydrobiopterin (BH₄) in *L. donovani* (Beck and Ullman 1991). BH₄ levels are important in controlling the extent of metacyclogenesis of *Leishmania*, which involves the transformation of poorly infective procyclic promastigotes into highly infective metacyclic promastigotes (Zakai *et al.* 1998). BH₄ levels decline over normal development, such that low BH₄ levels are associated with metacyclogenesis (Beck and Ullman 1991). Apart from its role in parasite differentiation, BH₄ has also been linked to oxidant scavenging, growth factor production and tyrosine production. The similarity between *ESAG10* and BT1, known to be involved in parasite differentiation, further supports the lines of evidence suggesting that *ESAG10* is a suitable TbPP69 receptor/transporter candidate.

ESAG10 is a transmembrane protein (and has an N-terminal signal peptide) which would position ESAG10 on the cell surface of the parasite where it would be capable of up-taking an external signal factor like T-GIF. ESAG10 is also predicted to be glycosylated which would enable it to bind to the tomato lectin that the TbPP69 factor also binds to. ESAG10 has significant similarity to BT1, a *Leishmania* protein involved in differentiation (Lemley *et al.* 1999). Although the predicted molecular weight of ESAG10 is larger than would be expected for the TbPP69, ESAG10 is similar to other transmembrane proteins, including BT1, which experience an anomalous migration on SDS-PAGE gels and run at a much lower molecular weight (Myler *et al.* 1999).

Several lines of evidence based on experiments in this chapter (and thesis) further suggest that ESAG10 is in fact the TbPP69 receptor/transporter. Although there was no difference in the level of mRNA expression in transfected induced and uninduced cells, a significant abundance of a 69 kDa protein (that bound to tomato-lectin) was observed in induced *T. b. brucei* ESAG10 transfected cells. This abundant 69 kDa protein displayed the same molecular weight as the TbPP69. No other protein bands were observed to be overexpressed in ESAG10 transfected and induced cells, strongly suggesting that ESAG10 is the TbPP69 transporter is likely implicated in differentiation of long slender to short stumpy forms of *T. b. brucei*.

Furthermore, co-localisation studies were also carried out to investigate the interaction between ESAG10 and another factor implicated in differentiation, trypanosomal Arf1 (TbArf1), which has been previously shown to bind TbPP69 (Murphy and Olijhoek 2001). These studies showed that overexpression of ESAG10 resulted in a completely novel pattern of expression and localisation of TbArf1. Expression of both ESAG10 and TbArf1 was found mainly along the flagellum in

transfected cells, not just located in the Golgi as in untransfected cells. Although the co-localisation of ESAG10 and TbArf1 does not prove a direct interaction, the evidence taken together suggests that ESAG10 is the TbPP69 receptor/transporter that specifically binds to TbArf1.

Chapter 4: Identification, Phylogenetic Classification and Initial Characterisation of TbArf1 Related Proteins and Effector Proteins

4.1 Summary

ADP-ribosylation factor 1 from *T. b. brucei* (TbArf1) is a vesicular trafficking protein that has been shown to be the intracellular target of the differentiation inducing factor (T-GIF). T-GIF binds to TbArf1 specifically in the alpha helix 3 region, a protein-binding region of TbArf1 (Murphy and Olijhoek. 2001). The previous chapter identified ESAG10 as a possible differentiation receptor/transporter that facilitated the uptake of T-GIF. TbArf1 was shown to be differentially located and upregulated within the cell upon overexpression of ESAG10. The purpose of this chapter was to bioinformatically identify, classify and analyse TbArf1, its related proteins and effector proteins in *T. b. brucei*.

Using this bioinformatics approach, *T. b. brucei* was found to have genes encoding TbArf1 and TbArf3. At least 8 Arf-like genes, TbArfs, were identified: *TbArf12*, *TbArf13*, *TbArf16*, *TbArf17*, *TbArf18*, *TbArf19*, *TbArf20* and *TbArf21*. Homologues of Arf- GTPase activating proteins (ArfGAPs) that negatively regulate

TbArf1 and Arf guanine nucleotide exchange factors (ArfGEFs) that positively regulate TbArf1 were also identified: five TbArfGAPs and three TbArfGEFs in *T. b. brucei*.

Phylogenetic classification of the identified proteins was carried out. This allowed the identification of genes most closely related to mammalian Arfs, Arls, ArfGEFs and ArfGAPs which were of known function. Alignments facilitated the assessment of levels of conservation and regions of significant divergence in the protein families.

4.2 Identification of Arf Proteins in *T. b. brucei*

4.2.1 Introduction to Arf, Arls and Effector Proteins

ADP- ribosylation factors (Arfs) are small GTPase proteins that have roles in intracellular trafficking (Balch *et al.* 1992) and vesicle biosynthesis (Moss and Vaughan 1995). Arf proteins cycle between active and inactive conformations dictated by the GTP status. Arf is inactive when GDP is bound and active when GTP is bound (Moss and Vaughan 1998). The switch 1 and switch 2 regions of Arf proteins bind tightly to the γ phosphate of GTP and poorly to the alternative GDP (Renault *et al.* 2002). The active status of Arfs enables other effector proteins of Arf to bind. In addition to this family of proteins, another subfamily of Arf-like proteins (Arls) exists (Tamkun *et al.* 1991). Arls have also been implicated in membrane trafficking (Huang *et al.* 1999; Lu *et al.* 2001; Rosenwald *et al.* 2002).

A trypanosomal Arf protein, TbArf1, has been implicated in differentiation of *T. b. brucei* (Murphy and Olijhoek 2001). In order to understand effectively any

involvement of TbArf1 in differentiation, it is important to examine the roles of related proteins in depth. Arf proteins have roles in vesicular trafficking; thus it is possible that this process has a significant role in differentiation.

Effector proteins play an important role in the regulation of Arfs; they regulate the active status of Arf proteins in conjunction with other activities. Arf-Guanine nucleotide exchange factors (ArfGEFs) positively regulate Arfs (Cherfils *et al.* 1998) and Arf-GTPase activating proteins (ArfGAPs) negatively regulate Arfs (Goldberg 1999). Other effectors of Arf include vesicle coat proteins, COPI (Sprang and Coleman 1998) Golgi-localized- γ -ear-containing, adenosine diphosphate ribosylation factor-binding proteins (GGA) (Bonifacino 2004) and clathrin (Puertollano *et al.* 2001).

4.2.2 Identification of Sequences in *T. b. brucei* Encoding Arf Proteins

ADP-ribosylation factors (Arfs) are important proteins in vesicular trafficking and in the cytoskeletal structure of the cell (Lu and Hong 2003). Six Arf proteins have been identified in humans (Arf1-6) (Kahn and Gilman 1986; Moss and Vaughan 1998). Human Arf proteins have been classified into three classes. Class I consists of human Arf1 (HsArf1), HsArf2 and HsArf3, while class II contains both HsArf4 and HsArf5, and HsArf6 is the only member of class III. An objective of this thesis was to identify and classify the homologues of the human Arfs and other related proteins in *T. b. brucei*. Two genes encoding putative Arf proteins, Arf1 and Arf3 (denoted as TbArf1 and TbArf3), were identified in the *T. b. brucei* genome, by comparing the human Arf protein to the *T. b. brucei* TIGR (<http://www.tigr.org/>) and Sanger (www.sanger.ac.uk/) databases. TbArf1, a 182 amino acid protein, was encoded by a

546 bp gene on Chromosome 9 of *T. b. brucei*. It was predicted to be a 21 kDa protein and to be myristoylated. Activated myristoylated proteins are usually found on the cell membrane (Franco *et al.* 1995). TbArf3, a 190 amino acid protein, is encoded by a 570 bp gene on chromosome 7 of *T. b. brucei*. This 21 kDa protein is also predicted to be myristoylated. A search of conserved domains using the Proscan programme (<http://expasy.org/-tools/scanprosite/>) characterised both TbArf1 and TbArf3 as ADP-ribosylation factors.

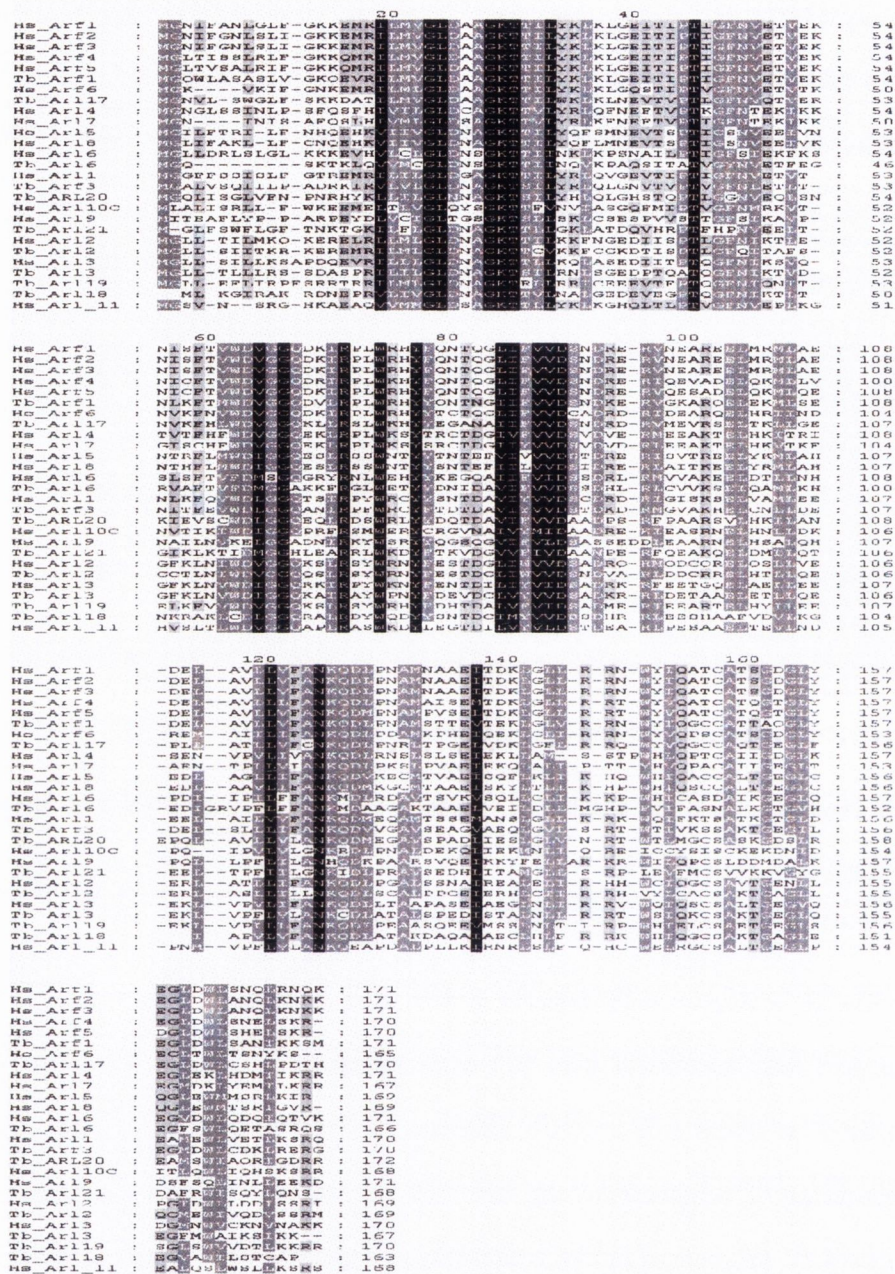
By searching the database for closely related TbArf1 and TbArf3 proteins other putative proteins similar to the TbArfs, denoted as TbArls, were identified in the *T. b. brucei* genome. Arf-like proteins (Arls) are closely related to Arf proteins and some Arls, such as human Arl1, have also been implicated in vesicular trafficking (Munro 2005). At least 16 Arl proteins have been identified in the human genome (Kahn *et al.* 2006). Eight putative Arls were identified in *T. b. brucei*, a finding which has recently been confirmed by another group (Field 2005). The difference in the number of Arfs and Arls in humans and in *T. b. brucei* suggests that this family has expanded in mammals or other vertebrates. The number of amino acids and the molecular weight of the putative TbArl (and TbArf) proteins were determined (Table 4.1). Though these Arl proteins were similar to the TbArf proteins in size and sequence, not all TbArls have myristoylation sites, which would imply that not all will be associated with membranes. To further characterise the putative TbArf and TbArl proteins, the sequences of proteins were aligned with sequences of homologous proteins from other species and classified phylogenetically.

Table 4.1. Putative TbArl proteins encoded in the *T. b. brucei* genome. The name, chromosome location and length of the Arl gene is shown. A myristoylation prediction program was used to predict whether each protein was N-myristoylated (<http://expasy.org/tools/myristoylator/>). The molecular weight and pI of the proteins were predicted using Compute pI/Mw program from Expasy (http://expasy.org/tools/pi_tool.html).

Name	Gene length	pI	Amino acids	Molecular weight	N-myristolyate
Tb_Arf1	546	8.8	182	21 kDa	Myristolyated
Tb_Arf3	570	5.2	190	21 kDa	Myristolyated
Tb_Arl2	558	8.9	186	21 kDa	Non-myristolyated
Tb_Arl3	534	5.3	178	20 kDa	Non-myristolyated
Tb_Arl6	567	8.9	189	21 kDa	Myristolyated
Tb_Arl17	567	6	189	21 kDa	Myristolyated
Tb_Arl18	549	6.6	183	20 kDa	No prediction
Tb_Arl19	570	5.5	190	22 kDa	Non-myristolyated
Tb_Arl20	579	6.5	193	21 kDa	Myristolyated
Tb_Arl21	582	7	194	22 kDa	Non-myristolyated

4.2.3 Phylogenetic Classification of Sequences in *T. b. brucei* Encoding Arf and Arf-Like Proteins (Arls)

To classify phylogenetically putative *T. b. brucei* Arf and Arl proteins a T-coffee alignment was generated (Figure 4.1) using known human Arfs and Arl sequences and the *T. b. brucei* Arfs and Arls identified in GeneDB (<http://www.genedb.org/>). Subsequently a neighbour-joining tree was constructed based on this alignment (Figure 4.2). Arf proteins formed a monophyletic clade with the exception of TbArf3. TbArf3 was found outside of other human Arf proteins, grouping with Arl proteins and was closely related to HsArl1, implying that the naming of TbArf3 as an Arf may be incorrect and that it is unlikely to have the same function as other Arfs. A *T. b. brucei* Arl protein (TbArl17) was found to group with Arf proteins and is a better Arf protein candidate.



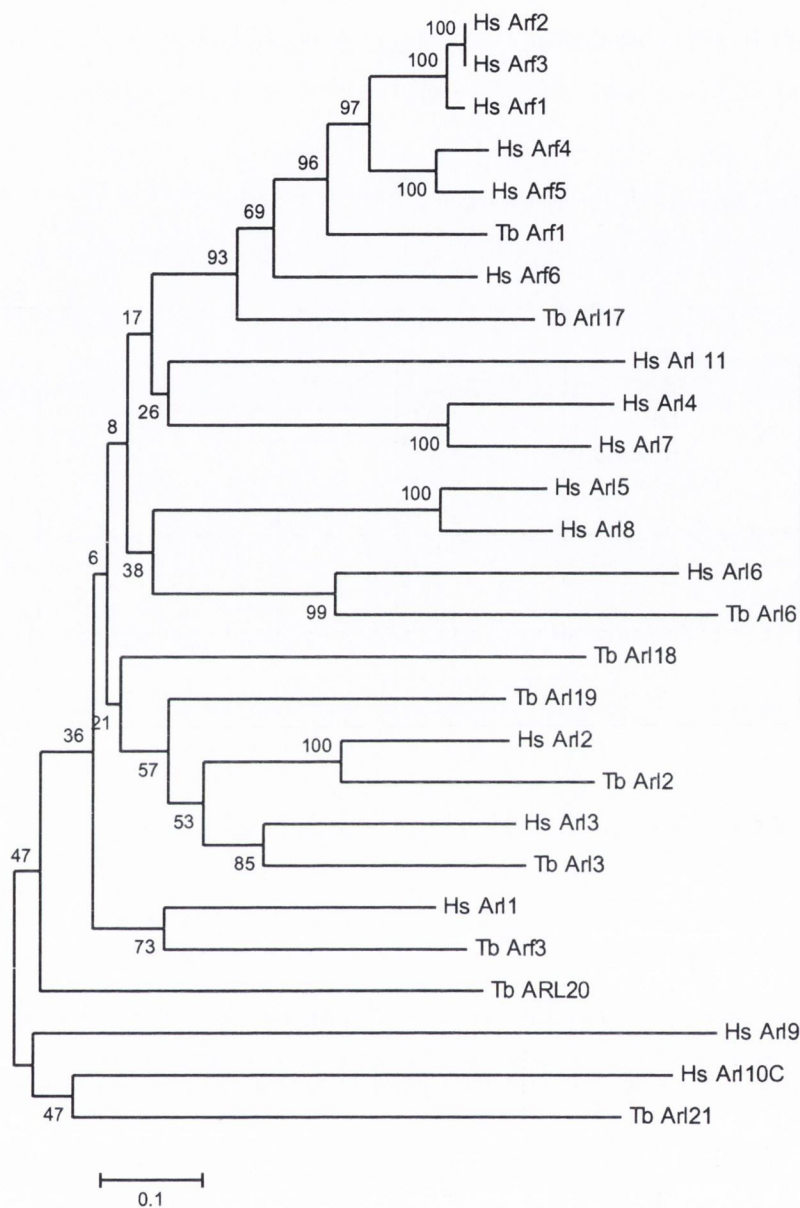


Fig 4.2. A neighbour-joining tree depicting T-Coffee alignment of human (Hs) and *T. b. brucei* (Tb) Arf and Arf-like proteins. 1000 bootstraps were carried out to assign support for each node. This neighbour-joining tree was constructed using the program Mega 3.1.

T. b. brucei Arls are more closely related to various human Arls than to Arfs. TbArl2 is closely related to HsArl2, TbArl3 is closely related to HsArl3, TbArl6 is closely related to HsArl6, and TbArl21 is closely related to HsArl10C. As the phylogenetic relationship between the various human and *T. b. brucei* Arls is not well resolved it is difficult to discuss these relationships in more detail.

TbArf1 is the *T. b. brucei* Arf protein most closely related to the human Arf proteins. TbArf1 although closely related to the human Arf proteins, it is not directly orthologous to any one human Arf. Given that the *T. b. brucei* genome contains only one or possibly two Arfs it seems likely that TbArf1 incorporates one or several of the functions which are independently carried out by each of the human Arfs. HsArf1 is associated with the Golgi and is involved in trafficking from Golgi to the ER and endosomal systems (Takeuchi *et al.* 2002). HsArf6 is involved in endosomal recycling to the plasma membrane and regulating secretion, such that Arf6 is often found on the plasma membrane (D'Souza-Schorey *et al.* 1998). In the pleomorphic *T. b. brucei* parasites (parasites that display differentiated bloodstream forms), TbArf1 is found both in the Golgi apparatus, as would be expected of an Arf1 protein, and also along the trypanosome flagellum, where Arf6 would be expected to be positioned, as HsArf6 localises to the plasma membrane of the cell (as shown in Figure 1.10).

4.2.4 Phylogenetic Classification of Arf1

TbArf1 may have several functions in *T. b. brucei*, fulfilling the roles that both HsArf1 and HsArf6 have in humans. TbArf1 has also been implicated in the long slender to short stumpy differentiation in *T. b. brucei* (Murphy and Olijhoek 2001). Though Arf1 proteins of other organisms have been described to possess numerous

functions, a role in differentiation has not been described. Therefore, there may be differences at the protein level between TbArf1 and Arf1 proteins of other organisms that account for the additional function of TbArf1 in differentiation. To investigate this, TbArf1 was compared to Arf1 from several other species by protein alignment analysis. These species included *Caenorhabditis elegans*, *Dictyostelium discoideum*, *L. major*, *T. b. gambiense*, *T. vivax*, *T. congolense*, *T. cruzi*, *Saccharomyces cerevisiae*, *Mus musculus*, *Homo sapiens*, *Xenopus laevis* and *Drosophila melanogaster* (Figure 4.3). A phylogenetic tree was generated from this alignment to investigate the evolutionary relationships of the Arf1 proteins from the various species (Figure 4.4).

The protein alignment revealed important differences between the African trypanosome Arf1 protein and those of other eukaryotes. Arf1 is a highly conserved protein, but clearly the African trypanosome Arf1 differs in some key functional areas, in the myristoylated N-terminus, the switch 2 binding region, the helix $\alpha 3$ region, and in the fourth GTP-binding site. Of these regions, the switch 1, switch 2 and helix $\alpha 3$ regions are important functional regions of Arf1 that are involved in GTPase-activation and guanine nucleotide exchange factor binding (Nie *et al.* 2003a).

The trypanosome species have a helix $\alpha 3$ region of Arf1 that is conserved between other trypanosomes but differs from *L. major* and the other eukaryotes. Conservation of this region within trypanosomes and the divergence of it compared to other eukaryotes suggest additional or altered functions for the trypanosomal Arf1 proteins in comparison to other eukaryotic Arf1 proteins, and may account for the additional functions in differentiation attributed to TbArf1.

The phylogeny of Arf1 is consistent with the currently accepted eukaryotic phylogeny (Cavalier-Smith 2004). Trypanosomes form a clade with the other kinetoplastid, *L. major* (see Figure 4.4).

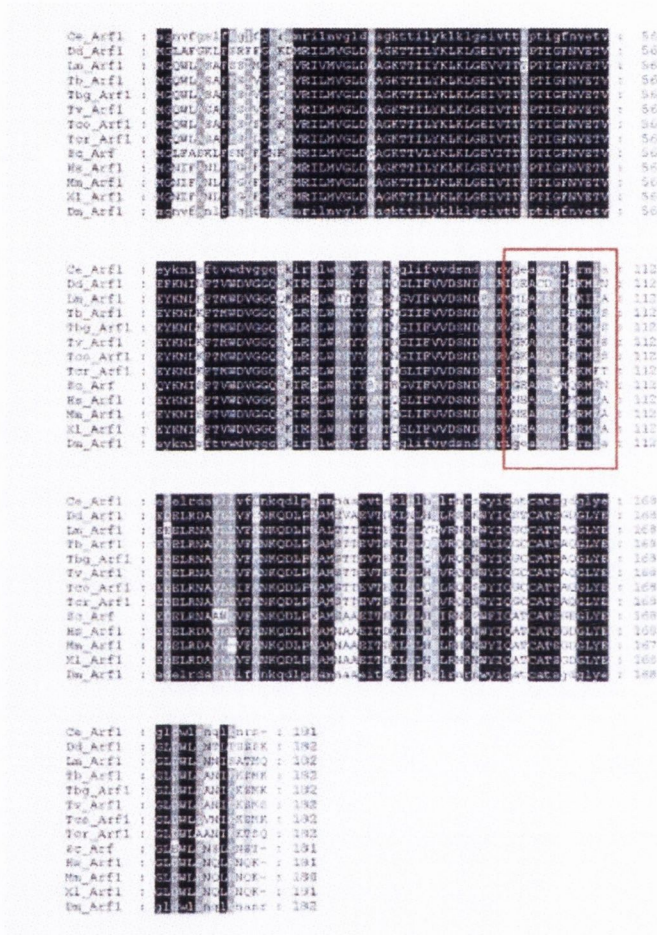


Fig 4.3. A T-coffee alignment of Arf1 sequences from trypanosomes and other eukaryotes. The figure depicts a T-coffee alignment of the Arf1 protein from a number of species including (Ce) *C. elegans*, (Dd) *D. discoideum*, (Lm) *L. major*, (Tb) *T. b. brucei*, (Tbg) *T. b. gambiense*, (Tv) *T. vivax*, (Tco) *T. congolense*, (Tcr) *T. cruzi*, (Sc) *S. cerevisiae*, (Mm) *M. musculus*, (Hs) *H. sapiens*, (XI) *X. laevis*, (Dm) *D. melanogaster*. High sequence conservation is indicated in black (highly conserved) and grey (less highly conserved). Poorly conserved regions are not highlighted. The box outlined in red depicts the helix $\alpha 3$ region of Arf, which is highly conserved in trypanosomes but divergent compared to other eukaryotes. The alignment was visualised using the GeneDoc program.

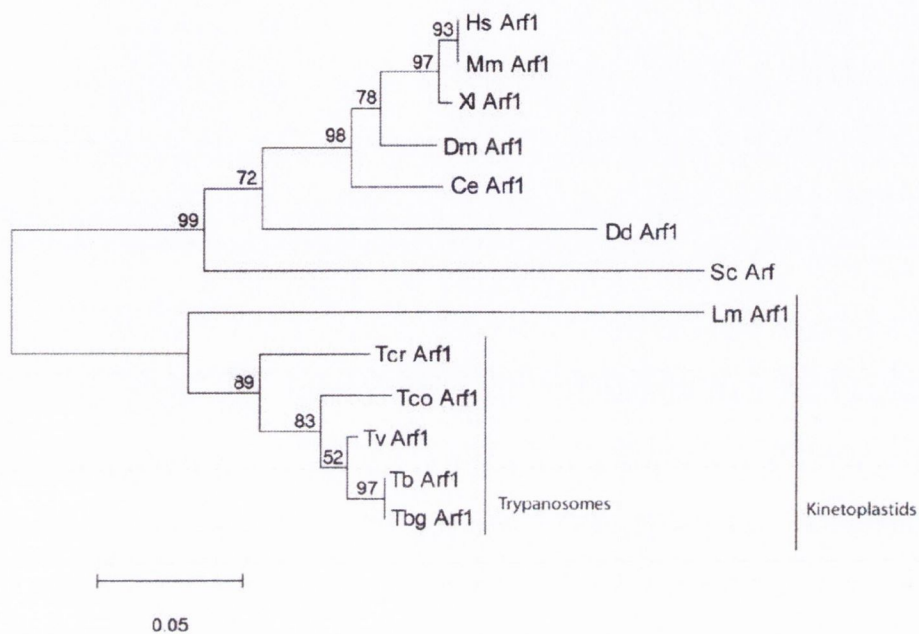


Fig 4.4. Neighbour-joining tree of Arf1 proteins from trypanosomes and other eukaryotes. The figure above depicts a neighbour joining phylogenetic tree of the Arf1 proteins from a number of species, including (Ce) *C. elegans*, (Dd) *D. discoideum*, (Lm) *L. major*, (Tb) *T. b. brucei*, (Tbg) *T. b. gambiense*, (Tv) *T. vivax*, (Tco) *T. congolense*, (Tcr) *T. cruzi*, (Sc) *S. cerevisiae*, (Mm) *M. musculus*, (Hs) *H. sapiens*, (Xl) *X. laevis*, (Dm) *D. melanogaster*. 1000 bootstrap replicates were carried out to assign support for each node. The neighbour-joining tree was constructed using the program Mega 3.1.

4.3 TbArf1 Genomic Organisation and Transcription

4.3.1 Analysis of the TbArf1 Contig

There are four copies of the *TbArf1* gene located in tandem on chromosome 9 of *T. b. brucei*. These genes are contained within the same transcription unit and are co-transcribed (Barry and Turner 1991). Comparative analysis was carried out to characterise the *Arf1* gene organisation in several related species (Table 4.2). In other kinetoplastids, the *Arf1* open reading frames were not organised as four tandem open reading frames suggesting that the organisation of the *TbArf1* transcriptional unit may be significant. For instance, even the closest relation to *T. b. brucei*, *T. b. gambiense* only two copies of *TbArf1* are located in tandem. Furthermore, *TbArf1* has been shown to be developmentally regulated with a five to seven fold upregulation of *TbArf1* in long slender cells as compared to short stumpy cells (Murphy and Olijhoek 2001). This raises questions as to whether each open reading frame contributes equally to produce stable transcripts and whether each copy is under the same developmental regulation.

The *TbArf1* transcription unit is composed of a promoter, and four *TbArf1* open reading frames (ORFs); ORF1, ORF2, ORF3 and ORF4 (Figure 4.5). There are identical 5' untranslated regions (UTRs) in ORF1, ORF2 and ORF3, and identical 3' UTRs in ORF1 and ORF2, and a unique 3' UTR on ORF3. There are also unique 5' and 3' UTRs on ORF4. The presence of alternative UTRs in the various ORFs was suggestive that the ORFs are alternatively regulated and expressed.

Table 4.2. Arrangement of Arf1 genes in *T. b. brucei* and related species. The table below depicts the number and arrangement of Arf1 copies in four different species, *T. b. brucei*, *T. cruzi*, *T. vivax*, *T. congolense* and *L. major*.

Organism	Number of Arf1 copies	Organisation
<i>T. b. brucei</i>	4	In tandem
<i>T. b. gambiense</i>	2	In tandem
<i>T. cruzi</i>	4	2 in tandem +2 in tandem
<i>T. vivax</i>	1 (incomplete genome)	Singular
<i>T. congolense</i>	2 (incomplete genome)	In tandem
<i>L. major</i>	2 (incomplete genome)	Singular

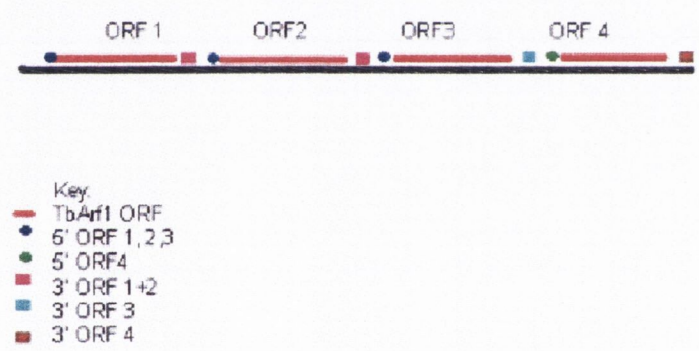


Fig 4.5. Schematic of the TbArf1 contig of *T. b. brucei*. ORF1, 2, 3 and 4 are depicted with a red line, the 5' UTRs are depicted as small circles and the 3' UTRs are depicted as small squares at either side of the ORFs. ORF 1, 2, and 3 have the same 5' sequence, ORF1 and 2 have the same 3' sequence, and ORF4 has unique 5' and 3' UTR sequences.

4.3.2 Differential Expression of TbArf1 ORFs in Different Life Cycle Stages of the Trypanosome

To investigate whether the four *TbArf1* ORFs are differentially expressed in different life cycle stages of *T. b. brucei*, oligonucleotides were designed to amplify the *TbArf1* ORFs from cDNA. A spliced leader forward primer was generated from the spliced leader addition that occurs at the mature 5' end of the mRNA. This spliced leader provides an unusual cap structure and common sequence to all trypanosome mRNAs, and consequently the use of the spliced leader primer in PCR reactions ensures that all PCR products have been generated from cDNA rather than genomic DNA. The spliced leader primer was used with specific reverse primers designed to amplify the individual *TbArf1* ORFs. (The primers are described in chapter 2, Table 2.2).

Using trypanosome cDNA derived from *T. b. brucei* long slender, short stumpy, and procyclic stages, amplification of the expressed *TbArf1* ORFs was carried out (Figure 4.6). A control gene, α -tubulin, with equal expression levels in these life cycle stages was used to characterise the level of expression of the *TbArf1* ORFs. ORF1, ORF2 and ORF4 of *Arf1* were detected in all life cycle stages, while ORF3 was detected only in long slender and short stumpy stages. The α -tubulin control, however, was also weaker in the procyclic stage in comparison to the long slender and short stumpy, which may account for undetection of ORF3 in procyclic forms. Therefore it cannot be conclusive said that the *TbArf1* ORFs were differentially expressed in different life cycle stages of *T. b. brucei*.

Further functional characterisation of *TbArf1* at the protein level is described in Chapter 5. To identify and characterise *Arf1* effector proteins in *T. b. brucei*, a bioinformatics approach similar to that described for *TbArf1* was employed.

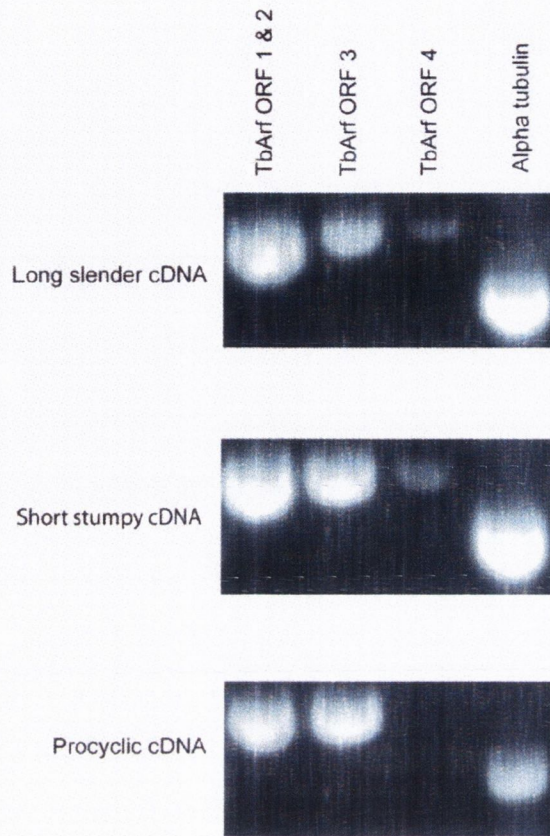


Fig 4.6. Expression of TbArf1 ORFs in the *T. b. brucei* life cycle stages. The above figure depicts a 1% agarose gel. Expression of TbArf1 ORF1, ORF2, and ORF3 was detected in long slender, short stumpy and procyclic stages. TbArf1 ORF4 was detected in long slender and short stumpy stages only and not in procyclic stages, this may be due to lower concentration of RNA. (TbArf1 ORF1 and ORF2 have identical sequences, so separate expression could not be achieved).

4.4 Effectors of Arf1: ArfGAPs

4.4.1 Identification of ArfGAPs in the *T. b. brucei* Database

Arf1 has numerous effector proteins that facilitate the many activities of Arf1. Arf GTPase-activating proteins (ArfGAPs) function as negative regulators of Arf1, hydrolysing Arf1 bound GTP to GDP, thereby inactivating TbArf1 (Goldberg 1999). ArfGAPs also have roles in vesicle coat production (Lewis *et al.* 2004).

To identify these important Arf1 regulators in *T. b. brucei*, known ArfGAPs from *S. cerevisiae*, Glo3p and GCS1, were used as queries to identify ArfGAP homologues in the Sanger (http://www.sanger.ac.uk/Projects/T_brucei/) and TIGR (<http://www.tigr.org/tdb/e2k1/tba1/>) databases. A ScanProsite motif search was used to confirm the presence of the ArfGAP domains in ArfGAP homologues (<http://www.expasy.ch/tools-/scanprosite/>). Five putative ArfGAPs were identified (Table 4.3, Figure 4.7). A sixth putative sequence was also identified, but was shown not to have a proper Arf-GAP domain.

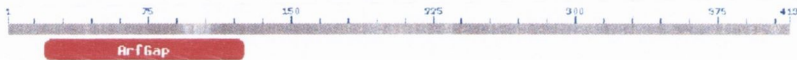
Table 4.3. Characterisation of TbArfGAP I, II, III, IV, and V. The gene length, chromosome number, and amino acid lengths were determined for the TbArfGAPs. The predicted pI and molecular weight were calculated using Compute pI/molecular weight (http://expasy.org/tools/pi_tool.html). The presence of the ArfGAP domain and other domains was also noted.

Name	Gene length	Chr Number	pI	Amino acids	Molecular weight	Arf domain	Other domain
TbArfGAP I	921 bp	11	6	307	34 kDa	Yes	No
TbArfGAP II	12,396 bp	9	5.9	413	45 kDa	Yes	No
TbArfGAP III	873 bp	11	8.7	291	31 kDa	Yes	No
TbArfGAP IV	875 bp	3	9.5	275	31 kDa	Yes	No
TbArfGAP V	1059 bp	11	6.7	353	45 kDa	Yes	No

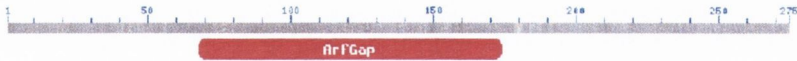
TbArfGAP I



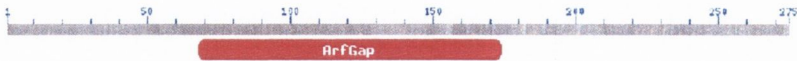
TbArfGAP II



TbArfGAP III



TbArfGAP IV



TbArfGAP V



Fig 4.7. Domain structure of TbArfGAP I, II, III, IV, and V. The ArfGAP domain is depicted in red in each of the TbArfGAPs. The figure was generated using a BlastP search of conserved domain database (CDD) (<http://www.ncbi.nlm.nih.gov/BLAST/>).

4.4.2 Phylogenetic Classification of TbArfGAPs

The ArfGAPs of *T. b. brucei* were all classified as ArfGAP type 1 class proteins, since they have no other conserved domains and the ArfGAP domain is located relatively close to the N-terminus of the proteins (Nie *et al.* 2003a). Putative ArfGAP proteins were identified using GeneDB (<http://www.genedb.org/>) in *T. b. gambiense*, *T. congolense*, *T. cruzi*, *T. vivax* and *L. major* using a similar strategy as discussed for *T. b. brucei*. Five ArfGAPs were identified in *T. b. brucei* and in *T. b. gambiense*, which was perhaps unsurprising as *T. b. brucei* and *T. b. gambiense* are both subspecies of *T. brucei*. Five ArfGAPs were also identified in *T. cruzi*, while only four were identified in *L. major* and in *T. vivax*. Only three putative ArfGAPs were identified in *T. congolense*. Failure to identify all of the ArfGAP orthologues in some species may be due to incomplete sequencing projects. In particular, the *T. congolense* project remains to be finished.

Trypanosome and *Leishmania* ArfGAP domain sequences were aligned using the T-Coffee alignment program (Figure 4.8) and a neighbour-joining phylogenetic tree was constructed (Figure 4.9). Based on this phylogenetic tree, putative ArfGAP sequences from the trypanosome and *Leishmania* were classified into five groups. Each group contained one *T. b. brucei* ArfGAP sequence. All identified clades were supported by very high bootstrap values (99-100%) indicating strong statistical support for each node.

A second phylogenetic tree was produced using human, yeast and kinetoplastid ArfGAP sequences (Figure 4.10). Type I human and yeast ArfGAPs (groups: ScGlo3, HsArfGAP I, HsArfGAP III) were located between groups KI and

KII of the kinetoplastids. Human ArfGAP1 clusters with a bootstrap value of 71 with group II of the trypanosome and *Leishmania* ArfGAP sequences. *T. b. brucei* ArfGAP I was found to be most closely related to human ArfGAP1 and is likely to be orthologous to it while TbArfGAP II was shown to be most closely related to human ArfGAP3. Orthologous genes were also identified in *L. major*, *T. b. gambiense*, and *T. cruzi*. The KIII and KIV groups of the kinetoplastids were next closely related to the human ArfGAP1 and 3. ArfGAP group III contains only ArfGAPs from *T. b. brucei*, *T. b. gambiense*, *T. congolense* and *T. cruzi*. Strikingly, *L. major*, a much studied organism is missing from this clade, along with *T. vivax*. ArfGAP IV contains an ArfGAP from all of the kinetoplastids.

The majority of the human ArfGAPs grouped outside the other kinetoplastid groups and do not appear to have orthologues in these species. The human ArfGAPs with ankyrin repeat, pleckstrin homology domain (ARAP), Arf GAP with GTP-binding protein-like, ankyrin repeat and pleckstrin homology domains 1 (AGAP1), Arf GAP with SH3 (Src homology 3), ANKs and (PH) pleckstrin homology domains (ASAPs), Arf GAP with coiled-coil, ankyrin repeat and pleckstrin homology domains 1 (ACAP1), and G protein-coupled receptor kinase-interacting target (GITs) all cluster in their own groups (H + Y II). These groups are commonly clustered together (Nie *et al.* 2003a). Group V trypanosome and *Leishmania* sequences were found to be distantly related to the rest of the kinetoplastid sequences. This implies that they are more divergent than the rest of the trypanosome and *Leishmania* sequences, and indicates the possibility of alternative functions for these putative ArfGAPs.

Further analyses were conducted, comparing the closely related ArfGAP1 human proteins and the putative trypanosome and *Leishmania* ArfGAP I sequences. An amino acid alignment of the ArfGAP domains was constructed with additional

ArfGAP1 proteins from other species. All ArfGAP domains were highly conserved (Figure 4.11). Outside of this ArfGAP domain there is, however, a significant level of diversity amongst the ArfGAP 1 proteins.

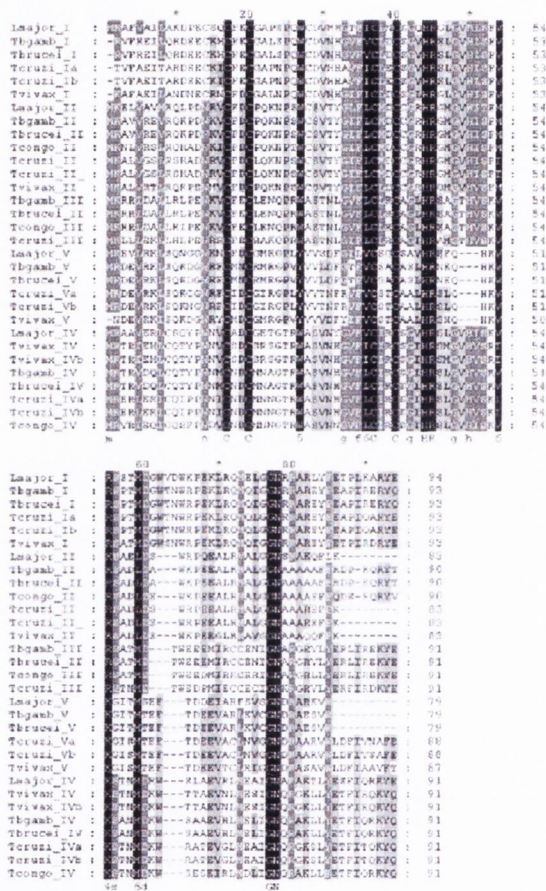


Fig 4.8. T-coffee alignment of Trypanosome and *Leishmania* ArfGAP domains. The alignment includes *L. major*, *T. b. gambiense*, *T. b. brucei*, *T. congolense*, *T. vivax* and *T. cruzi* putative ArfGAPs. High sequence conservation is indicated in black (highly conserved) and grey (less highly conserved). Poorly conserved regions are not highlighted.

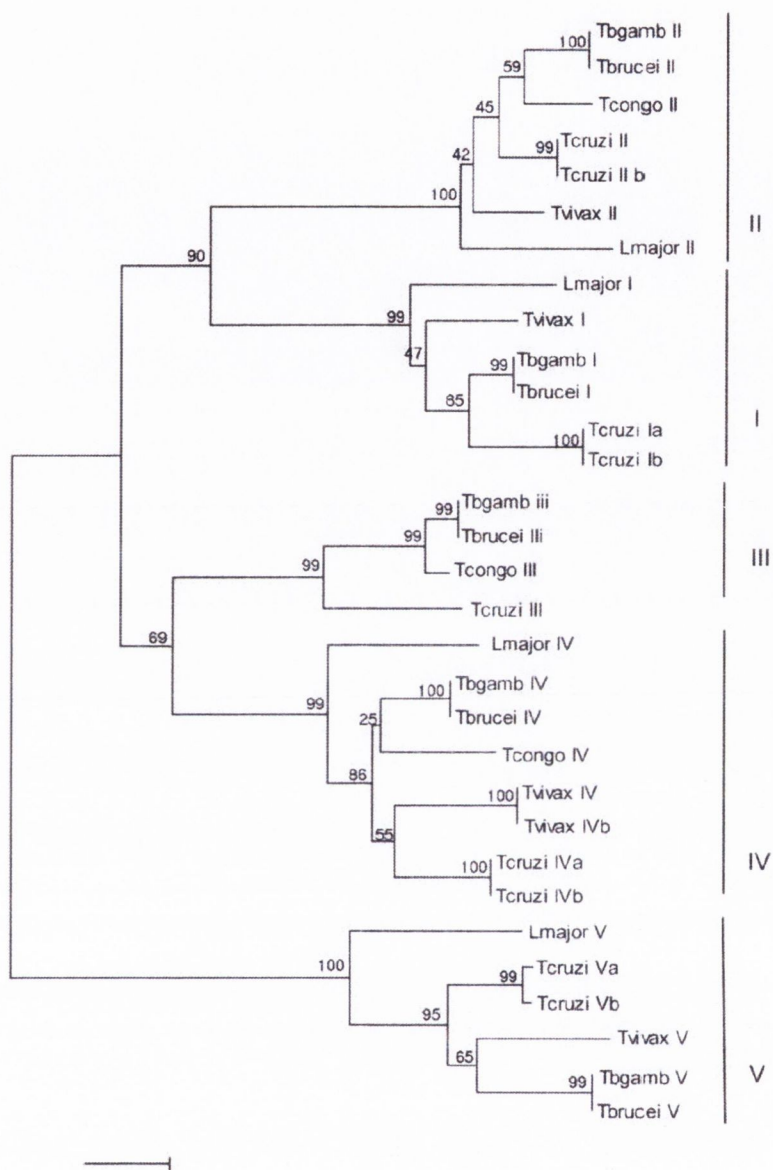


Fig 4.9. A neighbour-joining tree produced from a T-coffee alignment of Trypanosome and *Leishmania* ArfGAP domains. The phylogenetic tree includes *L. major*, *T. b. gambiense*, *T. b. brucei*, *T. congolense*, *T. vivax* and *T. cruzi* putative ArfGAPs. The tree was constructed using Mega 3.1. 1000 bootstrap replicates were carried out. Five different clades are defined.

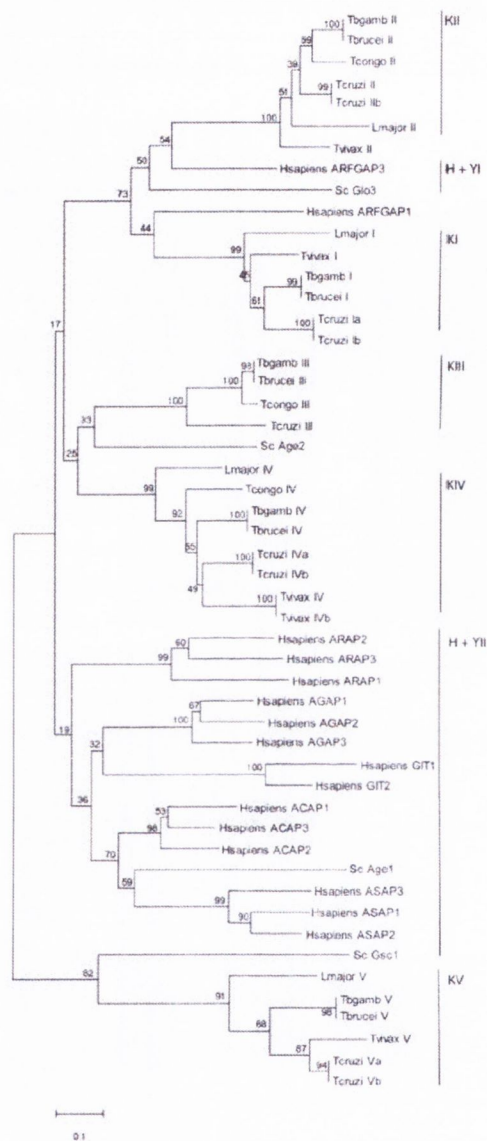


Fig 4.10. A neighbour-joining tree produced from a T-coffee alignment of ArfGAP domains of *H. sapiens*, *S. cerevisiae*, *L. major*, *T. b. gambiense*, *T. b. brucei*, *T. congolense*, *T. vivax* and *T. cruzi*. The tree was constructed using Mega 3.1. 1000 bootstrap replicates were carried out.

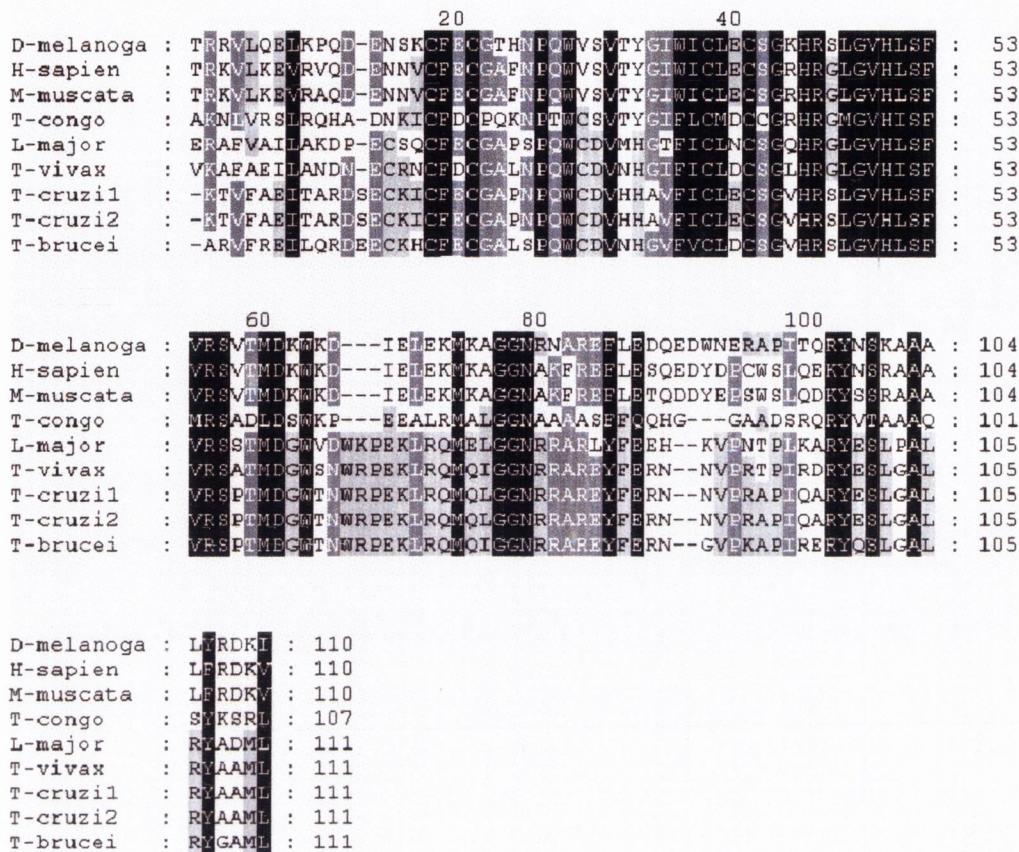


Fig 4.11. Alignment of the ArfGAP domains of known and putative ArfGAP 1 proteins. The figure depicts amino acid sequence alignment of human, mouse, *Drosophila* and *Xenopus* ArfGAP1 proteins, and putative ArfGAP1s from *T. congolense*, *T. b. brucei*, *T. cruzi*, *T. vivax*, and *L. major*. High sequence conservation is indicated in black (highly conserved) and grey (less highly conserved). Poorly conserved regions are not highlighted. This alignment was viewed using Genedoc.

4.4.3 Initial Characterisation of TbArfGAPs Based on Protein Sequence

To investigate the role of ArfGAP 1 proteins in the catalysis of the Arf1-GTP to Arf1-GDP reaction, protein sequences were superimposed on the resolved crystal structure of ArfGAP1 (Goldberg 1999). Crystallisation studies of human Arf1 complexed with HsArfGAP1 identified the specific residues involved in the interaction (Goldberg 1999). The orthologous residues important in the reaction between TbArf1 and TbArfGAPs were identified in *T. b. brucei*. Arf1 is a highly conserved protein, and there is some conservation of residues involved in protein-protein interactions between human and *T. b. brucei* ArfGAP proteins. Although Arf1 proteins are highly conserved, the Arf1 sites likely implicated in the interaction with ArfGAPs are partly conserved (10/14) when compared to between human and trypanosome (Table 4.4), residues differ at positions 105, 108, 109, 112, and 150.

ArfGAPs are highly divergent proteins, particular outside the ArfGAP domain. ArfGAP residues likely implicated in the interaction Arf1 were compared between human and trypanosome at homologous positions in TbArfGAP I, II, III, IV and V. There is much less conservation of residues (6/15) likely involved in interaction between human and trypanosome (Table 4.5). This data would suggest that there may be slight differences in residue involved in Arf1 and ArfGAP interaction between humans and *T. brucei*.

Table 4.4. Comparison of HsArf1 residues, known to be important in the Arf1/ArfGAP1 interaction, with the orthologous positions in TbArf1. Residues are numbered according to position in HsArf1. The domain in which the residue occurs is denoted, switch 2 domain (2, highlighted in blue) or the helix α 3 domain (3, highlighted in green). Residues highlighted in red are amino acids of the helix α 3 region that are unique to the trypanosome.

Residue	70	72	76	77	79	80	105	106	108	109	111	112	114	117	150
Domain	2	2	2	2	2	2	3	3	3	3	3	3	3	3	3
HsArf1	G	D	P	L	R	H	E	E	M	R	L	A	D	R	H
TbArf1	G	D	P	L	R	H	Q	E	E	K	L	S	D	R	Q

Table 4.5. Comparison of Human ArfGAP 1 residues, known to be important in the Arf1/ArfGAP interaction, with the orthologous positions in TbArfGAP I, II, III, IV, and V. Residue numbering is according to position of residues in the human ArfGAP1. The domain in which the residue occurs is denoted, specifically whether the residues occur in the switch 2 domain (2, highlighted in blue) and or the helix α 3 domain (3, highlighted in green). Empty boxes indicate that there is no equivalent residue, yellow shading of boxes indicates conservation of the human ArfGAP residue.

Residue	54	55	58	60	66	68	70	71	112	116	120	122	134	135	136
Domain	3	3	3	3	3	2	2	3	3	2	2	2	3	3	3
HsArfGAP 1	V	H	F	R	K	K	I	E	R	A	E	K	W	T	P
TbArfGAP I	V	H	F	R	N	R	E	K	G	E	L		W	A	P
TbArfGAP II	V	H	F	R	A	K	E	E	K	D			G	T	P
TbArfGAP III	T	H	K	R	T	E	E	M	R				Y	F	N
TbArfGAP IV	V	H	K	K	K	S	A	E	I	Y			F	S	V
TbArfGAP V	-	K	K	K	E	T	E	E	F	Y			A	S	S

4.4.4 Cloning of TbArfGAPs for Protein Expression Studies

Two ArfGAPs, TbArfGAP I and TbArfGAP V, were chosen for further study. TbArfGAP I was chosen as it was most closely related to the Human ArfGAP1, and therefore most likely to function in a similar manner to the Human Arf1GAP1. A second ArfGAP, TbArfGAP V, was also chosen for study as it was most distantly related to the human ArfGAP1, and may possibly function differently from TbArfGAP I. A full length TbArfGAP I and a partial length TbArfGAP V sequence (repetitive C-terminus was too difficult to express) were chosen for study to investigate the location of the catalytic activity in *T. brucei* ArfGAP proteins and these were cloned into the pET21a and subsequently into the pET51a Ek/LIC *E. coli* expression vector (Figure 4.12). Conditions for expression of the recombinant protein were optimised with respect to temperature, concentration of IPTG, time of induction and purification procedure. Reduction of temperature worked best for protein expression. Protein was produced for both TbArfGAP I (Figure 4.13) and TbArfGAP V (Figure 4.14). Once protein expression and purification had been achieved, co-expression recombinant TbArfGAP V with TbArf1 was attempted.

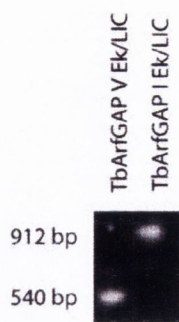


Fig 4.12. pET51a Ek/LIC Amplified PCR products for cloning. The figure depicts a 1% agarose gel displaying the 540 bp PCR product of the ArfGAP domain of TbArfGAP V and the 912 bp PCR product of the full sequence of TbArfGAP I (primers table 2.2) and was viewed under UV light.

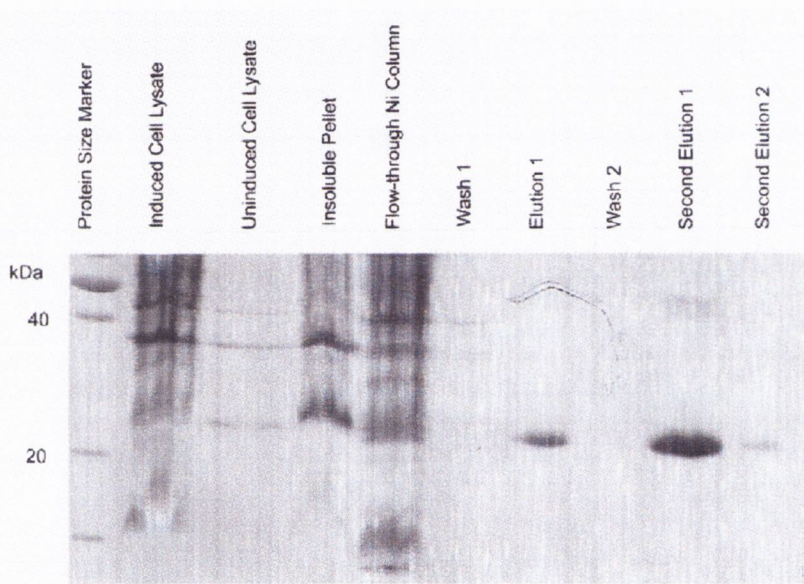


Fig 4.13. Expression of TbArfGAP I. The figure depicts an SDS-12% PAGE gel showing TbArfGAP I (approximately 30 kDa) recombinant protein expression in *E. coli* using the pET51a (Ek/LIC) system following various purification steps. Lane 1: protein size marker, Lane 2: induced unpurified lysate, Lane 3: uninduced lysate (no IPTG added), Lane 4: insoluble pellet from induced fraction, Lane 5: flow-through the Ni₂⁺ purification column, Lane 6: purification column wash 1, purification column eluant, Lane 7: wash 2 from a second purification step, Lane 8: second purification eluant 1, Lane 9: second purification elutant 2. Note the second elution 2 is always the most pure. The gel was stained with Coomassie blue.

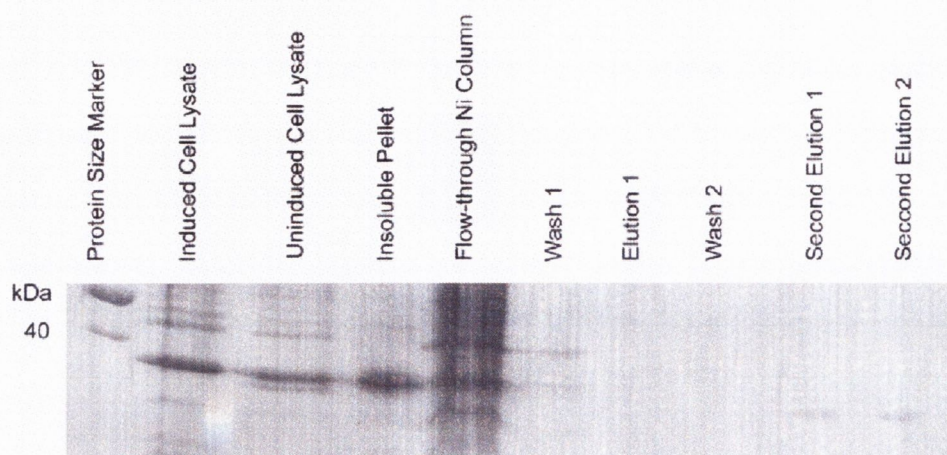


Fig 4.14. Expression of TbArfGAP V. The figure depicts an SDS-12% PAGE gel showing TbArfGAP V (approximately 25 kDa) recombinant protein expression in *E. coli* using the pET51a Ek/LIC system following various purification steps (see previous figure, 4.13). The gel was stained with Coomassie blue.

4.4.5 TbArf1 and TbArfGAP V Co-Expression Studies

A second pET vector was chosen to co-express TbArf1 with TbArfGAP for crystallisation purposes. A version of the *TbArfGAP V* gene, containing only the ArfGAP domain was cloned into pET28b kanamycin resistance plasmid. A truncated ArfGAP was chosen as a concentrated region containing only the catalytic domain had been shown to produce human Arf1-ArfGAP1 crystals (Goldberg 1999). TbArf1 was cloned into a pET21a plasmid encoding ampicillin resistance. Both plasmids were co-transformed into *E. coli* BL21 DE3 competent cells and clones possessing both vectors were selected using agar plates containing ampicillin and kanamycin. The co-expressed proteins were produced and purified. Co-expressed protein was visualised on a 1-D SDS-PAGE gel (Figure 4.17). Subsequently, the preparation was

further analysed to begin crystallisation studies. A mass spectrometry analysis was conducted to examine the purity of the complex (Amir Khan's lab, Trinity College Dublin). The results showed that there were two products evident and no complex had formed. Though co-expression was achieved the analyses indicated that TbArf1 and TbArfGAP V proteins did not form a complex. It was thought that the proteins may interact instantaneously, but do not form a stable complex.

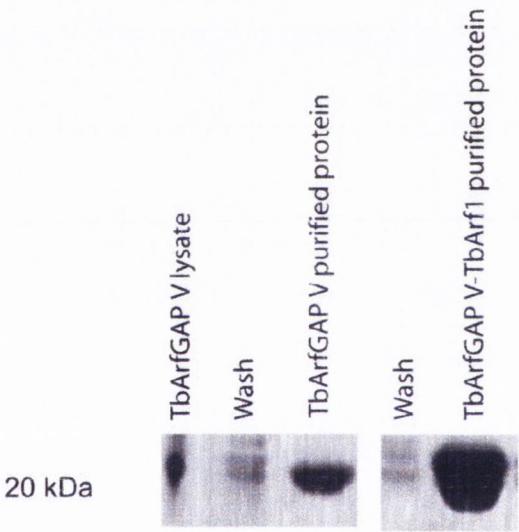


Fig 4.15. Co-expression of TbArf1 and TbArfGAP V. Co-expressed TbArf1 and TbArfGAP V were run on an SDS-12% PAGE gel and stained with Coomassie blue. Note the double band when proteins are co-expressed.

4.5 Effectors of TbArf1: TbArfGEFs

4.5.1 Identification of ArfGEFs in the *T. b. brucei* Sanger Database

Arf-guanine nucleotide exchange factors (ArfGEFs) are positive effectors of Arf1, and ensure a rapid exchange of GDP to GTP (as outlined in the introduction). All ArfGEFs identified to date possess a conserved domain, the Sec7 domain that comprises the catalytic unit of the protein (Mouratou *et al.* 2005). Homology searches using the Sec7 domain of three human ArfGEF proteins, Brefeldin nucleotide exchange factor 1 and 2 (BIG1, BIG2), and ArfGEF 1 identified three putative ArfGEF genes in the *T. b. brucei* GeneDB database (<http://www.genedb.org/>), TbArfGEFA, TbArfGEFB and TbArfGEFC (Figure 4.18). A ScanProsite motif search was used to confirm the presence of Sec7 domains in these putative proteins (<http://www.expasy.ch/tools-/scanprosite/>).

TbArfGEFA is encoded on a 6,192 bp sequence located on chromosome 8. TbArfGEFA encodes a 2063 amino acid protein. [The DNA sequence of the protein was translated using “Translate” (<http://expasy.org/tools/dna.html>)]. TbArfGEFA has a pI of 5.7 and a predicted molecular weight of approximately 225 kDa. [pI and predicted molecular weights were calculated using “Compute pI/ Mw” an internet based program from expasy (Bjellqvist *et al.* 1993) (http://www.expasy.org/tools/pi_tool.html)]. TbArfGEFB is encoded on a 5,016 bp sequence located on chromosome 4. TbArfGEFB (1671 amino acids) has a predicted molecular weight of 184 kDa. It has a pI of 5.3. TbArfGEFC was encoded by a 3,141 bp gene located on chromosome 11. TbArfGEFC (1046 amino acids) has the smallest predicted molecular weight of the TbArfGEFs of 117 kDa and it has a pI of 6.1. No

conserved domains, apart from Sec7, were identified in the *T. b. brucei* ArfGEFs. These novel *T. b. brucei* ArfGEFs were phylogenetically classified along with ArfGEFs from several other species.

TbArfGEFA



TbArfGEFB



TbArfGEFC



Fig 4.16. Characterisation of the domain structures of TbArfGEFA, TbArfGEFB and TbArfGEFC. The location of the Sec7 domain is shown. This figure was generated via a BLAST search of the conserved domain (CDD) database at NCBI (<http://www.ncbi.nlm.nih.gov/BLAST/>).

4.5.2 Phylogenetic Classification of TbArfGEFs

ArfGEFs from several other African and the American trypanosome species and *Leishmania* were identified based on similarity to the *T. b. brucei* ArfGEFs. Putative homologues of TbArfGEFA, B and C were identified in *T. b. gambiense*, *T. vivax*, *T. congolense*, *T. cruzi*, and *L. major* genomes, indicating that these proteins are conserved throughout kinetoplastids.

A T-coffee alignment was carried out to align the novel ArfGEFs from *T. b. brucei*, *T. b. gambiense*, *T. vivax*, *T. congolense*, *T. cruzi*, and *L. major*. From this alignment a neighbour-joining phylogenetic tree was generated using the programme Mega 3.1 as shown in Figure 4.19. The neighbour-joining tree depicts three major clades, groups I, II and III, which are supported by bootstrap values of 99-100 %. This phylogenetic tree groups all the putative ArfGEFA sequences together in group I, all the putative ArfGEFB sequences together in group II, and all the ArfGEFC sequences together in group III. The phylogeny is consistent with the relationships between various kinetoplastid species. Based on this evolutionary relationship it is concluded that orthologous ArfGEFs have been identified in the various species and that these genes are likely to have a very similar function in the different species.

Subsequently, the putative kinetoplastid ArfGEFs were compared to other eukaryotic ArfGEFs with defined functions. Human and yeast ArfGEF sequences were identified in the GenBank database (<http://www.ncbi.nlm.nih.gov/>), and a comparison was carried out with the kinetoplastid ArfGEFs. A T-coffee alignment was generated using the Sec7 domains from all of the identified sequences, and a neighbour-joining phylogenetic tree was constructed (Figure 4.20).

The neighbour-joining tree was composed primarily of six groups where groups I, III and V comprised the kinetoplastid ArfGEFs B, A and C respectively. Group II kinetoplastid ArfGEFBs were shown to be most closely related to the human ArfGEF proteins (BIG1, BIG2, ARNO) and yeast SEC7. This implies that TbArfGEFBs and the human ArfGEFs (BIG1, BIG2 and ARNO) are likely to have similar functions acting as guanine nucleotide exchange factors, and as such are likely to function as ArfGEFs for TbArf1. Group III kinetoplastid ArfGEFAs and Group V kinetoplastid ArfGEFCs were shown to be less closely related to human ArfGEFs, and more like several yeast ArfGEFs. Group VI contained only EFA6, a human ArfGEF, and was separated from all other ArfGEF clades. EFA6 has been shown to interact mainly with HsArf6, rather than HsArf1 (Franco *et al.* 1999). As there is no Arf6 orthologue in *T. b. brucei*, it is not surprising that there was no EFA6 equivalent in *T. b. brucei*.

Characterised ArfGEFs have been shown to interact with Arf1-GDP and operate as guanine nucleotide exchange factors (Cherfils *et al.* 1998). Based on the evolutionary relationship it is concluded that the orthologous ArfGEFs that have been identified in *T. b. brucei* are likely to have a very similar function. More information regarding the mechanisms of how these kinetoplastid ArfGEFs operate was gathered by comparing TbArfGEFs to the resolved crystal structures of human ArfGEFs.

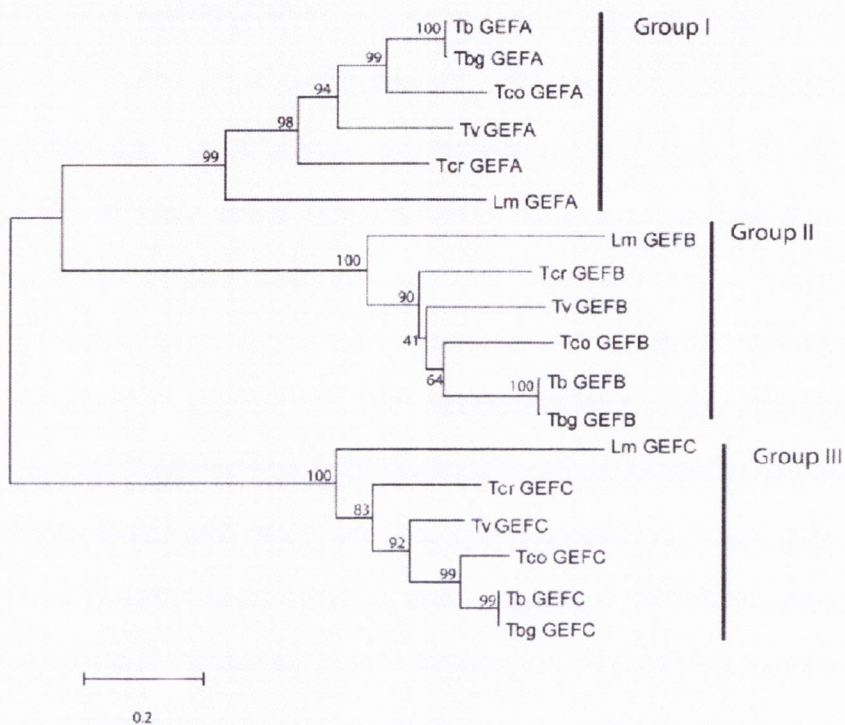


Figure 4.17. A neighbour-joining tree depicting the evolutionary relationship of putative trypanosome and *Leishmania* ArfGEF proteins. The neighbour-joining tree was generated from a T-coffee alignment of the conserved Sec7 domain of ArfGEFs. The alignment includes the following Sec7 domains: *T. b. brucei* ArfGEFA, B, C, *T. b. gambiense* ArfGEFA, B, C, *T. congolense* ArfGEFA, B, C, *T. vivax* ArfGEFA, B, C, *T. cruzi* ArfGEFA, B, C, and *L. major* ArfGEFA, B, C. The neighbour-joining tree was generated using the program Mega 3.1. 1000 bootstrap replicates were carried out.

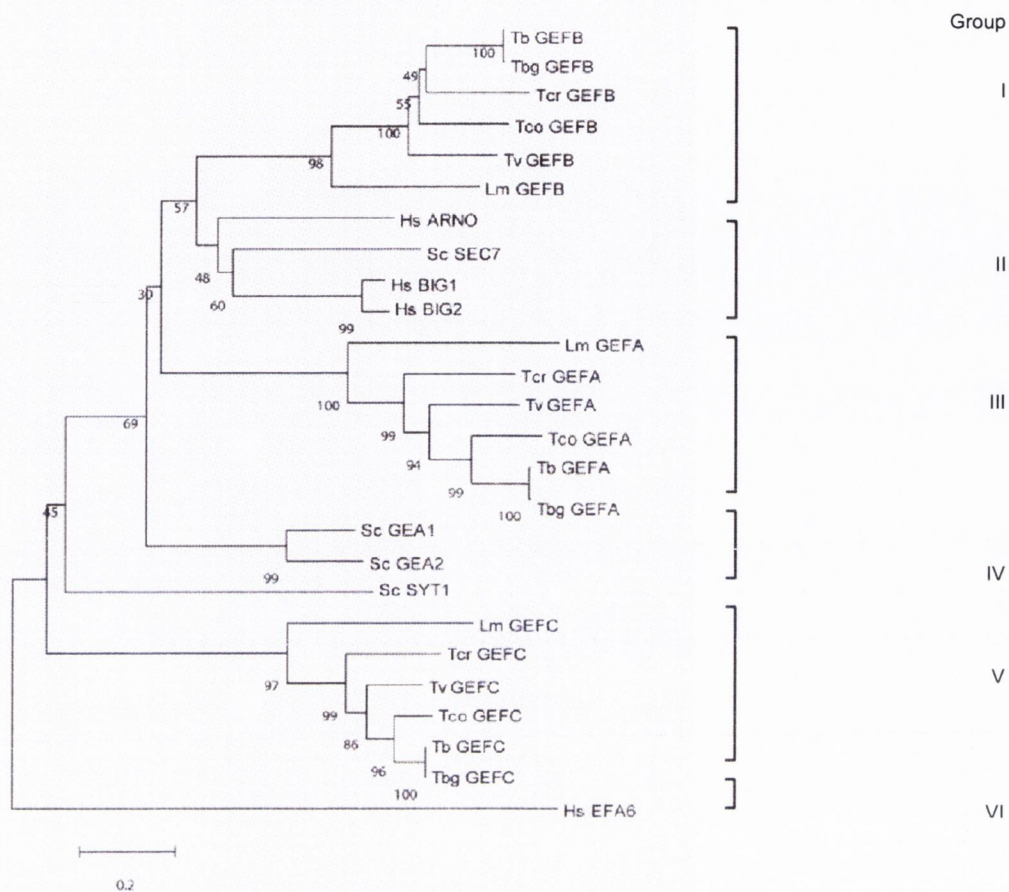


Figure 4.18. A neighbour-joining tree depicting the evolutionary relationship of human, yeast and kinetoplastid ArfGEF proteins. The neighbour-joining tree was generated from a T-coffee alignment of the Sec7 domain of ArfGEFs. The tree includes the following Sec7 domains of *H. sapiens*: BIG1, BIG2, ARNO, EFA6 proteins, *S. cerevisiae* GEA1, GEA2, SYT1, Sec7 proteins, *T. b. brucei* ArfGEFA, B, C, *T. b. gambiense* ArfGEFA, B, C, *T. congolense*: ArfGEFA, B, C, *T. vivax*: ArfGEFA, B, C, *T. cruzi*: ArfGEFA, B, C, and *L. major*: ArfGEFA, B, C. The neighbour-joining tree was generated using the program Mega 3.1. 1000 bootstrap replicates were carried out.

4.5.3 Initial Characterisation of TbArfGEFs Based on Sequence Composition

To elucidate the structural and functional relationships between the *T. b. brucei* and human ArfGEFs, trypanosomal TbArfGEFA, TbArfGEFB and TbArfGEFC protein sequences were superimposed on the resolved crystal structure of the HsARNO Sec7 domain (Cherfils *et al.* 1998; Mossessova *et al.* 1998). The HsARNO Sec7 model structure consists of 10 alpha-helices (A-J) arranged in a right-handed superhelix. The C-terminus of the Sec7 domain mediates the interaction with Arf1 through hydrophobic and polar residues. Specifically, alpha helices F, G and H form a deep hydrophobic groove, and a hydrophilic loop between helices F and G which act as the binding site for nucleotide free Arf1 (Cherfils *et al.* 1998; Mossessova *et al.* 1998). The sequence of the F-G loop (FRLPGE) is a highly conserved motif in the Sec7 domain. The F-G loop from human and *T. b. brucei* is depicted in Figure 4.21A, where the FRLPGE loop is highlighted in yellow. A schematic of the HsARNO sites of interaction is shown in Figure 4.21B. The loop is based around an invariant glutamate residue that plays a key role in the interaction between Arf1 and ArfGEFs, which is conserved in TbArfGEFA and TbArfGEFB, but not in TbArfGEFC.

Sequence conservation within TbArf1 and within TbArfGEF A-C is consistent with the model of interaction between the human ArfGEF (loop F-G) and Arf1 (switch 1 and 2) proposed by Cherfils *et al.* (1998). The switch 1 region of TbArf1 and HsArf1 are conserved, and there are only two substitutions in the switch 2 region of TbArf1 (K73V, I74L). The conservation of the F-G loop in the trypanosome ArfGEFs suggests that trypanosomal ArfGEFs may interact in a similar manner to that of ARNO.

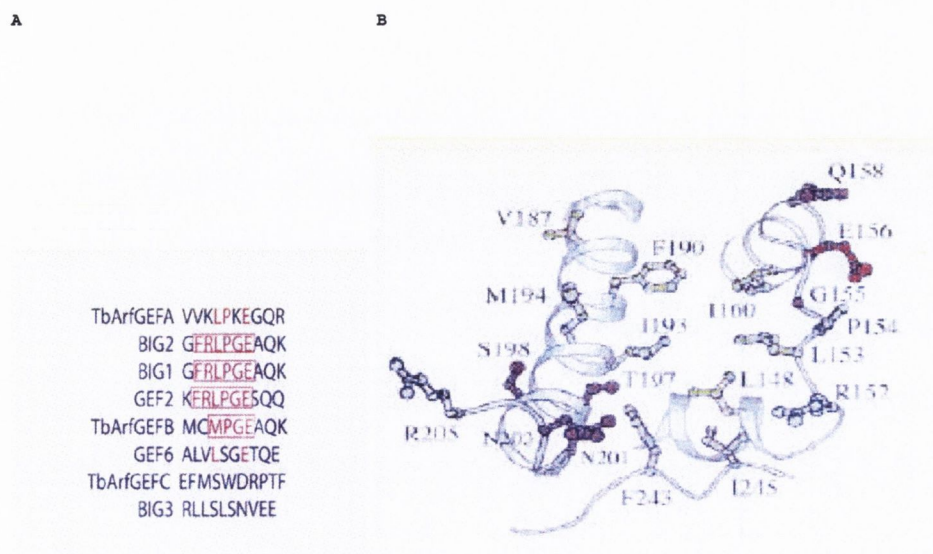


Fig 4.19. The FRLPGE loop of the Sec7 domain of ArfGEFs. Figure A depicts an alignment of the FRLPGE motif in TbArfGEF A, B, and C, and HsBIG1, 2, 3, HsGEF2, and HsGEF6. Residues identical to that of the human FRLPGE loop are highlighted in red, and in a red box. The alignment was produced using Multialin (Corpet 1998). (B) Figure B is a schematic depicting the putative Arf1 binding site in HsARNO. The figure depicts the FRLPGE loop of the Sec7 domain of the ArfGEF. This figure is taken from Cherfils *et al.* (1998).

4.6 Other Effectors of TbArf1

4.6.1 Identification of Other Effectors of TbArf1

As outlined in the introduction, Arf1 operates in conjunction with several other factors that enable it to function with greater specificity. These other effectors of Arf1

have been divided into two groups depending on whether they interact with Arf1 in its GTP- or GDP-bound form.

Apart from ArfGEFs, which have previously been discussed, members of the p24 family also interact with Arf1-GDP. P24 family members were searched for and identified in the *T. b. brucei* database. Other than ArfGAPs, other Arf-GTP interacting proteins include vesicle coat components (COPI, AP1/3/4, and GGA1/2/3), phospholipid kinases, and other factors such as Arfaptin. These Arf-GTP interacting partners were searched for in the *T. b. brucei* database, putative homologues were identified and sequence analysis was carried out.

4.6.2 Identification of the P24 Family in *T. b. brucei*

P24 proteins are a family of membrane-bound proteins that have been shown to interact with Arf1-GDP, and are implicated as possible cargo receptors in vesicular trafficking (Barr *et al.* 2001). A literature search was carried out to identify key p24 proteins in humans. Homologues were identified in the *T. b. brucei* database using the OmniBlast function of GeneDB (<http://www.genedb.org/>) as outlined in the materials and methods section. One p24 precursor protein homologue was located in the *T. b. brucei* Sanger database, COP-coated vesicle membrane protein Erv25. This protein was predicted to be 253 amino acids, with a predicted molecular weight of 28 kDa, and a predicted pI of 10. These parameters were predicted using compute pI/Mw (http://expasy.org/tools/-/pi_tool.html). A transmembrane protein program, using TMHMM (<http://www.cbs.dtu.dk/services/TMHMM-2.0/>), predicted that COP-coated vesicle membrane protein Erv25 had two transmembrane domains. An N-terminal

signal peptide was also predicted using the SignalP 3.0 Server (Bendsten *et al.* 2004, <http://www.cbs.dtu.dk/services/-SignalP/>).

4.6.3 Identification of COPI-Arf-GTP Interacting Components in *T. b. brucei*

COPI is a major component of the coatamer complex (Kirchhausen 2000). The protein sequences of known COPI components that interact with Arf1, β and γ COPI, were downloaded from NCBI (<http://www.ncbi.nlm.nih.gov/>). Homologues in the *T. b. brucei* GeneDB database were identified using the OmniBlast function of GeneDB (Table 4.6). Putative proteins for both β and γ components of COPI that interact with the switch 1 region of Arf1 were located in the *T. b. brucei* GeneDB database.

4.6.4 Identification of Adaptin-Arf-GTP Interacting Components in *T. b. brucei*

Adaptin proteins are part of the coatamer of vesicles and have been shown to interact with Arf1- GTP. Adaptin components AP-1, AP-3: β , γ and δ and AP-4: ϵ and μ are thought to interact with switch 1 of Arf1 (Nie *et al.* 2003b; Nie *et al.* 2003a). Protein sequences of each human adaptin were downloaded from NCBI (<http://www.ncbi.nlm.nih.gov/>). Again, searches using the OmniBLAST function of GeneDB (<http://www.genedb.org/>) were used to identify homologous *T. b. brucei* genes (Table 4.7). Not all these components were identified in the *T. b. brucei* Sanger database, although many (11 of the 24 components) of the AP-1, AP-3 and AP-4 components were identified. AP-2 was missing completely.

4.6.5 GGAs

In yeast and mammals, GGA proteins are Golgi localised, γ -ear-containing, monomeric clathrin adaptors, that interact with Arf1 (Bonifacino 2004). GGAs possess 4 domains; a VHS domain, a GAT domain, a hinge-linker domain and a GAE domain. Homology searches of the *T. b. brucei* GeneDB (<http://www.genedb.org/>) database were carried out using yeast Gga1 and Gga2, and human GGAs (GGA1, GGA2, and GGA3). No significant homologues were found in the Sanger *T. b. brucei* database. Searches of individual domains were also undertaken. The *T. b. brucei* database was searched for proteins with homology to the VHS domain, the GAT domain, and the GAE domain respectively. Three proteins were identified with GAE domains, and two proteins were found to contain VHS domains. No significant results were found with the GAT domain and no proteins were identified with more than one domain. GGAs appear to be missing from the *T. b. brucei* genome.

4.6.6 Phospholipid Kinases

Phospholipids are important in the activation of Arf1 and in coating of the clathrin coated vesicles (Paris *et al.* 1997). Although not much is known about how phospholipids interact with Arf, phosphatidylinositol has been shown to be an important effector of Arf1 (Beemiller *et al.* 2006). Two phosphatidylinositol-4-phosphate 5-kinases (PIP5KII- α) were identified on chromosome 4 and 10 of *T. b. brucei*.

4.6.7 Other Factors that Interact with Arf-GTP

Two other factors that interact with Arf-GTP, Arfaptin 2 and Arfophilin, were sought in the *T. b. brucei* database (<http://www.genedb.org/>). Arfaptin is also known as ADP-ribosylation factor interacting protein 2 or POR1. Arfophilin is a known target protein for GTP-bound Arf (Nie *et al.* 2003a). Homology searches were carried out using the human Arfaptin2 and Arfophilin proteins. No significant homologues for either protein were found in the Sanger *T. b. brucei* database.

Table 4.6. Putative COPI- Arf1 interacting proteins in *T. b. brucei*. The table indicates whether known COPI components that interact with Arf1 were present in the *T. b. brucei* GeneDB database (<http://www.genedb.org/>). The gene I.D. and molecular weight are given also.

COPI component	Present in <i>T. b. brucei</i>	Annotation	Size
β	Yes	Tb927.1.2570	110 kDa
α	Yes	Tb11.01.3740	97 kDa

Table 4.7. Putative Adaptin proteins in *T. b. brucei*. The table indicates known adaptin components and whether they were present in the *T. b. brucei* GeneDB database.

Chain						
Adaptin	β	γ	δ	ε	μ	φ
AP-1	Present	Present	Absent	Absent	Present	Absent
AP-2	Absent	Absent	Absent	Absent	Absent	Absent
AP-3	Present	Absent	Present	Absent	Present	Present
AP-4	Present	Absent	Absent	Present	Present	Present

4.7 Discussion: Arf1 and Effectors of Arf1 in *T. b. brucei*

Arf1 is an important protein in cytoskeleton structure and vesicular trafficking in the cell (Brown *et al.* 1993). This is also likely to be true of Arf1 in *T. b. brucei* (TbArf1). In addition, TbArf1, has also been implicated in the differentiation of long slender to short stumpy *T. b. brucei* cells (Murphy and Olijhoek 2001). Therefore, TbArf1 and related factors are of interest in understanding the biology of African trypanosomes and related kinetoplastids. A bioinformatics approach was taken to identify and phylogenetically classify putative Arf1, related proteins and effector proteins in *T. b. brucei*.

Two genes encoding putative Arf proteins, TbArf1 and TbArf3 were identified in *T. b. brucei* (<http://www.genedb.org/>), along with eight putative Arf-like proteins, Arls (TbArl2, TbArl3, TbArl6, TbArl17, TbArl18, TbArl19, TbArl20 and TbArl21), as confirmed by Field *et al.* (2005). All of the putative Arf and Arl proteins were in the 20-22 kDa range, and some were also predicted to be myristoylated. Phylogenetic classification studies that were carried out on TbArf and TbArl proteins showed that TbArf1 was closely related to the human Arf proteins, but not to any protein in particular, such that it is likely that TbArf1 incorporates the functions several of the Arf proteins. Further phylogenetic classification of Arf1 from *H. sapiens*, *M. musculus*, *C. elegans*, *D. discoideum*, *S. cerevisiae*, *X. laevis*, *L. major*, *T. b. gambiense*, *T. vivax*, *T. congolense* and *T. cruzi* followed. This revealed that the helix $\alpha 3$ region of Arf1 was observed to be highly conserved in kinetoplastids but divergent in comparison to other eukaryotes, suggesting the helix $\alpha 3$ as a reason for the additional/altered function of TbArf1. Also, examination of the genomic organisation

of TbArf1 showed that one of the four alternative ORFs encoding TbArf1 was differentially expressed at different life cycle stages.

This divergence in the helix $\alpha 3$ region of Arf1 has great significance as a possible additional function of TbArf1 and also impacts the localisation of TbArf1 within the cell. Through use of chimera studies fusing C-terminal regions of human Arf1 and Arf6 to N-terminal regions of human Arf1 and Arf6 Donaldson *et al.* (2005) showed that a 16 amino acid region around the helix $\alpha 3$ was key to localisation of human Arf1 to the Golgi. The same is not true for human Arf6, where both sequences in the N- and C-terminals play roles in targeting human Arf6 to the plasma membrane (Donaldson and Honda 2005). This suggests that the divergence of trypanosomal Arf1 from Human Arf1 in this helix $\alpha 3$ region may promote targeting of *T. b. brucei* Arf1 to the Golgi membrane in addition to the plasma membrane (as seen in Chapter 1, Figure 1.9), where TbArf1 plays a role in differentiation of long slender to short stumpy bloodstream forms.

Subsequently, effector proteins of TbArf1 that positively and negatively regulate TbArf1 were investigated. Five putative ArfGAP proteins, negative regulators of Arf1, were identified; TbArfGAP I, TbArfGAP II, TbArfGAP III, TbArfGAP IV and TbArfGAP V. Phylogenetic classification studies found TbArfGAP I to be most closely related to the human ArfGAP1. Initial characterisation studies, superimposing the protein sequence of TbArfGAP I on the resolved ArfGAP1 crystal structure (Goldberg 1999), suggested that TbArfGAP I most likely functioned in a similar manner to the human counterpart, ArfGAP1. Co-expression studies were attempted, to confirm the details of this TbArf1/TbArfGAP V interaction; however, as the complex was unable to form, the model could not be tested.

Genes encoding putative ArfGEFs, positive regulators of Arf1, were identified in the *T. b. brucei* database (<http://www.genedb.org/>). Three putative ArfGEFs were identified in *T. b. brucei*, TbArfGEFA, TbArfGEFB and TbArfGEFC. Three ArfGEFs were also identified in the other kinetoplastid sequence databases investigated. Of the ArfGEFs, ArfGEFBs were the most like human ArfGEFs. An initial characterisation, based on superimposing the TbArfGEF protein sequence on the resolved crystal structure of human ARNO ArfGEF, was carried out. It showed that there was high sequence conservation in the F-G loop of TbArfGEFs with ARNO, suggesting that trypanosomal ArfGEFs also function in a similar way to human ArfGEFs.

Other putative effectors of TbArf1 were identified in the *T. b. brucei* Sanger database (<http://www.genedb.org/>). These included a putative member of the p24 family, COP-coated vesicle membrane protein Erv25, which may have a role as a cargo receptor. Components of the COPI coatomer, and adaptins were also identified, both with roles in construction of vesicles. However, other proteins with roles in vesicle construction were missing; there were no GGA homologues present. Arfaptin2 and Arfophilin were also missing from the Sanger *T. b. brucei* database, though two putative phosphatidylinositol 4-phosphate 5-kinases were present.

The presence/absence of putative homologues of known trafficking proteins in the *T. b. brucei* database can be used to develop a model for vesicle trafficking in *T. b. brucei*. Of particular note is the absence of homologues of GGA in the *T. b. brucei* database. GGAs play a large role in the production of clathrin coated vesicles from the Golgi, thus the construction of clathrin-coated vesicles from the Golgi in African trypanosomes must occur by a different mechanism to that of the GGA clathrin-coated mechanism. Instead the trypanosome may use adaptor-molecules to facilitate

the construction of its clathrin-coated vesicles, such as AP-1 and AP-2 which are required for endocytosis (Morgan *et al.* 2001).

Chapter 5: Signalling in the African Trypanosome, *T. brucei*

5.1 Summary

Differentiation of long slender *T. b. brucei* bloodstream forms occurs via a cell density dependent mechanism. In this chapter, the role of TbArf1, a factor previously implicated in this differentiation process, was further investigated. An assay was developed to examine the effect of signalling factors, implicated in this differentiation process, on effector proteins of TbArf1. Specifically, inhibition of interactions between TbArf1 and its effector proteins were investigated.

Two signalling factors, cAMP and T-GIF, both previously implicated in differentiation, were shown to inhibit interactions of TbArf1 and its effector proteins. This assay was also used to examine the expression of TbPP69 which has previously been shown to interact with TbArf1. Overexpression of ESAG10 resulted in increased production of a protein with properties similar to TbPP69 further implicating ESAG10 in the differentiation process.

5.1.1 Introduction to Cell Signalling in the African Trypanosome, *T. b. brucei*

Differentiation in bloodstream forms of the African trypanosome, *T. b. brucei*, involves a cell density signalling pathway that controls the number of cells in the

population that are in the long slender actively-dividing and short stumpy non-dividing form (Vassella *et al.* 1997). This signalling pathway results in the differentiation of the long slender cells via a secreted signalling factor, the trypanosome growth inhibitory factor, (T-GIF) (Murphy and Olijhoek 2001), also referred to as SIF in the case of *T. brucei* (Vassella *et al.* 1997). Lab-derived strains which cannot undergo this long slender to short stumpy differentiation step (monomorphic strains) are unable to detect and respond to this factor (Vassella *et al.* 1997). Pleomorphic strains (which can undergo this differentiation process) are able to detect and respond to this signalling factor possibly by import via the TbPP69 receptor or transporter (a putative receptor upregulated in pleomorphic strains that has been implicated in differentiation) (Murphy and Olijhoek 2001). This differentiation signal must then be propagated throughout the cell via a cell signalling cascade. As has been discussed in previous chapters, TbPP69 has the same subcellular distribution as TbArf1, a protein involved in vesicle formation, and TbArf1 appears to be an internal target in the cell for the signalling factor.

Proteins implicated in the long slender to short stumpy signalling event include adenylate cyclase and protein kinase A, both of which have been previously shown to be located along the flagellum of *T. brucei* (Paindavoine *et al.* 1992), suggesting that the flagellum is a site of cell signalling. In addition, both TbArf1 and TbPP69 have been shown to be co-located in this region.

5.1.2 cAMP is Implicated in Differentiation in *T. b. brucei*

cAMP induces quiescence (a G₀-like state, a pause in cell development) in long slender forms and subsequently initiates differentiation to short stumpy forms

(Vassella *et al.* 1997). Artificial pressure such as a change in pH and temperature, that can force cells from the G₁ stage to the G₀-like state may also be sufficient to initiate the differentiation process (Vassella *et al.* 1997). As with higher eukaryotic cells where cAMP generally acts through a cascade of events involving cAMP-binding proteins, cAMP-dependent protein kinases and phosphorylated nuclear factors (Vandromme *et al.* 1994), the ultimate role of cAMP in *T. b. brucei* may be to modulate the level of expression of genes which are more directly involved in differentiation.

A derivative of cAMP, 8-(4-Chlorophenylthio)-cAMP, pCPTcAMP, has previously been shown to induce differentiation in *T. brucei* cells in a manner similar to SIF (Vassella *et al.* 1997). This suggested that cAMP was indeed the secondary messenger mediating the SIF signal in differentiation. This concept was further tested by measuring the intracellular cAMP content of long slender cells following short incubation in conditioned medium (medium extract from cells grown at high density) (Vassella *et al.* 1997). A 2-3 fold elevation of cAMP was observed 15 minutes after the addition of conditioned medium, followed by a slight decrease after 30 minutes. This observation was compatible with a direct receptor-mediated effect, the decrease in cAMP being due to some kind of desensitisation. This further supported the theory that SIF signals density information via the cAMP pathway. Low concentrations of a phosphodiesterase inhibitor, etazolate, had the same effect as SIF or the cAMP analogue, suggesting that endogenous production of cAMP by adenylate cyclases is sufficient to induce differentiation (Vassella *et al.* 1997).

Compartmentalisation of phosphodiesterase has been shown to have a role in the regulation and specificity of cAMP signalling in mammals (Houslay and Milligan 1997) and the possibility that this occurs in kinetoplastids was investigated. cAMP-

specific phosphodiesterases, TbPDE1 and TbPDE2 were examined in *T. brucei* (Gong *et al.* 2001; Zoraghi *et al.* 2001; Zoraghi and Seebeck 2002) and the TbPDE2 family was shown to be crucial to survival. PDE1 was also examined in *T. cruzi* (D'Angelo *et al.* 2004).

In *T. cruzi*, cAMP has been shown to be important in allowing the parasite to invade the mammalian host cell. In particular, cAMP agonists promote the movement of lysosomes towards the cell periphery and facilitate Ca^{2+} -regulated lysosome exocytosis which enhances *T. cruzi* invasion of fibroblasts (Rodriguez *et al.* 1999). Furthermore, infective forms of *T. cruzi* activate adenyl cyclase in mammalian cells to increase the levels of cAMP (Rodriguez *et al.* 1999).

5.1.3 Programmed Cell Death in the African Trypanosome

Cell differentiation from long slender to short stumpy cells is described as a 'terminal differentiation step' (Vassella *et al.* 1997) and is coupled to growth arrest in the mammalian host. Several signalling factors have been implicated in this terminal step, which is also referred to as a type of programmed cell death. Figarella *et al.* (2005) demonstrated that prostaglandin D_2 (PGD_2) can induce a cell density-dependent programmed cell death in African trypanosomes. They showed that trypanosomes produce different prostaglandins in the course of development: prostaglandin $\text{F}_{2\alpha}$ ($\text{PGF}_{2\alpha}$) was mainly produced by fast dividing long slender and procyclic forms, whereas PGD_2 was mainly produced in short stumpy parasites. Prostaglandin E_2 (PGE_2) was also detected in other life cycle stages.

Figarella *et al.* (2005) showed that PGD_2 is not the SIF factor, as it is not produced by long slender cells, but by short stumpy cells. Also, PGD_2 has a short

half-life and works only in the immediate environment, rendering the PGD₂ molecule ineffective after triggering cell death. The effect of PGD₂ on the differentiation/cell death of *T. b. brucei* will be investigated further in the course of this chapter.

The focus of this chapter is on the internal signalling events that are triggered during the initial stages of differentiation and to elucidate whether cAMP and other signalling factors play a role in TbArf1-mediated differentiation.

5.2 Effects of Signalling Factors on the Growth of Long Slender Trypanosomes

Using cell culture techniques, a series of assays were undertaken to investigate the effects of several differentiation signalling factors on monomorphic long slender bloodstream form *T. b. brucei* 328.114 cells. Several experiments were carried out using different concentrations of cell signalling factors, which have been previously shown to affect the growth of *T. b. brucei* cells in a concentration dependent manner. The effect of these signalling factors on growth rate and viability were recorded based on cell counts with a haemocytometer.

5.2.1 The Effect of cAMP on the Growth of Bloodstream Form Trypanosomes

cAMP, a known signalling factor, implicated in the differentiation of long slender to short stumpy bloodstream forms, was added to cell cultures. Addition of cAMP to pleomorphic and monomorphic cells has previously been shown to cause growth arrest and differentiation (Vassella *et al.* 1997; Breidbach *et al.* 2002). More recently,

hydrolysed analogues of membrane permeable cAMP have been shown to prevent proliferation of long slender parasites (Laxman *et al.* 2006). In this chapter, the effect of cAMP on differentiation was further investigated in comparison to a control signalling factor, cGMP, which has not been implicated in this differentiation. cGMP has several properties similar to those of cAMP.

0, 1, 2 and 4 mM concentrations of cGMP (Figure 5.1) and membrane permeable pCPTcAMP (Figure 5.2) were added to bloodstream form *T. b. brucei* 328.114 cells, and cells were monitored over 4 days. Cells were counted daily at specific times and growth rates and viability of the cells were noted. As expected, no difference in growth rate was noted comparing cell growth with addition of 1, 2, and 4 mM cGMP, to the growth of cells without the addition of cGMP. Unlike cAMP, cGMP had not previously been implicated in the differentiation of long slender to short stumpy cells, and no impact was expected or observed.

pCPTcAMP is a lipophilic derivative of cAMP, which is resistant to phosphodiesterases. Addition of pCPTcAMP to pleomorphic AnTat1.1 cells has been shown to initiate short stumpy morphology in cells under axenic conditions (Vassella *et al.* 1997). A similar morphology was also observed in monomorphic cells (culture-adapted 221a *T. b. brucei* cells) upon addition of pCPTcAMP (Breidbach *et al.* 2002). Arrest of cell growth was therefore expected in the *T. b. brucei* 328.114 cells. Unexpectedly, the addition of membrane permeable cAMP to the cell culture revealed no differences compared to the control cells (cells without addition of cAMP or cGMP). After 3 days, no difference in growth rate was noted and cells were diluted down to 10^4 cells/ml. The growth rate was then observed for another 2 days to allow time for the addition of pCPTcAMP to impact on cell growth. Cells continued to

proliferate at the same rate as the untreated control. Using three times the amount of cAMP that Breidbach *et al.* (2002) used did not have an effect on the cell growth.

These results differed greatly from that of Breidbach *et al.* (2002) where at lower concentrations of a cAMP analogue (0.1, 0.5 and 1 mM cAMP) a decline in the growth rate and viability of the cells was observed. The reason for the different results in the Breidbach experiment and this experiment is unclear. Breidbach *et al.* (2002), used 221a *T. b. brucei* cells compared to the 328.114 cells used in this experiment and a different medium was used (Baltz medium not HMI-9). Baltz medium does not differ greatly from HMI-9 medium and it is unlikely that the medium used would have such an effect on the result of the experiment. It is more likely that the strain used had an effect on the outcome of the experiment, although *T. b. brucei* 221a and *T. b. brucei* 328.114 cells are both monomorphic cell lines. In the strain *T. b. brucei* 221a the addition of pCPTcAMP to the medium induces differentiation (1 mM pCPTcAMP); this does not appear to be the same for *T. b. brucei* 328.114 cells, where differentiation was not initiated. These results highlight that findings in one cell line are not necessarily relevant for all cell lines.

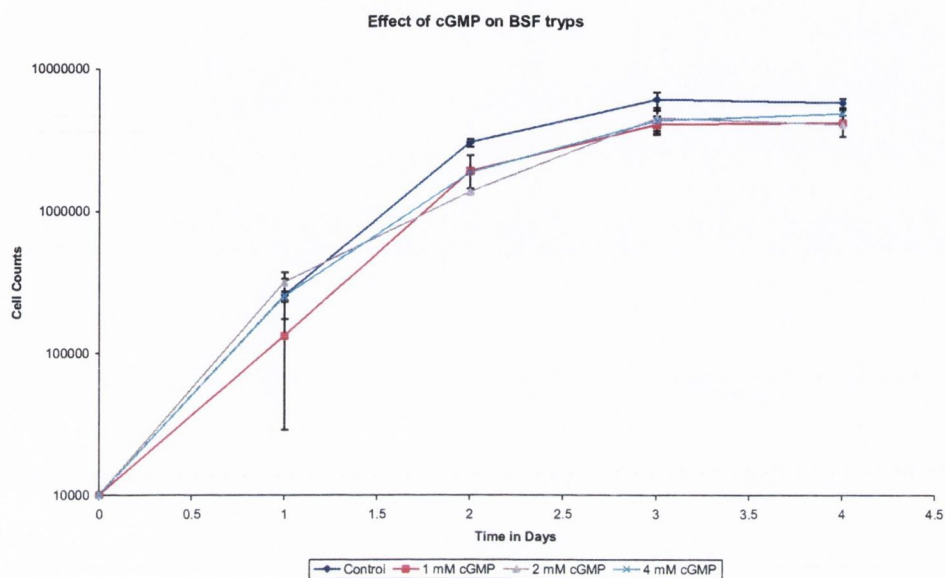


Fig 5.1. The effect of cGMP on bloodstream form *T. b. brucei* cells. The above figure depicts a logarithmic graph displaying the effects of cyclic GMP on the growth of monomorphic 328.114 *T. b. brucei* cells, as counted daily on a haemocytometer. The four lines shown represent the control cells (dark blue), cells with addition of 1 mM cGMP (pink), cells with addition of 2 mM cGMP (grey), and cells with addition of 4 mM cGMP (light blue). Cells proliferated until day 3, and then began to die out. There was no difference in the pattern of growth in cells with or without the addition of cGMP.

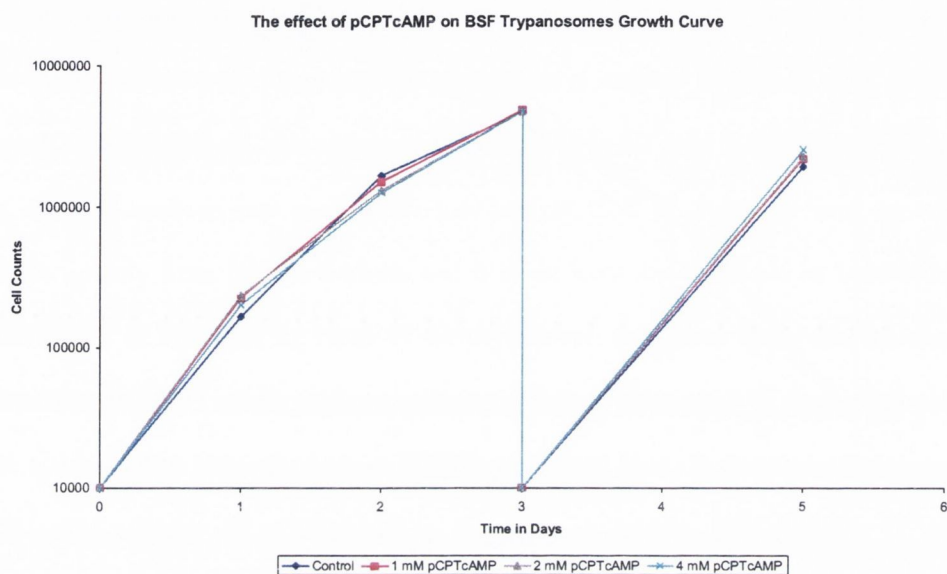


Fig 5.2. The effect of pCPTcAMP on the growth of bloodstream form *T. b. brucei* cells. Cells were grown for three days, before dilution back to 1×10^4 cells/ml. Cells were then grown for a further 2 days. Four lines are shown representing the control cells (dark blue), cells with the addition of 1 mM pCPTcAMP (pink), cells with the addition of 2 mM pCPTcAMP (grey), and cells with the addition of 4 mM pCPTcAMP (light blue). No difference between the control cells and the cells with the addition of pCPTcAMP was observed.

5.2.2 The Effect of Prostaglandins on the Growth of Bloodstream Form *T. b. brucei*

Following up on evidence that the levels of prostaglandins increased in patients with sleeping sickness, it was shown that trypanosomes produced several prostaglandins, including prostaglandin D₂, E₂ and F₂ from arachidonic acid during infection (Kubata *et al.* 2000; Kabututu *et al.* 2002). To investigate the effect of prostaglandins on rates of *T. b. brucei* cell growth in culture, Figarella *et al.* (2005) added PGD₂ to *T. brucei* MITat1.2 (VSG variant 221) monomorphic strain EATRO 427 parasites at concentrations of 0, 2.5, 5 and 7.5 µM. Cells were seeded at 2x 10⁵ cells/ml and the growth rates of the cells were monitored over 72 hours. In addition, the IC₅₀, the concentration necessary to inhibit cell growth by 50%, was calculated for PGD₂, PGE₂ and PGF₂.

In the current study a similar experiment was carried out using a different isolate of *T. b. brucei*, 328.114, using PGD₂, PGE₂, PGF₂ over a range of concentrations, and their effects on cell growth were monitored. The concentrations chosen were based on the previously determined IC₅₀ of PGD₂, PGE₂ and PGF₂ (Figarella *et al.* 2005). 0, 2.5, 5 and 7.5 µM PGD₂, and 0, 100, 200 and 300 µM PGE₂ and PGF₂ were incubated with cells seeded at 1x 10⁴ cells/ml and monitored over 96 hours.

The addition of 2.5 µM PGD₂ had no effect on the growth of *T. b. brucei* 328.114 cells, which grew at a rate similar to that of wild-type cells (Figure 5.3). Addition of higher concentrations of PGD₂ had a more dramatic effect where the addition of 5 µM and 7.5 µM amounts of PGD₂ caused inhibition of growth of cells. These results were similar to that of Figarella *et al.* (2005) where both 5 and 7.5 µM of PGD₂ caused inhibition of cell growth, such that a similar effect of PGD₂ was

observed on the growth rate of both 328.114 (by our lab) and MITat 1.2 (by Figarella) *T. b. brucei* cells. IC₅₀ values were also consistent between the strains.

In the case of PGE₂, concentrations of 0, 100, 200 and 300 µM PGE₂, chosen around the previously determined IC₅₀ value of PGE₂ (159 µM) (Figarella *et al.* 2005), were added to bloodstream form 328.114 cells. At all concentrations growth inhibition was immediate and complete (Figure 5.4). As a consequence, the concentrations of PGE₂ were lowered, so that a concentration dependent effect could be investigated. PGE₂ was added at 0, 2.5, 5 and 7.5 µM PGE₂ concentrations. PGE₂ was shown to have a concentration dependent effect on inhibition of cell growth at these concentrations (Figure 5.5) in contrast to the findings of (Figarella *et al.* 2005). Thus for *T. b. brucei* 328.114 PGE₂ displays similar inhibitory effects on the growth of bloodstream forms to that of PGD₂ where increasing amounts of PGE₂ from 2.5 to 5 to 7.5 µM successively increased the inhibition of growth on the cells.

The IC₅₀ value in this experiment differed greatly from the value given in the Figarella *et al.* (2005) paper. Instead of 50% of cell growth inhibition occurring at 150 µM concentrations of PGE₂, the IC₅₀ was observed to be 5 µM in the *T. b. brucei* 328.114 strain. This result showed that the growth inhibition effect of PGE₂ differs between *T. b. brucei* cell lines.

A similar experiment was carried out for PGF₂, where 100, 200 and 300 µM concentrations of PGF₂ (based on PGF₂ having an IC₅₀ of 199 µM) were added to the cell culture (Figure 5.6). No effect on growth inhibition was noted, as cells proliferated at equivalent rates to the control cells. This result also differed from the IC₅₀ value of PGF₂ given in the Figarella *et al.* (2005) paper (199 µM). The actual IC₅₀ for *T. b. brucei* 328.114 strain is likely to be higher, showing that the effect on growth by PGF₂ differs between strains of *T. brucei*.

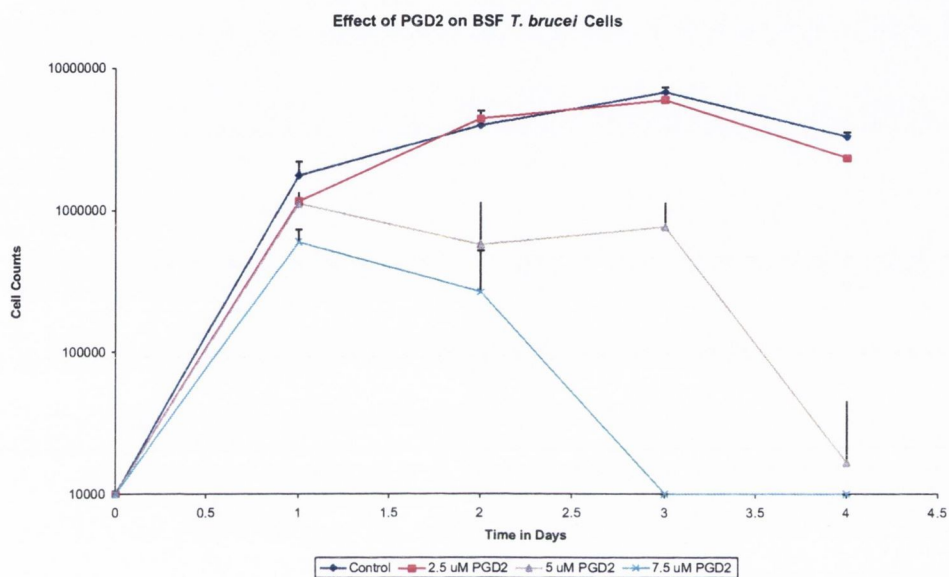


Fig 5.3. The effect of PGD₂ on bloodstream form *T. b. brucei* 328.114 cells. The effect of PGD₂ on the growth of monomorphic 328.114 *T. b. brucei* cells was monitored using a haemocytometer. Control cells are represented in dark blue, 2.5 μ M PGD₂ (pink), 5.0 μ M PGD₂ (grey), and 7.5 μ M PGD₂ (light blue). The graphs shows that concentrations $\geq 5 \mu$ M have a deleterious effect on cell growth.

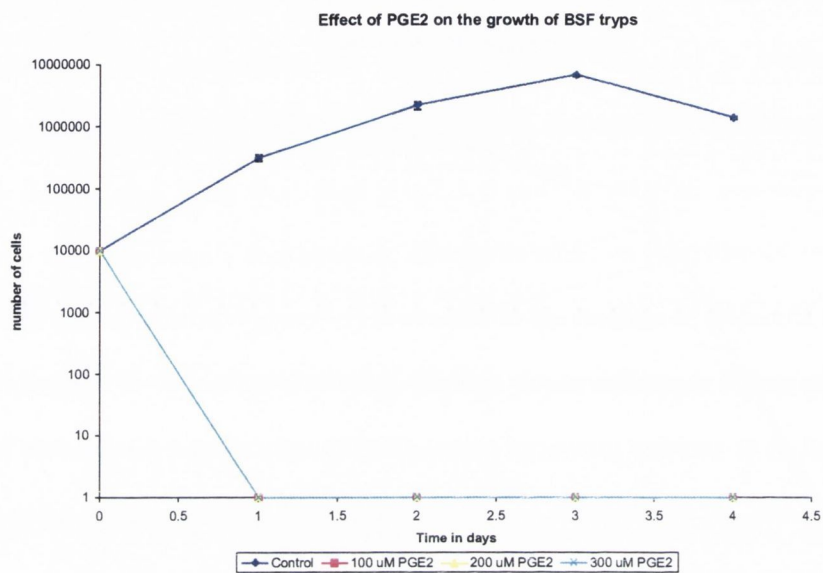


Fig 5.4. The effect of 100-300 μ M PGE₂ on bloodstream form *T. b. brucei* 328.114 cells. The effect of PGE₂ on the growth of monomorphic *T. b. brucei* 328.114 cells. Control cells (dark blue), 100 μ M PGE₂ (pink), 200 μ M PGE₂ (grey), and 300 μ M PGE₂ (light blue). Cells had all died out on day 1 with PGE₂ concentrations ≥ 100 μ M.

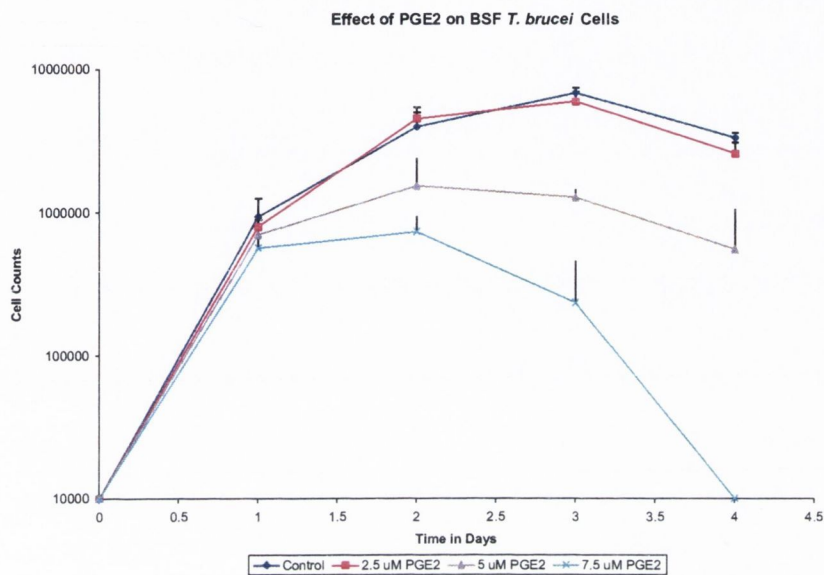


Fig 5.5. The effect of PGE₂ on bloodstream form monomorphic *T. b. brucei* 328.114 cells. Control cells (dark blue), 2.5 μ M PGE₂ (pink), 5.0 μ M PGE₂ (grey), and 7.5 μ M PGE₂ (light blue). Cells grew exponentially for the first day, and subsequently, the effect of the addition of PGE₂ on growth rate was observed, as cells began to die.

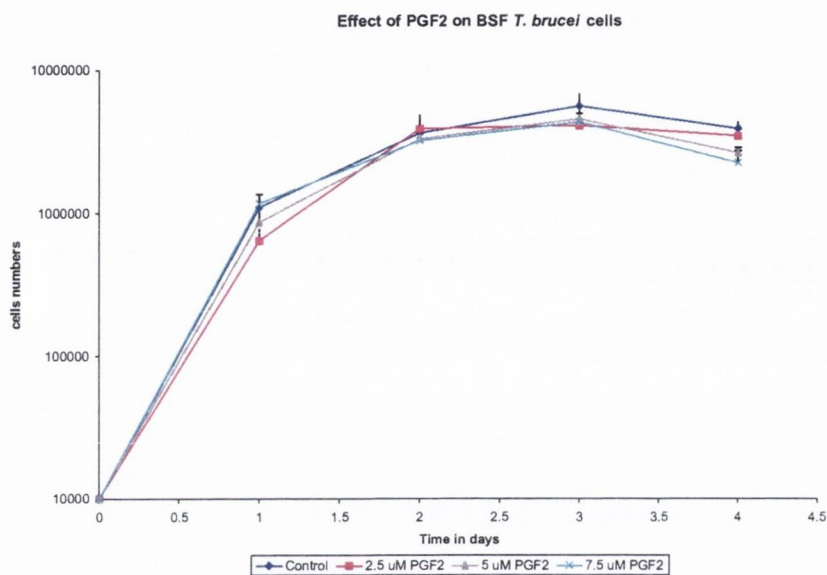


Fig 5.6. The effect of PGF₂ on bloodstream form monomorphic *T. b. brucei* 328.114 cells. Control cells (dark blue), 2.5 μ M PGF₂ (pink), 5.0 μ M PGF₂ (grey), and 7.5 μ M PGF₂ (light blue). Cells grew exponentially similar to the control cells and no effect on growth rate was apparent following addition of PGF₂ to the culture.

5.2.3 The Effect of Arf1 as an Inhibitor of Signalling Factors

Investigating the effect that signalling factors (prostaglandins and cAMP) have on differentiation and the role, if any, that Arf1 (TbArf1) has to play is the focus of this chapter. We postulate that the putative target of the T-GIF signalling factor, TbArf1 interacts with the differentiation signalling factors. An experiment was carried out to determine whether TbArf1 had an effect on the ability of prostaglandin to inhibit growth of the bloodstream form cells.

TbArf1 has previously been identified as the target of T-GIF, the trypanosome differentiation factor. T-GIF was shown to bind to a column containing TbArf1 (see introduction) (Murphy and Olijhoek 2001). The composition of the T-GIF factor, however, has not been deduced. As shown in the previously section, prostaglandin D₂ and E₂ cause inhibitory growth effects on bloodstream form cells. We speculated that prostaglandins may affect the growth of cells via a TbArf1 dependent pathway.

To investigate this, the effect of PGD₂ (5 µM concentration) on cell growth was monitored in the presence and absence of excess TbArf1 (Figure 5.7). 5 µM PGD₂ was chosen as a suitable concentration as it produced adequate growth inhibitory effects when added to the cell culture (Figure 5.3). Recombinant TbArf1 alone had no effect on the growth of the cells. Once PGD₂ was added to the cells, the cells all died within two days, even in the presence of recombinant TbArf1. Though, on day 1 there was a minor proliferative effect (75%), cells quickly died away by day 2. This showed that the PGD₂ inhibitory effect on the growth of cells was not reversed by the addition of TbArf1. A control of non-specific protein should be added to PGD₂ and this experiment repeated.

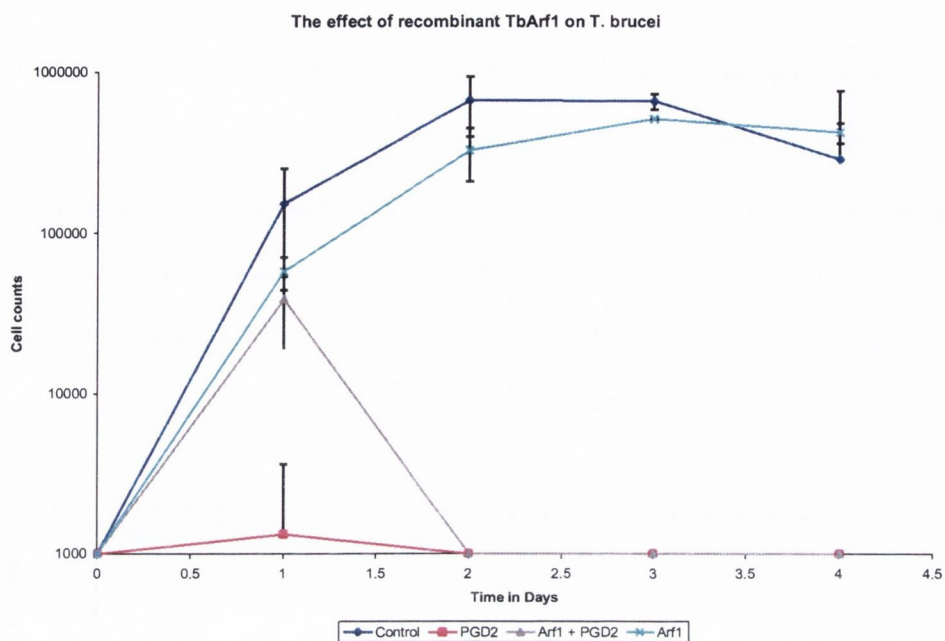


Fig 5.7. The effect of recombinant TbArf1 on bloodstream form *T. b. brucei* cells inhibited with 5 μ M PGD₂. The graph displays the effects of TbArf1 added to the cells (light blue line). This follows a similar trend to the control, untreated cells (dark blue line). The addition of PGD₂ to TbArf1 (grey line) displayed a similar trend to that of PGD₂ alone (pink); death occurred within two days. Note that the grey and pink lines follow the same trend. The addition of TbArf1 to the cell had a proliferative effect on day 1, but cells quickly died by day 2, suggesting that there is no significant influence on the inhibitory effect of PGD₂.

5.3 Detection of TbArf1-Binding Proteins

5.3.1 An Assay to Demonstrate the Effect of Signalling Factors on TbArf1, *in vitro*

A different approach was subsequently taken to elucidate the effect of several signalling factors on the role, if any, of TbArf1 in the differentiation process. An assay was developed to examine TbArf1 interactions with other trypanosomal proteins in the absence and presence of signalling factors. To do this purified recombinant TbArf1 protein was bound to a nickel resin column via its His-tag domain. Bound TbArf1 was incubated with signalling factors and the ability to bind other trypanosomal proteins was investigated. Only proteins that bound to TbArf1 would be recovered. Recombinant TbArf1 was treated with the following signalling factors, PGD₂, PGE₂, PGF₂, cAMP, cGMP and T-GIF.

5.3.2 Production of Recombinant TbArf1

A pET21a expression vector containing the full open reading frame of TbArf1, with a C-terminal His₆-tag, has previously been generated (Murphy and Olijhoek 2001). This expression vector was transformed into *E. coli* BL21 DE3 competent cells for protein expression. Subsequently, the protein was purified through binding of the His₆-tag to a Ni₂₊ agarose column and the recombinant protein further purified using an Amicon size exclusion column for proteins less than 50 kDa. Proteins were visualised on a 1D SDS-PAGE gel (Figure 5.8). This purified recombinant TbArf1 protein was used in

the TbArf1 interaction assay described in the following section, and to confirm that TbArfGAPs have a phosphatase function.

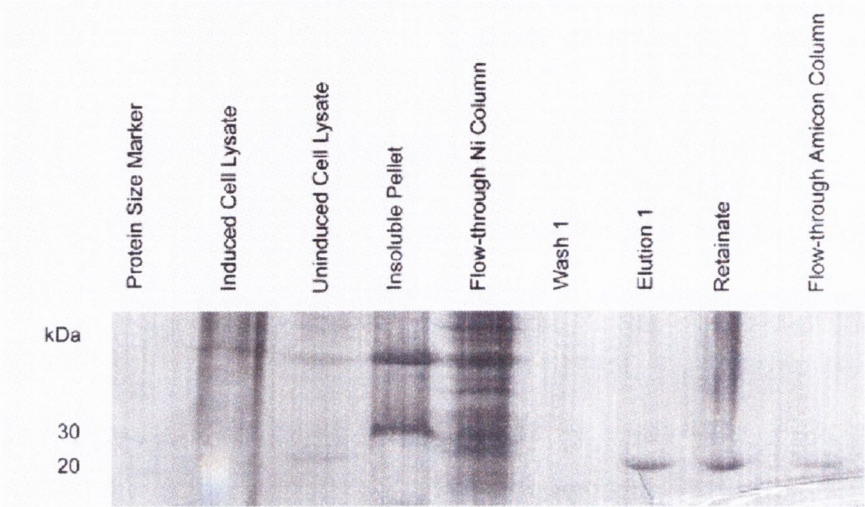


Fig 5.8. Expression of recombinant TbArf1. The figure depicts an SDS-12% PAGE gel with recombinant TbArf 1 stained with Coomassie blue. Lane 1: protein size marker, Lane 2: induced unpurified lysate, Lane 3: uninduced lysate (no IPTG added), Lane 4: insoluble pellet from induced fraction, Lane 5: flow-through the Ni₂ purification column, Lane 6: purification column wash 1, purification column elutant. The protein was further purified using the Amicon protein purification filter. Lane 7: retainate from amicon column, Lane 8: Flow-through the amicon column. The gel was stained with Coomassie blue.

5.3.3 TbArf1 Treated with PGD₂ and cAMP

PGD₂ and cAMP were the signalling factors of most interest. cAMP has been implicated in differentiation of long slender to short stumpy cells and PGD₂ has been implicated in a programmed cell death process that follows the differentiation step. PGE₂ was not of interest as it is produced only in short stumpy forms of *T. brucei*, and as such would have no impact on the triggering of differentiation (Figarella *et al.*

2005). To examine whether PGD₂ and/or cAMP influenced the binding of TbArf1 to other trypanosomal proteins, the purified recombinant TbArf1 was incubated in the presence of signalling factors, PGD₂ or cAMP at 4°C overnight. After extensive washing (see figure 5.9) the following day, the recombinant TbArf1 proteins were incubated with a total soluble protein extract generated from lysed *T. b. brucei* bloodstream form cells (as described in the materials and methods, section 2.8). The columns were extensively washed to remove all unbound proteins, and subsequently TbArf1 binding proteins were eluted with a 250 mM imidazole buffer.

The primary elution containing the majority of the TbArf1 bound-proteins in 250 mM imidazole elution buffer was analysed on an SDS-12% PAGE gel (Figure 5.10). No difference in proteins bound to TbArf1 were observed in the case of cells treated with PGD₂ compared to those which were untreated, suggesting that PGD₂ did not interfere with TbArf1 binding to other effector proteins *in vitro*.

This was not the case for cAMP. Differences in the protein banding pattern of TbArf1 bound proteins were observed when TbArf1 was treated with cAMP when compared to untreated TbArf1. At least two proteins were blocked from binding to TbArf1 in the presence of cAMP; one of about 37 kDa, and a second of about 50 kDa (Figure 5.10). These could be ArfGAPs, which bind to Arf1, in particular TbArfGAP I (34 kDa), TbArfGAP V (37 kDa) or TbArfGAP III (45 kDa). There was also a more pronounced band of about 40 kDa compared to the control untreated TbArf1, or TbArf1 pre-incubated with PGD₂. This showed that cAMP has significant effects on the proteins that interact with TbArf1. To investigate these further, the eluted proteins were examined by 2-D gel electrophoresis.

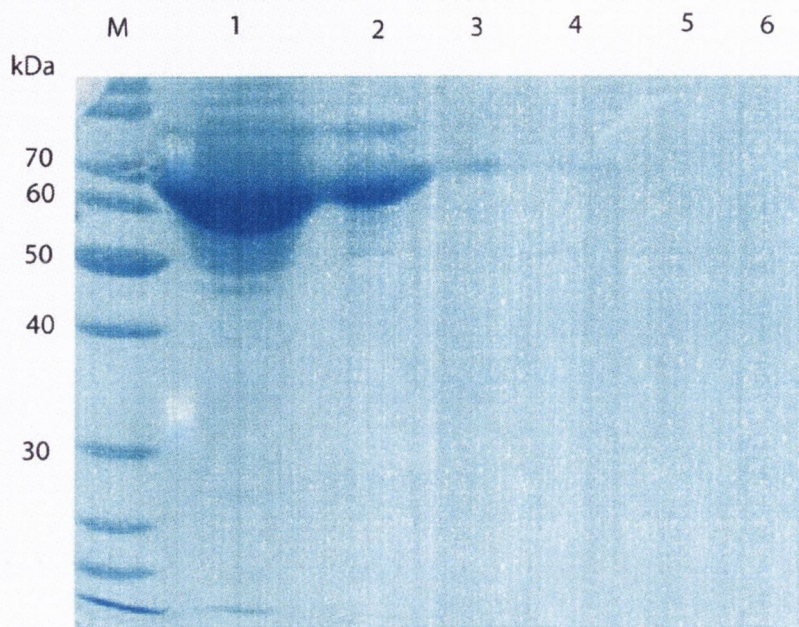


Fig 5.9. Demonstrating the extensive washing prior to elution of TbArf1-bound proteins. The above figure depicts a SDS-12% PAGE gel. The following samples are represented: Lane M: Peq labs protein marker. Lane 1: flow-through column, Lane 2: Low salt wash 1 (5 ml Tris buffer, section 2.8), Lane 3: Low salt wash 2, Lane 4: High salt wash 1 (5ml Tris buffer with added NaOH, section 2.8), Lane 5: High salt wash 2, Lane 6: High salt wash 3. The gel was stained with coomassie blue. Note that there are no traces of proteins in the final three washes. Similar wash results were achieved in all of the TbArf1 assays in the following sections.

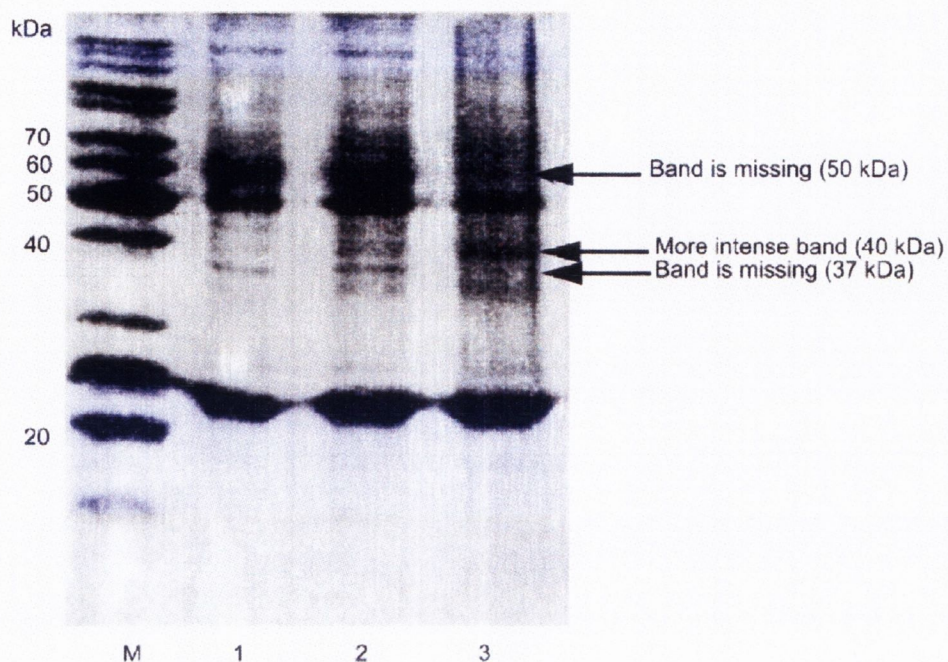


Fig 5.10. Comparison of proteins bound to TbArf1 treated with signalling factors. The figure above depicts trypanosomal proteins bound to recombinant His₆-tag TbArf1 from Ni₂₊-agarose columns following elution with 250 mM imadazole buffer to remove the proteins from resin. The following samples are represented: Lane M: Peq labs protein marker. Lane 1: Recombinant untreated TbArf1 protein (bound to His₆-tag column) was incubated with a protein lysate extract from bloodstream form 328.114 *T. b. brucei* cells. Lane 2: As for Lane 1 but TbArf1 protein was pretreated with PGD₂. Lane 3: As for Lane 1 but TbArf1 protein was pretreated with cAMP. Note the bands absent at 50 kDa and 37 kDa and the more intense band at 40 kDa in Lane 3.

5.3.4 2-D Analysis of Arf1-Bound Proteins

To further characterise the proteins identified in the previous experiment, the eluted proteins from the control and treated samples were concentrated via an acetone protein precipitation reaction and subsequently run on 2-D gels (Figure 5.11). This enabled further separation of the proteins both by pI (pI is the pH at which a protein has neutral charge), and by molecular weight. Individual proteins of similar molecular weight that co-migrate on 1D gels can often be separated and visualised on 2-D gels.

Untreated TbArf1 and TbArf1 treated with PGD₂ depicted similar protein spot patterns on the 2-D gels. In the upper right side of the gels (indicated by an ellipse, Figure 5.11), both untreated and PGD₂ treated TbArf1 retained several protein spots. These protein spots are visibly reduced in the case of cAMP treated TbArf1. In particular, the 37 kDa protein that was missing in the 1-D gels is again absent in the cAMP treated TbArf1 2-D gel. The 40 kDa protein band that had increased expression in the 1-D gel is not apparent in the 2-D gel of cAMP treated TbArf1. We can conclude that these proteins are unable to bind to or have reduced affinity for TbArf1 in the presence of cAMP. It is likely that these proteins are effectors of TbArf1 (such as ArfGAPs or ArfGEFs). In the case of all three gels, TbArf1 can be clearly identified at 21 kDa and with a pI of 8.2 on the right of the gels. (This has been confirmed using mass spectrometry analysis in a similar sample preparation). The identities of the other proteins on the gels have not been deduced to date.

To understand the value of these results further experiments were carried out whereby TbArf1 was pretreated with PGD₂, PGE₂, and PGF₂. Bound TbArf1 protein was then incubated with a soluble protein lysate from bloodstream forms *T. b. brucei* 328.114. Proteins that bound to TbArf1 were eluted from the column together with

the recombinant protein by addition of 250 mM imidazole, and were examined on a 1-D SDS PAGE gel (Figure 5.12). A similar banding pattern to that produced from untreated TbArf1, and TbArf1 treated with PGD₂ was observed. This result showed that PGD₂, PGE₂ and PGF₂ also had no effect on the proteins that interact with TbArf1.

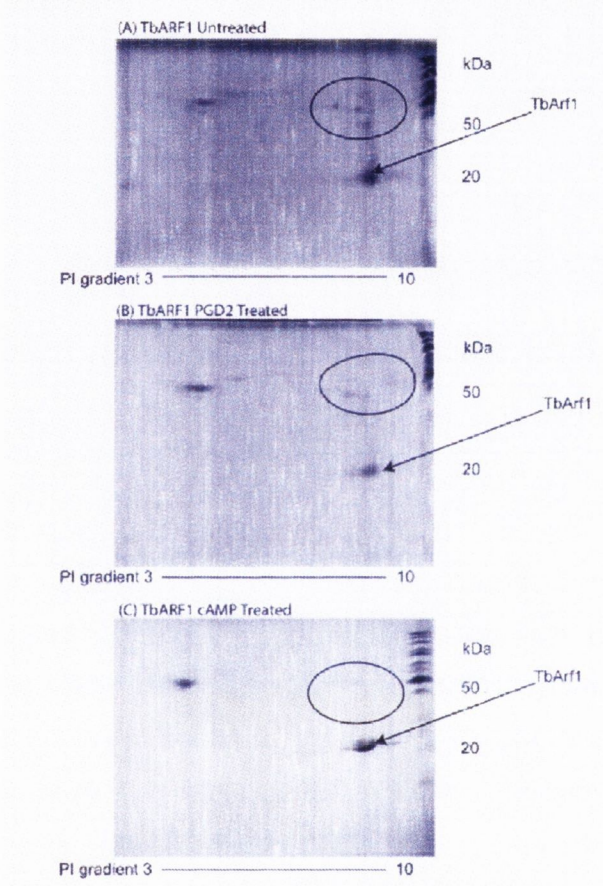


Fig 5.11. 2-D gels of treated and untreated TbArf1 elutions. The above figure depicts 3 2-D gels of (A) untreated TbArf1 and bound trypanosomal lysate proteins, (B) PGD₂ treated TbArf1 and bound trypanosomal lysate proteins and (C) cAMP treated TbArf1 and bound trypanosomal lysate proteins. Note that in all 3 gels the TbArf1 can be clearly identified at 20 kDa, and there are several high molecular protein spots (> 50 kDa) with pIs around 3. Note the circled protein spots. These are equivalent in untreated TbArf1 and PGD₂ treated TbArf1, however, many of these protein spots are missing in cAMP treated TbArf1. It is likely that the interaction of cAMP with TbArf1 has inhibited these proteins from interacting with TbArf1.

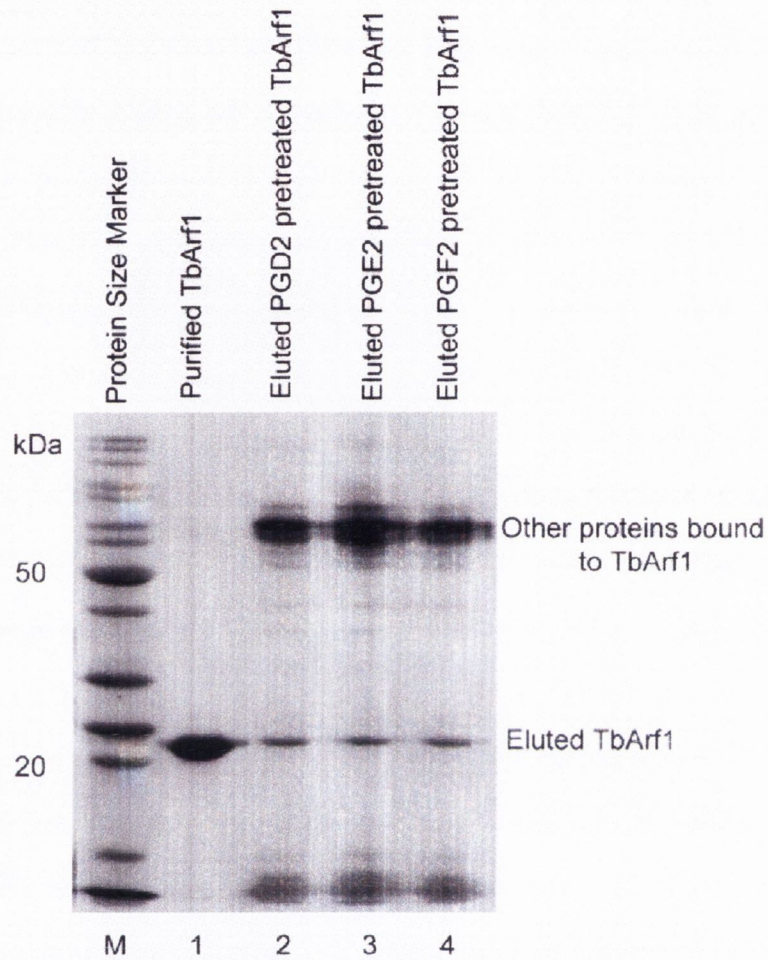


Fig 5.12. Trypanosomal proteins binding to TbArf1 following treatment with prostaglandins. The above figure depicts the banding pattern of the trypanosomal proteins from bloodstream form *T. b. brucei* 328.114 that bound to TbArf1 following treatment with prostaglandin D₂, E₂ and F₂. Note that all the samples had an identical banding pattern.

5.3.5 T-GIF Blocks TbArf1 Protein Binding

The trypanosome differentiation inducing factor (T-GIF) is a signalling factor that is released and sensed by long slender parasites and initiates a cell density dependent differentiation process (Murphy and Olijhoek 2001). TbArf1 has been shown to bind to T-GIF, specifically in the helix $\alpha 3$ region. In order to investigate this further, an experiment was carried out to investigate the effect of T-GIF on TbArf1 protein binding.

TbArf1 was treated with an aliquot of the T-GIF signalling factor. [T-GIF was isolated from *T. congolense* cell medium using a Ni_{2+} column to which recombinant TbArf1 was bound (Murphy and Olijhoek. 2001)]. A trypanosomal protein lysate generated from bloodstream form 328.114 *T. b. brucei* cells was subsequently incubated with the untreated TbArf1, cGMP treated TbArf1 and T-GIF treated TbArf1 (Figure 5.13). TbArf1-bound proteins were eluted and the protein banding pattern was visualised on a 1-D SDS PAGE gel. Pretreatment of TbArf1 with cGMP produced a similar protein banding pattern to that of untreated TbArf1 implying that cGMP had no effect on the trypanosomal proteins binding to TbArf1. In the case of T-GIF treated TbArf1 an alteration in the binding pattern of proteins to TbArf1 was noted. No proteins other than TbArf1 were eluted when the trypanosome protein lysate was pretreated with T-GIF suggesting that T-GIF blocked several if not all proteins binding to TbArf1.

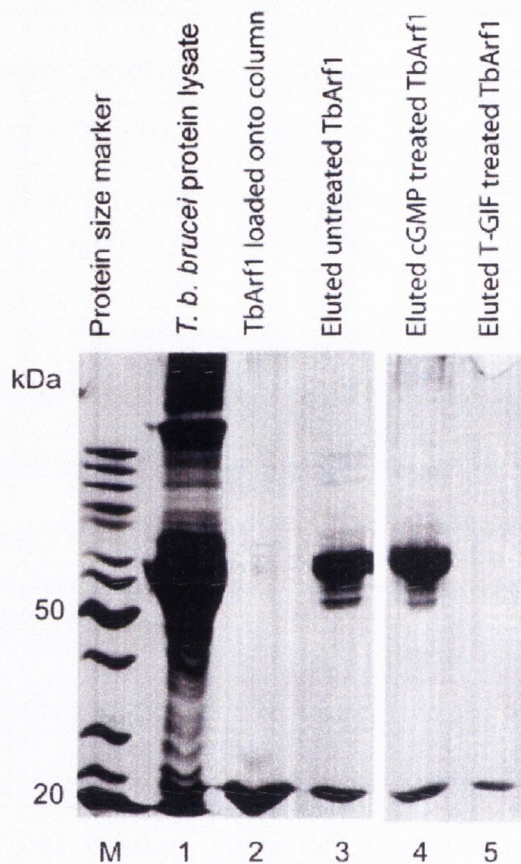


Fig 5.13. Effect on binding of trypanosomal proteins to TbArf1 of treatment with T-GIF and cGMP. The figure above depicts an SDS-10% PAGE gel. Lane M: protein size marker; Lane 1: *T. brucei* protein lysates; Lane 2: TbArf1 that was loaded onto the column; Lane 3: untreated TbArf1 elution; Lane 4: cGMP treated TbArf1 elutate; Lane 5: T-GIF treated TbArf1 elutate. Both cGMP treated TbArf1 and untreated TbArf1 depict the same banding pattern. No proteins were observed to bind T-GIF treated TbArf1.

5.4 TbArf1 Interacts with TbArfGAP I and TbArfGAP V

Several effector proteins of TbArf1 are expected to bind to TbArf1. In particular, mammalian and yeast ArfGAPs (Goldberg 1999) and ArfGEFs (Peyroche *et al.* 1996; Cherfils *et al.* 1998; Goldberg 1998) have well defined interactions with Arf1. Recombinant proteins of two of the effectors of TbArf1 were available, TbArfGAP I and TbArfGAP V. A similar protein interaction assay to that previously described was carried out to confirm the expected interaction of TbArf1 and TbArfGAP I and TbArfGAP V.

TbArfGAP I and TbArfGAP V were purified (as shown in Chapter 4, Figure 4.15 and Figure 4.16), and bound to a nickel column via their His₆-tags and incubated over night at 4°C. The following day unbound protein was washed off, and a protein lysate produced from bloodstream form *T. b. brucei* 328.114 cells were incubated with the column. Following several low and high salt washes, to remove unbound proteins (similar to figure 5.9) so that only proteins interacting with the recombinant protein bound to the Ni₂₊ agarose column were eluted in 250 mM imidazole. Eluted proteins were electrophoresed on SDS-10% PAGE gel (Figure 5.14). TbArf1 was expected to be recovered in the eluted fraction, along with additional proteins that TbArfGAP I and TbArfGAP V interact with. The resultant elutions displayed a very similar protein binding pattern to TbArf1 bound proteins. This is likely due to the TbArfGAPs binding to TbArf1, and subsequently, TbArf1 binds to its effector proteins. Both TbArfGAP I and TbArfGAP V were observed to bind to a protein of similar molecular weight to TbArf1 (Figure 5.14, 20 kDa protein band), suggesting that both TbArfGAPs are likely to function as effector molecules of TbArf1 in a similar manner to that observed in the mammalian and yeast systems.

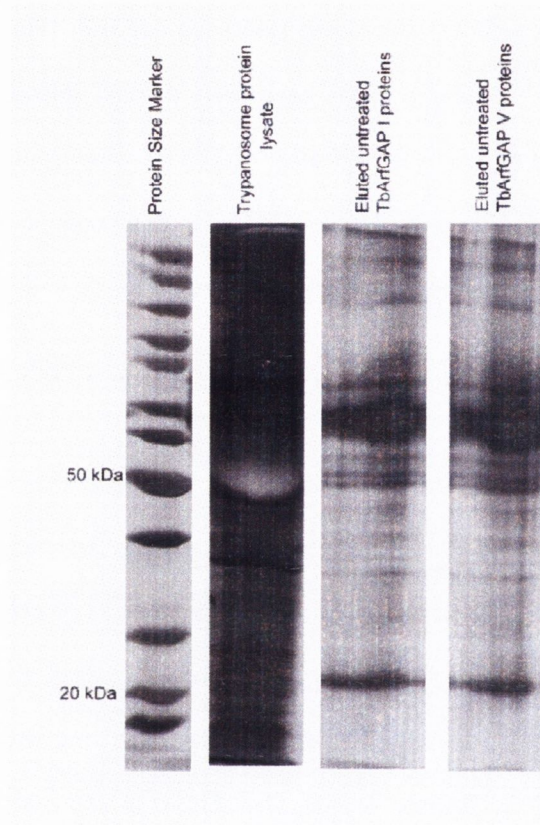


Fig 5.14. TbArfGAP I and TbArfGAP V bound trypanosomal proteins. The figure above depicts the elutions of proteins bound to TbArfGAP I and V after incubation with trypanosomal protein lysate. Note the presence of a band at 20 kDa that migrates on the gel at the same position as TbArf1 and is suspected of being TbArf1. The protein banding patterns are similar to that of the TbArf1-binding proteins from bloodstream forms of *T. b. brucei*.

5.5 Interaction between TbArf1 and ESAG10

Recombinant TbArf1 was incubated with protein lysates generated from ESAG10 over expressing *T. b. brucei* 328.114 cells (that were produced in chapter 3). The aim of this experiment was to show that overexpression of ESAG10 results in increased production of a cell surface receptor/transporter (TbPP69) that has been previously implicated in differentiation and that this protein interacts with TbArf1.

This experiment was carried out as described previously (Section 5.3.1), with the exception that TbArf1 was not pre-treated with any signalling factor. Protein lysates were extracted from wild-type cells (where wild-type refers to 328.114 untransfected cells) and cells transfected with pLEW100 containing the *ESAG10* gene. The pLEW100 plasmid allows tetracycline inducible expression of a protein of interest from an integrated plasmid in the *T. brucei* genome. Eluted fractions were run on a 1-D SDS-PAGE gel (Figure 5.15). The banding pattern of proteins binding to TbArf1 from the transfected and untransfected cells were similar except that a protein band between 60 and 70 kDa displayed increased abundance in the transfected cells with the greatest abundance in transfected cells incubated in the presence of tetracycline. The 60-70 kDa band that increases when *ESAG10* transfected cells were used is suspected to be ESAG10, although there is greater quantity of ESAG10 available when ESAG10 transfected cells were used. This suggests that TbArf1 binds ESAG10 and supports the previous findings that ESAG10 is likely the TbPP69 cell surface receptor/transporter (see chapter 3). There is also an increase in amount of protein in uninduced cells which is likely due to a leaky promoter.

To further investigate this differential pattern of protein abundance in the 60-70 kDa band, the eluted fractions were concentrated by acetone precipitation and

examined on 2-D gels (Figure 5.16). Three proteins of about 60-70 kDa displayed differential abundance between the three 2-D gels. These proteins were less abundant in the protein lysate produced from wild-type cells, displayed greater abundance in the protein lysate produced from uninduced *ESAG10* transfected cells, and the greatest abundance in the protein lysate produced from the tetracycline-induced *ESAG10* transfected cells. The composition of these proteins has not been deduced, but it is likely that at least one represents *ESAG10* and is the suspected TbPP69 transporter which is upregulated in induced transfected cells.

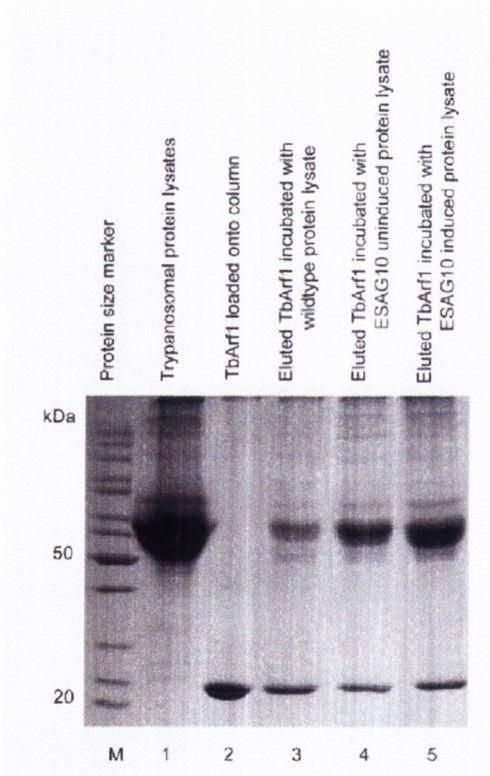


Fig 5.15. Comparison of TbArf1 interacting proteins using protein lysates generated from induced and uninduced *ESAG10* transfected and wild type cells. The figure above depicts an SDS-10% PAGE gel stained with Coomassie blue. Note the presence of the 60-70 kDa bands in all cases, this band is least pronounced where TbArf1 was incubated with wild type cell protein lysate (Lane 3), and greatest in the case of incubation with induced *ESAG10* transfected cell protein lysate (Lane 5).

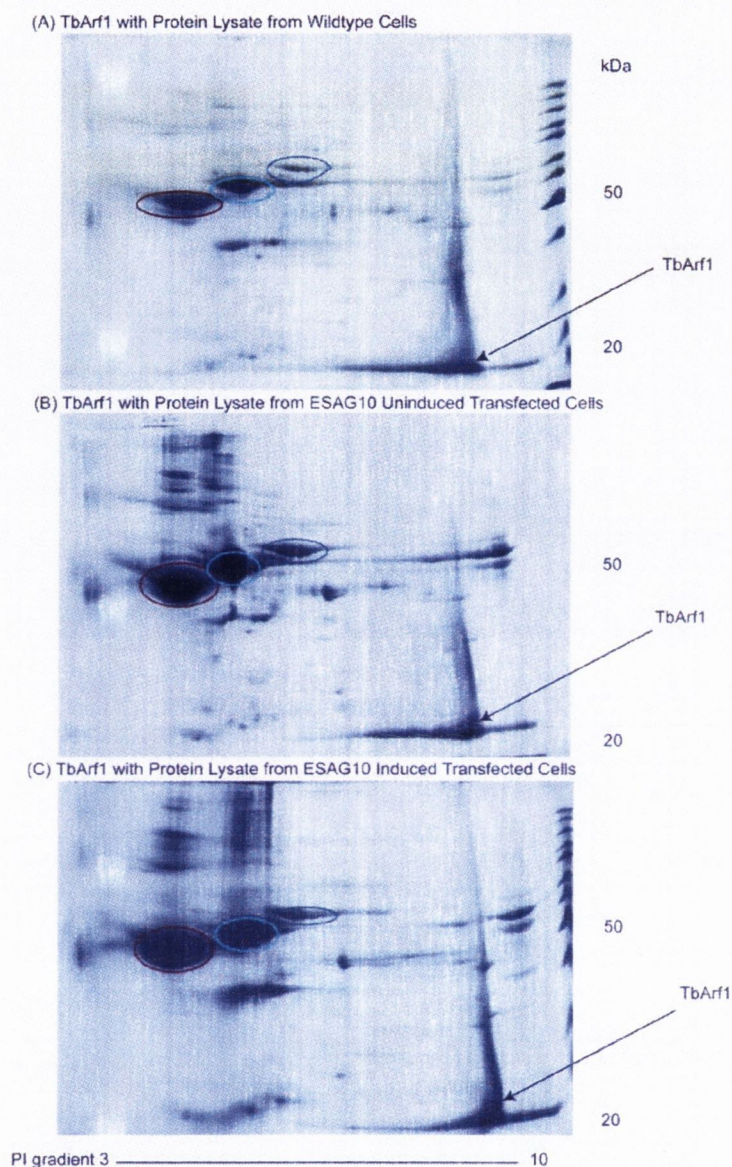


Fig 5.16. Comparison of TbArf1 interacting proteins from protein lysates generated from induced and uninduced *ESAG10* transfected and wild type cells via 2-D gel electrophoresis. The figure above depicts three 2-D gels stained with colloidal blue. Note the red, blue and purple circles which depict three proteins which are differentially expressed in the 3 gels. These proteins are most abundant in ESAG10-induced transfected cells.

5.6 Discussion: Signalling Factors Implicated in TbArf1 Mediated Differentiation

Differentiation of long slender to short stumpy bloodstream forms of *T. b. brucei* occurs via a cell density dependent signalling mechanism (Vassella *et al.* 1997). T-GIF, the trypanosome differentiation inducing factor, is secreted and sensed by long slender bloodstream form cells and triggers their differentiation to the short stumpy form (Murphy and Olijhoek 2001). The mechanism of this differentiation event has not been fully deduced, however, several signalling factors have been implicated in the differentiation process.

cAMP is one of several signalling factors that has been implicated in the long slender to short stumpy cell differentiation process. The addition of membrane permeable analogues of cAMP to long slender cells has been shown to trigger differentiation in both pleomorphic (cells that can differentiate) and monomorphic cells (lab-derived cells that have lost the ability to differentiate) (Vassella *et al.* 1997; Breidbach *et al.* 2002). In order to investigate these results further these experiments were repeated using the *T. b. brucei* 328.114 monomorphic lab strain. In this *T. b. brucei* strain membrane permeable cAMP did not induce a short stumpy phenotype and cells continued to proliferate. It appears that the ability of membrane permeable cAMP to induce cessation of growth is strain specific. Recently, it has come to light that the previously observed effect of membrane permeable cAMP on long slender cells was not due to cAMP itself, but actually due to the hydrolysed products of analogues of cAMP (Laxman *et al.* 2006), which may explain why cell growth arrest was not achieved in the above experiment. Specifically, the authors found that non-hydrolysable cAMP analogues were unable to induce a significant antiproliferative

cell growth responses. Only metabolic derivatives of hydrolysable cAMP analogues were able to induce antiproliferative effect on cell growth (Laxman *et al.* 2006). As a result the authors doubted the role of non-hydrolysed cAMP's in long slender to short stumpy *T. brucei* differentiation.

A similar approach was taken to investigate the effects of other signalling factors, primarily the prostaglandins. *T. b. brucei* produce prostaglandins at different stages throughout the life cycle, including prostaglandins D₂, E₂ and F₂ (Figarella *et al.* 2005). Of these, PGD₂ has been implicated in a programmed cell death process (Figarella *et al.* 2005). PGD₂ is produced by and sensed by short stumpy cells, triggering a programmed cell death response (Figarella *et al.* 2005). The mechanism by which this occurs has not been deduced at present. Although the programmed cell death process has not been directly linked to long slender differentiation, this process is initiated in short stumpy cells following differentiation. Recent studies have demonstrated that addition of prostaglandins, 3.7 μ M PGD₂, 159 μ M PGE₂ and 199 μ M PGF₂ to *T. b. brucei* cultures induced growth inhibition of cells (Figarella *et al.* 2005). These experiments were repeated using the *T. b. brucei* 328.114 strain which is derived from the 427 wildtype strain expressing 221 VSG, whereas Figarella *et al.* (2005) used the MITat 1.2 *T. b. brucei* strain. The addition of PGD₂ and PGE₂ to actively-dividing bloodstream forms of *T. b. brucei* 328.114 was observed to cause growth inhibition of these cells. PGE₂ caused growth inhibition at similar levels to PGD₂ which differed to the results presented by Figarella *et al.* (2005) where PGE₂ had a minor growth inhibitory effect. This greater effect of PGE₂ on cell growth may be strain specific. PGE₂, however, may play a bigger role in programmed cell death than previously thought, as PGE₂ has also been implicated in triggering programmed cell death in erythrocytes (Lang *et al.* 2005). PGE₂ is secreted throughout the long

slender and short stumpy stages of bloodstream form cells (Kubata *et al.* 2000), whilst patients and animals suffer from an anaemic condition (Kobayashi *et al.* 1976; Facer *et al.* 1982) the production of PGE₂ might contribute to this condition.

To further investigate if these signalling factors have an impact on protein interactions of TbArf1, a vesicular protein which has also been implicated in differentiation (Murphy and Olijhoek 2001), an assay was carried out to examine interactions of TbArf1 with whole cell protein extracts under different conditions (treatment of TbArf1 with cAMP, cGMP, PGD₂, PGE₂ or PGF₂). Of these signalling factors only cAMP has been implicated in the long slender to short stumpy differentiation but no mechanism for cAMP triggering long slender differentiation has been characterised. Another of the signalling factors, PGD₂, has been implicated in short stumpy programmed cell death process. Experiments were designed to show whether any of these signalling factors bound to TbArf1 and subsequently, whether they blocked TbArf1 from interacting with other proteins. The assay involved the treatment of column bound TbArf1 with a signalling factor (either cAMP, cGMP, PGD₂, PGE₂ or PGF₂), and subsequently incubation with a whole protein cell extract, extensive washing and comparison of resultant protein banding pattern with untreated TbArf1 on a 1-D SDS-PAGE gel. The assay revealed that untreated TbArf1 and TbArf1 incubated with PGD₂ had a similar banding pattern of proteins that interact with TbArf1 indicating that PGD₂ did not affect other TbArf1 binding proteins. This was also true in similar assays involving PGE₂, PGF₂ and cGMP, suggesting that none of these signalling factors influenced the interactions of other trypanosomal proteins with TbArf1.

A significant effect was, however, observed in assays involving the addition of cAMP. TbArf1 incubated with cAMP displayed a different protein banding pattern

compared to untreated TbArf1. Several proteins appear to be missing, particularly around 37 to 50 kDa. To further investigate this, 2-D gel electrophoresis was carried out to separate proteins by pI and molecular weight and achieve a more detailed resolution of the proteins present. An absence of at least two basic proteins in the 37-50 kDa range were confirmed in the case of cAMP treated TbArf1. These proteins were inhibited from binding to TbArf1 in the presence of cAMP. A possible explanation for this is that cAMP interacts with TbArf1. This further suggests that TbArf1 is involved in the differentiation process, and identification of these proteins may lead to further characterisation of the cAMP signalling cascade. cAMP levels have been shown to alter during the process of differentiation (Vassella *et al.* 1997), implicating cAMP as part of a signalling pathway. To induce this effect *in vitro* it seems that hydrolysed analogues of cAMP are required (Laxman *et al.* 2006).

To understand how the cAMP may be interacting with TbArf1 the *Dictyostelium discoideum* cell density sensing cAMP model may be used to explain. The *Dictyostelium* slime mould undergoes a cell density mediated differentiation process from unicellular to multicellular via sensing and relaying cAMP pulses using a cell surface cAMP receptor that activates its associated G proteins (Theibert and Devreotes 1986; Van Haastert *et al.* 1987; Van Haastert *et al.* 1989). In the *T. b. brucei* case TbArf1 may be acting like a G protein, setting off a cAMP signalling cascade through activation of adenylate cyclase. This triggering of cAMP signalling cascade by TbArf1 likely occurs via an effector protein of TbArf1, possibly an ArfGEF protein.

Another signalling factor that has been clearly implicated in the *T. brucei* differentiating pathway is T-GIF, the trypanosome growth inhibitory factor (known as SIF in the case of *T. brucei*). This factor is produced and sensed by long slender cells

triggering differentiation of cells into a short stumpy phenotype. A similar assay to that previously described for cAMP was carried out to investigate the effect T-GIF on proteins binding to TbArf1. This result suggests that T-GIF binds to TbArf1, as discussed in the introduction, section 1.1.7, and prevents TbArf1 from interacting with several other effector proteins. We propose that following entry into the cell, T-GIF triggers differentiation to short stumpy cells possibly by preventing TbArf1 from interacting with other effector proteins (possibly TbArfGAPs or TbArfGEFs) and that this in turn activates a cAMP signalling cascade triggering alterations in gene expression and ultimately resulting in growth arrest and differentiation.

Through the information obtained in these TbArf1 signalling assays described above we have been able to link the known cAMP signalling pathway involved in long slender to short stumpy differentiation with the TbArf1 protein (cAMP may block binding of effector proteins to TbArf1) and the T-GIF factor (T-GIF blocks binding of effector proteins to TbArf1), thus, advancing towards our goal of producing a new model of long slender to short stumpy differentiation in *T. b. brucei*. However, these results may be an artifact of the TbArf1 assay, and cAMP which may normally interact downstream (interacting with protein kinase A, triggering gene activation) maybe interfering with earlier in the signalling cascade, interacting with TbArf1 effector proteins.

Very little progress has been made with regards to characterisation of the T-GIF (or SIF) differentiation factor in the literature. It was first described in 1996 where it was shown to induce differentiation in *T. b. brucei* long slender monomorphic cells (Vassella *et al.* 1996). Following much effort by Borst's group and Engstler's group the T-GIF (or SIF) molecule failed to be characterised, there are only suggestions that it may be a folate-like molecule, or a cAMP analogue. Presently, groups investigating

the long slender differentiation step in *T. b. brucei* have focused on other aspects of the differentiation process and used other differentiation steps within the life cycle of *T. b. brucei* as models for this process.

ArfGEFs and ArfGAPs are highly studied effectors of Arf1. The *T. b. brucei* ArfGAPs, TbArfGAP I and TbArfGAP V, were used in an interaction assay to examine their possible interactions with TbArf1. Both of the TbArfGAPs were suggestive of binding to TbArf1 in the interaction assay, along with a number of other proteins, producing a protein binding pattern of TbArfGAP interacting proteins similar to that of TbArf1 interacting proteins. It is likely that these TbArfGAPs are binding to the TbArf1 present in the whole cell extract and are subsequently also binding to proteins that interact with TbArf1, thus producing a similar protein binding pattern to that of TbArf1 interacting proteins.

In the course of this thesis ESAG10 has been shown to most likely be the TbPP69 receptor/transporter protein that has been implicated in *T. b. brucei* differentiation (see chapter 3). TbPP69 has been shown to specifically bind to TbArf1 (Murphy and Olijhoek 2001). To confirm that ESAG10 also binds to TbArf1, protein lysates from wild-type ESAG10 transfected induced cells were assayed for binding to TbArf1. TbArf1 was shown to bind to three proteins with acidic to neutral pH in the molecular weight region of 60-70 kDa, which is the expected size of the TbPP69 receptor/transporter that is upregulated in pleomorphic cells. These proteins were differentially expressed and showed highest abundance in ESAG10 transfected induced cells. This assay suggests that TbArf1 binds ESAG10 and that ESAG10 is a strong candidate for the TbPP69 receptor/transporter. Further investigation should focus on elucidation of down stream steps in the long slender differentiation, to build a complete working model of the long slender to short stumpy differentiation process.

Chapter 6: Conclusions

6.1.1 Cell Density Dependent Differentiation of *T. b. brucei* Bloodstream Form

In the mammalian host stage of the life cycle of *T. b. brucei*, the bloodstream stage, the trypanosomes undergo a cell-density dependent differentiation step (Vassella *et al.* 1997). This differentiation step is triggered by a secreted differentiation inducing factor, T-GIF (Murphy and Olijhoek 2001) (also known as SIF, Vassella *et al.* 1997). T-GIF is likely taken up by the long slender cells via a cell surface receptor or transporter protein, which subsequently initiates a signalling cascade, inducing differentiation of actively-dividing long slender cells into non-dividing short stumpy cells, which are pre-adapted for uptake by the tsetse fly (Murphy and Olijhoek 2001). This is a terminal differentiation event; short stumpy cells undergo a programmed cell death like process if not taken up by the tsetse fly within three days.

A glycosylated surface protein named TbPP69 is suspected to be the T-GIF receptor/transporter as it is upregulated in pleomorphic cells (cells that can undergo the differentiation step from long slender to short stumpy) compared to lab-derived monomorphic cells that do not undergo this differentiation event (Murphy and Olijhoek 2001). This hypothesis is based on the fact that monomorphic cells that produce the SIF signalling factor, though, are unable to sense and respond to it (Vassella *et al.* 1997); suggesting that there is a receptor/transporter that is defective in monomorphic cells.

6.1.2 Molecular Characterisation of the TbPP69 Receptor/Transporter

To identify the TbPP69 receptor/transporter, the upregulated 69 kDa band of tomato-lectin purified preparations from pleomorphic *T. b. brucei* strain ILTat 1.1 was excised, purified and analysed by mass spectrometry. The results showed that the 69 kDa band was contaminated with large amounts of the variant surface glycoprotein, ILTat 1.4 VSG, so, it was not possible to characterise the TbPP69 receptor/transporter from this preparation. Instead, a candidate based approach was taken to identify the TbPP69 receptor/transporter.

6.1.3 ESAG10 as a Candidate for the TbPP69 Receptor/Transporter

Switching of pleomorphic to monomorphic cell types has been reported following changes in expression of VSGs (Barry and Turner 1991). This suggested that pleomorphism (the ability to differentiate) may be linked to the expression sites (ESs); in particular, the expression site associated genes (ESAGs). Following analysis of several ESAGs, ESAG10 was identified as a suitable candidate for the TbPP69 receptor/transporter. ESAG10 was predicted to possess an N-terminal signal peptide, 12 predicted transmembrane domains and a predicted surface N-glycosylation site, all features expected of the TbPP69 receptor/transporter. Furthermore, ESAG10 was homologous to a *Leishmania* biopterin transporter, BT1, which was implicated in differentiation of *Leishmania donovani* (Myler *et al.* 1994). In *Leishmania*, BT1 regulates BH₄ (tetrahydrobiopterin) levels which may act as a co-factor in controlling differentiation (Ouellette *et al.* 2002).

One potential problem with ESAG10 as a candidate for TbPP69 was that its predicted molecular weight exceeded the apparent molecular weight observed for the TbPP69 receptor/transporter. The homologous protein in *Leishmania*, BT1, and several other related proteins, however, have been shown to display anomalous migration on SDS-PAGE gels, such that they migrate at a much lower molecular weight than predicted (Mueckler *et al.* 1985; Lemley *et al.* 1999). Overexpression of ESAG10 in *T. b. brucei* bloodstream form cells resulted in increased abundance of a tomato-lectin binding protein (which also includes ESAG10 as compared to the control) that migrated at the same position as the TbPP69 receptor/transporter (69 kDa), suggesting that ESAG10 was possibly the TbPP69 receptor/transporter.

If the TbPP69/ESAG10 receptor/transporter were sufficient to allow induction of the differentiation process by T-GIF, it would be expected that *T. b. brucei* cells overexpressing this protein would show a marked reduction in cell growth rate, associated with the differentiation process. Furthermore, morphological evidence of the differentiation process would also be expected to be observed. No differences in cell growth rates or morphological changes were observed in *T. b. brucei* cells overexpressing this protein, indicating that although ESAG10 has properties that suggest it is the differentiation receptor/transporter, and that it appears to interact with TbArf1, overexpression of ESAG10 alone is not sufficient to allow the differentiation process to occur. There are two possible reasons for this: either ESAG10 is not the differentiation receptor/transporter, or there may be a secondary defect in the cell line that prevents T-GIF from functioning. Repetition of this experiment with use of another cell line would clarify this.

Labelled TbPP69 receptor/transporter has previously been shown to bind to a column containing TbArf1, an internal target of T-GIF (Murphy and Olijhoek 2001).

TbArf1 is a protein involved in vesicular trafficking in the cell (Price *et al.* 2007) and has been implicated in the long slender differentiation process (Murphy and Olijhoek 2001). In the course of this thesis, ESAG10 was also implicated with TbArf1 as would be expected if ESAG10 acts as the TbPP69 receptor/transporter. Comparative analyses were carried out on effector proteins of TbArf1 following incubation with protein lysates produced from cells overexpressing ESAG10. Overexpression of ESAG10 resulted in upregulation of several TbArf1 binding proteins suggesting that differentiation is a complex process involving several, as yet unidentified proteins. Expanding on these studies, co-immunofluorescence studies were carried out to show the effect of overexpression of ESAG10 on TbArf1. Cells overexpressing ESAG10 were stained with tomato lectin conjugated to Texas red. (Though ESAG10 and other tomato lectin binding proteins are stained red in this procedure, the increase in tomato lectin staining in cells overexpressing ESAG10, when compared to the untransfected control cells, indicates where upregulation of ESAG10 expression has occurred). In addition, a secondary antibody stain was used to investigate the localisation of TbArf1. Overexpression of ESAG10 in induced cells displayed an altered pattern of cellular localisation of TbArf1. Expression of TbArf1 should be located in the Golgi and endosomal system, however, TbArf1 was found throughout the cell and along the flagellum in cells overexpressing ESAG10, and was co-localised with ESAG10. This expression pattern differed from that of untransfected cells where TbArf1 was located in the Golgi, endosomal and flagellar pocket region.

In monomorphic cells, tomato lectin binds to proteins in the flagellar pocket and endosomal system but not along the flagellum (Nolan *et al.* 1999). Similarly, TbArf1 is located in the Golgi and endosomal system in monomorphic cells (Price *et al.* 2007), but, in pleomorphic cells, tomato lectin binds along the flagellum as well as in

the flagellar pocket and endosomal system (Murphy and Olijhoek 2001). Likewise, TbArf1 in pleomorphic cells is located in the Golgi, endosomal system and along the flagellum (Murphy and Olijhoek 2001). Thus, the altered location of TbArf1 following overexpression of ESAG10 and their co-staining signifying co-localisation along the flagellum of the parasite suggests an interaction between these two proteins in bloodstream forms of *T. b. brucei*. This is strong evidence that ESAG10 interacts with TbArf1 and further implicates ESAG10 as the TbPP69 receptor/transporter of T-GIF.

6.1.4 Characterisation of Arfs/Arls and Effector Proteins

To further characterise the role of TbArf1 in differentiation, a bioinformatic approach was taken to identify Arf proteins, Arf related proteins (Arls) and their effector molecules in *T. b. brucei*. Two genes encoding putative Arfs were identified in the *T. b. brucei* database, TbArf1 and TbArf3.

TbArf1 was shown to be the putative *T. b. brucei* Arf protein most closely related to the human Arfs by phylogenetic analysis, but was not orthologous to any one Arf in particular, indicating that TbArf1 may incorporate several of the functions of the human Arf proteins. This differs from the recently suggested role of TbArf1 put forward by Price *et al.* (2007) where TbArf1 was suggested to be most closely related to human Arf6 and that it fulfils the functions of both human Arf1 and human Arf6. The work in this thesis reveals the benefit of a phylogenetic perspective which shows that TbArf1 is not in fact orthologous to human Arf6, but rather, likely incorporates several of the functions of the paralogous human Arf proteins.

The importance of TbArf1 has been recently investigated by several other groups. TbArf1 RNAi studies were lately carried out and resulted in a grossly enlarged flagellar pocket and a rapidly lethal phenotype (Price *et al.* 2007). In addition, TbArf1 overexpression was shown to result in rapid cessation of cell division and increased cell death (Price *et al.* 2007). Similarly, a constitutively active mutant (TbArf1 Q71L) caused cell death within 24 hours (Price *et al.* 2007), as did the constitutively active *Leishmania donovani* Arf1 (LdArf1) mutant (Porter-Kelley *et al.* 2004). The constitutively inactive mutant of TbArf1 (T31N) has also been produced, also causing cell death within 24 hours (Price *et al.* 2007). Together, these studies demonstrate the importance of TbArf1 as an essential protein that ensures the survival of the cell, and indicates that TbArf1 must be tightly regulated to ensure cell longevity.

TbArf3, the other putative *T. b. brucei* Arf protein, was shown to be distantly related to the human Arf proteins, and more closely related to human Arl1, an Arf related protein. RNAi studies have recently been conducted on this protein (TbArl1) by Price *et al.* (2005a, b) who showed that it was essential for cell viability and Golgi integrity in the bloodstream form of *T. b. brucei*. This suggests that TbArf3 may also be important in vesicular trafficking or cytoskeletal structure organisation, but no role in differentiation has been suggested (Price *et al.* 2005a; Price *et al.* 2005b).

TbArl17, a putative *T. b. brucei* Arl protein, was shown to be more closely related to the human Arfs than to the Arls, indicating a possible role as an Arf protein. Of the other seven putative Arl proteins identified in *T. b. brucei*, TbArl2, TbArl3, TbArl6, TbArl18, TbArl19, TbArl20 and TbArl21, several were shown to be closely related to their human orthologues (TbArl2, TbArl3, TbArl6 and TbArl21). This

suggests that many of these TbArfs are likely to have functions similar to their human counterparts.

In this thesis, to further characterise the role of TbArf1 in differentiation, a comparative analysis of TbArf1 and other eukaryotic Arfs was undertaken. In particular the helix $\alpha 3$ region of TbArf1 was identified by phylogenetic analyses as an important region likely involved in the additional functions of TbArf1 in differentiation, a role not implicated in other eukaryotes. This region was shown to be highly conserved in kinetoplastids, but divergent from other eukaryotic species. This is a classical example of functional divergence, and suggests a reason for this additional function of TbArf1. Also, studies by Donalson *et al.* (2005) have shown that the helix $\alpha 3$ domain of Human Arf1 and human Arf6 were involved in transport to the Golgi (human Arf1) and to the plasma membrane (human Arf6). It is likely that the TbArf1 helix $\alpha 3$ domain has a role in determining its location, similar to its human counterparts. In addition, the differentiation factor, T-GIF, also binds to the helix $\alpha 3$ region of TbArf1 (Murphy and Olijhoek. 2001), providing evidence that this region is important in *T. b. brucei* differentiation.

Examination of the genomic organisation of TbArf1 was also carried out. It showed that TbArf1 was encoded by four open reading frames (ORFs) located in tandem on chromosome 9. In mammals, genes are regulated utilising complex promoters which can be upregulated or inhibited depending on the binding of transcription factors (Chen and Rajewsky 2007). In the trypanosome, most gene regulation is carried out post-transcriptionally, and a simpler mechanism accounts for differential expression at the mRNA level (Vassella *et al.* 2000). TbArf1 ORFs are found in tandem in the genome, and although they encode identical protein coding sequences, unique untranslated regions (UTRs) allow for differential expression of

TbArf1 in different life cycle stages and may facilitate the additional function of TbArf1 in *T. b. brucei* differentiation. Work described in Chapter 4 of this thesis established that TbArf1 was differentially expressed, in that there was less TbArf1 detected in procyclic forms than in long slender and short stumpy forms.

Several TbArf1 interacting proteins likely facilitate the role of TbArf1 in differentiation. To better characterise these, putative TbArf1 effector proteins were identified and phylogenetically analysed. Arf GTPase activating proteins, ArfGAPs, are negative regulators of Arf1 that hydrolyse GTP-bound Arf. Five putative ArfGAPs were identified in *T. b. brucei*; TbArfGAP I, TbArfGAP II, TbArfGAP III, TbArfGAP IV and TbArfGAP V. Phylogenetic classification studies showed that TbArfGAP I and TbArfGAP II were most closely related to human ArfGAP1 and human ArfGAP3. TbArfGAP III and TbArfGAP IV were less closely related to the human ArfGAPs 1 and 3, whilst TbArfGAP V was most distant from the human ArfGAP1 and 3. The TbArfGAPs were suggested to function in a manner similar to human ArfGAPs based on structural comparison to the resolved human ArfGAP1 crystal structure (Goldberg 1999).

Arf guanine nucleotide exchange factors, ArfGEFs, are positive regulators of TbArf1 catalysing the exchange of Arf1-GDP for Arf1-GTP. Three putative ArfGEFs were identified and characterised in the *T. b. brucei* Sanger database; TbArfGEFA, TbArfGEFB, and TbArfGEFC. TbArfGEFB was shown to be most closely related to the human ArfGEFs. In addition, the *T. b. brucei* ArfGEFs were suggested to function in a manner similar to their human counterparts based on structural comparison to the resolved crystal structure of a human ArfGEF (Goldberg 1998; Mossessova *et al.* 1998). In mammals, ArfGEF proteins have also been implicated in signalling (Li *et al.* 2003). If this were also the case in *T. b. brucei*, then TbArf1 could trigger the cAMP

cascade through ArfGEFs. This ability of mammalian ArfGEFs has not been fully characterised, therefore it is not possible to deduce whether *T. b. brucei* ArfGEFs also possess the same activity.

Homologues of other mammalian and yeast proteins shown to interact with Arf1 were also identified in the *T. b. brucei* Sanger database (<http://www.genedb.org/>), including components of the clathrin vesicle, COPI coatomer and adaptin molecules, suggesting that other interactions between Arf1 and effector proteins are likely conserved in *T. b. brucei* as well. Homologues of other molecules known to interact with TbArf1 such as GGAs, however, were not found, suggesting that in other cases interactions are not conserved.

It is likely that the additional role of TbArf1 in differentiation is partly due to the differences in the helix $\alpha 3$ region of TbArf1 (to which T-GIF binds), and possibly an effector protein may also facilitate the down-stream cAMP cascade triggering the differentiation process.

6.1.5 cAMP Signalling in Differentiation

cAMP has been previously shown to mediate signal transduction of the differentiation signal throughout the *T. b. brucei* cell, which subsequently triggers the changes in gene expression associated with differentiation (Vassella *et al.* 1997). A two-three fold elevation of intracellular cAMP occurred within long slender bloodstream form cells upon induction of differentiation (Vassella *et al.* 1997). In addition, regulators of cAMP have been shown to be involved in relaying the differentiation signal throughout the cell (Domenicali Pfister *et al.* 2006).

A simple experiment, where membrane permeable cAMP was added to pleomorphic and more recently monomorphic cell culture induced differentiation has been repeated (Vassella *et al.* 1997; Breidbach *et al.* 2002). On repeating this experiment in the course of this project, differentiation was not induced. Of late, however, this apparent induction of differentiation has been shown to be due to hydrolysed analogues of cAMP, not addition of cAMP to the medium (Laxman *et al.* 2006), which may explain why induction of differentiation did not occur in this case, (though the authors did question the role non-hydrolysable cAMP in differentiation, suggesting previous results implicating cAMP in differentiation could have been misinterpreted). Similarly, this induction of differentiation is not conclusive, as growth arrest was also demonstrated in monomorphic cell culture following the addition of the prostaglandin programmed cell death inducers. Both PGD₂ and PGE₂ were shown to induce growth arrest following addition to monomorphic cell culture both in literature (Figarella *et al.* 2005) and in the course of this thesis.

A different approach was subsequently taken to elucidate the effect of several signalling factors on the role, if any, of TbArf1 in the differentiation process. An assay was developed to examine TbArf1 interactions with other trypanosomal proteins in the absence and presence of cAMP and other signalling factors. cAMP was shown to block the binding of basic proteins (37-50 kDa) and enhance the binding of another protein (40 kDa); indicating that cAMP possibly interacts with TbArf1. This differed from the control experiments where no differences in the pattern of TbArf1 effector protein expression occurred following addition of cGMP, PGD₂, PGE₂, or PGF₂.

This is similar to the cAMP signal transduction process utilised by the unicellular eukaryote *Dictyostelium discoideum* (slime mould) that undergoes a cell-

density mediated differentiation (Mato *et al.* 1977a; 1977b; 1977c; 1977d). In the *D. discoideum* system cAMP is sensed by a G-protein, cAR1, which relays a signal throughout the cell via adenylyl cyclase (Theibert & Devreotes, 1986; Van Haastert *et al.* 1987, 1989). In the case of *T. b. brucei*, we predict that TbArf1 acts like a G-protein and through interaction with cAMP it facilitates the propagation of the differentiation signal throughout the cell. Alterations in gene expression patterns are initiated as a result, and cells are morphologically changed as they differentiate from of long slender cells into short stumpy cells.

6.1.6 The Role of T-GIF in Differentiation

Subsequently, a similar approach was used to investigate the impact of T-GIF binding to TbArf1. T-GIF has been previously shown to bind to the helix $\alpha 3$ region of TbArf1 (Murphy and Olijhoek 2001). T-GIF was shown to block the binding of all effector proteins to TbArf1, following incubation with TbArf1. We propose that following entry into the cell, T-GIF binds to the helix $\alpha 3$ region of TbArf1, and TbArf1 effector proteins and bound molecules are expelled from TbArf1 binding sites. This expulsion of bound proteins and molecules likely triggers a cAMP cascade, triggering differentiation.

6.1.7 A Model for Differentiation in *T. b. brucei*

Proliferative long slender *T. b. brucei* cell populations produce and export a low molecular weight factor, T-GIF (or SIF), which builds up in the media as cell density increases (Vassella *et al.* 1997). As peak parasitaemia approaches, the T-GIF factor is

taken up into long slender cells via a receptor/transporter, the TbPP69 surface protein that is present in abundance on pleomorphic cells and has been lost from monomorphic cells (Murphy and Olijhoek 2001). ESAG10 is likely the TbPP69 receptor/transporter, allowing uptake of the T-GIF factor. Upon entry into the cell, the T-GIF factor interacts strongly with TbArf1 which is located at the plasma membrane (Murphy and Olijhoek 2001). This interaction between TbArf1 and T-GIF is very strong, and expels several bound TbArf1 effector proteins and molecules from TbArf1 binding sites. Subsequently, a signalling cascade is triggered, likely through an effector of TbArf1 such as an ArfGEF, as ArfGEFs have been implicated in protein kinase A (PKA) signalling in mammals (Li *et al.* 2003), triggering adenylate cyclase. This results in alterations in gene expression patterns which ultimately result in long slender to short stumpy cell differentiation.

Compartmental analyses of this model show how this model fits with the novel information discussed in this thesis. Upregulation of ESAG10 expression in ESAG10 overexpressing cells results in greater tomato lectin staining along the flagellum of the cell (Chapter 3, Figures 3.15 and 3.16), suggesting that ESAG10 is expressed along the flagellum. Although ESAG receptor proteins are normally found within the flagellar pocket, overexpression of ESAG6 and ESAG7 (the two subunits of the transferrin receptor) leads to saturation of the flagellar pocket, and the transferrin receptor is subsequently found along the flagellum of the cell (Mussmann *et al.* 2003). It is likely that overexpression of ESAG10 also results in saturation of the flagellar pocket, so that ESAG10 is located along the flagellum as well. In addition, the genes encoding the Transferrin receptor can also regulate its own expression due to its promoter proximal position (Mussman 2003). Likewise, the gene encoding ESAG10 is also promoter proximal and this may allow self regulation of

ESAG10 expression. Upon overexpression of ESAG10 the location of TbArf1 within the cell is altered; TbArf1 is found along the flagellum as well as in the Golgi in cells overexpressing ESAG10 (Chapter 3, Figure 3.15 and 3.16). Unlike in monomorphic cells where TbArf1 is exclusively located in the Golgi and surrounding region (Price *et al.* 2007), in pleomorphic cells TbArf1 it is located at the flagellum (Chapter 1, Figure 1.8). Thus within the monomorphic ESAG10 overexpressing cells a situation is found comparable with pleomorphic cells.

Our model suggests that within pleomorphic cells ESAG10 and associated TbArf1 are located along the flagellum of the cell in addition to other important signalling factors such as cAMP phosphodiesterases and PKA (Oberholzer *et al.* 2007), making the flagellum an important region in cell signalling. Once ESAG10 and the associated TbArf1 have taken up their new position along the flagellum they can function as a kind of G-protein coupled-like receptor, GPCR. The ESAG10-TbArf1 G-protein coupled-like receptor subsequently transfers the signal from the transported T-GIF ligand to trigger the cAMP cascade that results in the differentiation event.

To understand how ESAG10 in conjunction with TbArf1 could operate in *T. b. brucei* as a GPCR the cell-density mediated differentiation process of *Dictyostelium discoideum* can be used as a model. When a *Dictyostelium* cell is starving in the presence of a high density of other starving cells, it receives a pulse of cAMP and it relays this pulse, by releasing a burst of cAMP itself. The amoebae then move towards the source of cAMP and expresses specific classes of genes (Firtel *et al.* 1989; Loomis 1993). The incoming cAMP pulse is sensed by cAR1, a cell surface cAMP receptor that activates its associated G proteins (Theibert and Devreotes 1986; Van Haastert *et al.* 1987; Van Haastert *et al.* 1989); this role would be assumed by ESAG10 in the case of *T. b. brucei*. The G $\beta\gamma$ subunit of the G-receptor CRAC

(cytosolic protein) transiently activates adenylyl cyclase; likewise the role of TbArf1 in the model that interacts with T-GIF, and transiently activates adenylate cyclase through an effector protein, likely an ArfGEF (Figure 6.1).

The findings of this thesis are consistent with cAMP being the T-GIF factor, such that cAMP or an analogue of cAMP is secreted by the parasite as the T-GIF factor. Tom Seebeck's group has identified two ATP-binding cassette (ABC) transporters of the multidrug resistance-associated proteins (MRP-like) type in the *T. b. brucei* that actively export cAMP from the cell. At present, Tom Seebeck's group suggest that these transporters are involved in removing excess cAMP from the cell; however, the major role of these transporters may be secreting the T-GIF factor that triggers differentiation (Marti and Seebeck 2007). Following exportation of cAMP or an analogue from the cell, the ESAG10-TbArf1 G-protein coupled-like receptor transports the cAMP or cAMP analogue inside the cell where it interacts with TbArf1. (Note that TbArf1 was suggested to possibly interact with cAMP in this thesis, Chapter 5, Figure 5.9 and 5.10). Subsequently, TbArf1 interacts with adenylate cyclase triggering a signalling cascade that ultimately alters the gene expression patterns resulting in the change from long slender cells into short stumpy cells.

If the T-GIF factor were cAMP or an analogue of cAMP then ESAG10 would have to import cAMP into the cell. ESAG10 is a biopterin transporter (Myler *et al.* 1994), and biopterin transporters have not been linked with importation of cAMP; however, a cAMP transporter, MRP 4, that ordinarily transports cAMP can also transport folate (Chen *et al.* 2002), so it may be possible that folate/biopterin transporters can also transport cAMP or an analogue of cAMP.

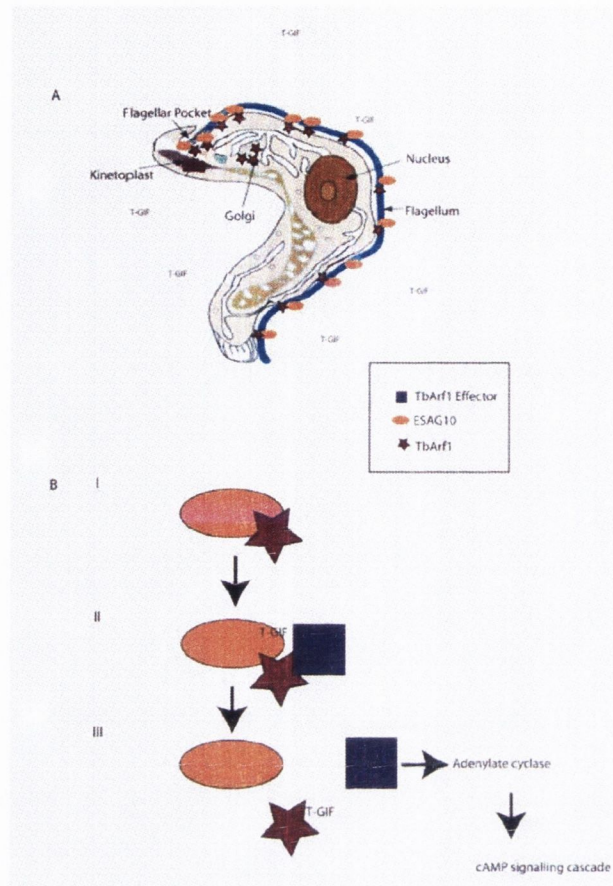


Fig 6.1. Schematic of long slender to short stumpy differentiation in *T. b. brucei* cells. (A) ESAG10 is located in the flagellar pocket and along the flagellum. TbArf1 which is usually found in the Golgi region associates with ESAG10 along the flagellum surface. (B) A schematic of the events that occur at the flagellar membrane. (I) ESAG10 is associated with TbArf1. (II) ESAG10 imports T-GIF in through the plasma membrane where it interacts with TbArf1. (III) TbArf1 can no longer associate with ESAG10. T-GIF binds to the helix $\alpha 3$ region to TbArf1, displacing other bound TbArf1 effector proteins, possibly TbArfGEF which triggers a cAMP cascade through activation of adenylate cyclase.

In order to show that this model is accurate it will require testing. The ESAG10 bioprotein transporter requires testing for importation of cAMP or a cAMP analogue. TbArf1 requires testing for triggering a cAMP cascade, through activation of adenylate cyclase through an ArfGEF, and most importantly, cAMP or an analogue of cAMP should be tested as the T-GIF factor.

This new model for differentiation is also consistent with the subsequent programmed cell death-like process that follows long slender to short stumpy differentiation when cells are not ingested in Tsetse fly blood meal within 2-3 days. As a similar programmed cell death-like process occurs in *Dictyostelium discoideum* following a similar differentiation event, this suggests support for this novel differentiation pathway in *T. b. brucei*.

6.2 Future Work

Several aspects of the differentiation process were not addressed in the course of this project, however, and addressing these other issues would lead to further understanding of the differentiation process. Although ESAG10 was suggested to be the T-GIF differentiation receptor/transporter, this has not been shown conclusively, and further studies are required to confirm this finding. In addition, ESAG10 was shown to associate with TbArf1 along the flagellum of the cell where this differentiation/signalling event is thought to occur. T-GIF was shown to bind to TbArf1 preventing several other TbArf1 effector proteins (ArfGAPs and ArfGEFs) from binding to TbArf1. These proteins that were prevented from binding to TbArf1 in the presence of T-GIF require identification and mass spectrometry studies should be carried out to obtain the identities of these proteins. This information would aid

understanding of how the cAMP cascade is triggered. Similarly, the proteins prevented from binding to TbArf1 in the presence of cAMP require identification; this information would help to explain if cAMP was the T-GIF factor, etc.

New drugs are required to treat trypanosomiasis as the existing drugs are dangerous and outdated. In this thesis, two new drug targets were identified. Firstly, the ESAG10 receptor/transporter has been identified as a possible drug target as during the life cycle of *T. b. brucei* in the mammalian host, the trypanosomes undergo a developmental transition from long slender stage to short stumpy forms. ESAG10 appears to be involved in the differentiation step as the TbPP69 receptor/transporter that imports the differentiation factor, T-GIF, into the cell. As this differentiation step is a terminal differentiation step, i.e. if cells are not uptaken by tsetse fly they undergo a programmed cell death (PCD)-like death process. If a drug or vaccine against ESAG10 could trigger this process, it would lead to growth arrest in the mammalian host. Similarly, TbArf1 presents a similar scenario; this essential internal protein could be used as a drug target. Although TbArf1 is a highly conserved protein in eukaryotes, it differs in helix $\alpha 3$ region in trypanosomes to other eukaryotes. The drug would target with domain of TbArf1, and therefore toxicity should not be an issue. Rapid death of the trypanosome cells would result.

Chapter 7: Appendix ILTat 1.1 VSG versus ILTat 1.4 VSG Expression

7.1 Further Investigation of the Presence of the ILTat 1.4 VSG in the Preparation of Tomato Lectin Binding Proteins Isolated from ILTat 1.1

The TbPP69 protein that is upregulated in pleomorphic *T. b. brucei* cells (cells that can differentiate to short stumpy bloodstream forms) in comparison to monomorphic cells (cells that cannot undergo this differentiation event) was prepared from pleomorphic *T. b. brucei* ILTat 1.1 serodeme cells and visualised on a 1-D SDS-PAGE gel (Chapter 3, Figure 3.1). To elucidate the identity of the TbPP69 protein, the 69 kDa band was cut from the gel and sent for mass spectrometry analysis. The resultant peptide sequences were compared to the *T. b. brucei* Sanger database (<http://www.genedb.org/>), and to the non-redundant GenBank database (<http://www.ncbi.nlm.nih.gov/BLAST/>) (Chapter 3, Section 3.3). The TbPP69 receptor/transporter mass spectrometry derived peptide sequences were matched to several partially sequenced ILTat 1.4 VSG *T. brucei* sequences from GenBank. This was surprising as the trypanosome strain used to make this TbPP69 kDa preparation was the ILTat 1.1 clone, which predominantly expresses ILTat 1.1 VSG, not the ILTat 1.4 VSG that was detected in the experiment.

The tomato lectin isolation technique used to prepare the TbPP69 protein should ensure that no VSG is isolated with the protein extract (Nolan *et al.* 1999). If, however, some VSG was co-isolated with the protein extract, one would expect ILTat

1.1 VSG contamination, the VSG that is predominantly produced by that strain. This was not the case; instead ILTat 1.4 VSG was a major protein present in the TbPP69 preparation. Either (1) there could have been a mix up and that the tomato lectin preparations were from ILTat 1.4 cells-however, this is unlikely as the cell line was checked by an antibody against the ILTat 1.1 VSG prior to protein preparation-or (2) there was a minor component of the population that had switched VSG and that this was predominantly expressing ILTat 1.4 VSG. If this switch was carried out at a rate of 1 in 10^4 in a population of 10^{10} , then 10^6 will have a different VSG, which may have been enriched in the tomato lectin purification process. It is difficult to understand why ILTat 1.4 VSG would contaminate the preparations rather than ILTat 1.1, but possibly ILTat 1.4 is processed differently and may be glycosylated in a manner that causes it to bind to the tomato lectin. Another possibility exists: (3) in all ILTat 1.1 cells there is a low level of expression of ILTat 1.4 VSG (possibly localised in a specific nuclear compartment preparing for switching) and that this form of expression results in a different type of processing that causes linear chains of poly-N-acetyllactosamine (pNAL) to be added, causing the protein to bind to tomato lectin.

RT-PCR can be used to show if other VSGs are being expressed at a lower level to the predominant VSG, but, this does not enable distinguishing of possibilities (2) and (3). With this in mind RT-PCR studies were conducted to determine whether the ILTat 1.4 gene was transcribed in preparations of ILTat 1.1 cells (Figure 7.1).

7.2 Investigation of ILTat VSG Gene Expression in Different Life Cycle Stages of *T. b. brucei*

VSG gene expression was investigated in long slender and short stumpy forms of *T. b. brucei* ILTat 1.1 serodeme to understand why ILTat 1.4 VSG contamination was apparent in the TbPP69 receptor/transporter preparations. mRNA was isolated from long slender and short stumpy *T. b. brucei* ILTat 1.1 cells and the mRNA was further purified with streptavidin-coated paramagnetic particle (SA-PMP®) magnetic beads. Reverse transcription was carried out to produce cDNA as described in the materials and methods (Chapter 2, Section 2.2.3) which was then amplified with four sets of VSG oligonucleotide primers that amplified ILTat 1.1 VSG, ILTat 1.2 VSG, ILTat 1.3 VSG, and ILTat 1.4 VSG, (Table 7.1, Figure 7.1). DNase treated preparations and genomic DNA controls were also amplified with the specified oligonucleotide primers.

The resultant PCR products showed expression of the surface coat VSG ILTat 1.1 gene in the long slender forms, with low-level expression of the ILTat 1.3 VSG gene. In the short stumpy forms, expression of the ILTat 1.1 VSG gene was not detected, while there was expression of the ILTat 1.3 VSG gene. None of the transcripts were detected in the DNase I treated genomic control, as expected, as the mRNA was pretreated with RNase-free DNase to remove any contaminating genomic DNA to demonstrate that the DNase treatment was effective. ILTat 1.2 and ILTat 1.4 VSG transcripts were not detected in long slender and short stumpy, only in the positive genomic control, where ILTat 1.1 VSG, ILTat 1.2 VSG, ILTat 1.3 VSG and ILTat 1.4 VSG were detected.

Table 7.1. Table of the ILTat 1.1 VSG, ILTat 1.2 VSG, ILTat 1.3 VSG and ILTat 1.4 VSG primer names, PCR product names and expected sizes.

Primer name	PCR product	PCR product size
ILTat 1.1 VSG F	ILTat 1.1 VSG	642 bp
ILTat 1.1 VSG R		
ILTat 1.2 VSG F	ILTat 1.2 VSG	618 bp
ILTat 1.2 VSG R		
ILTat 1.3 VSG F	ILTat 1.3 VSG	590 bp
ILTat 1.3 VSG R		
ILTat 1.4 VSG F	ILTat 1.4 VSG	538 bp
ILTat 1.4 VSG R		

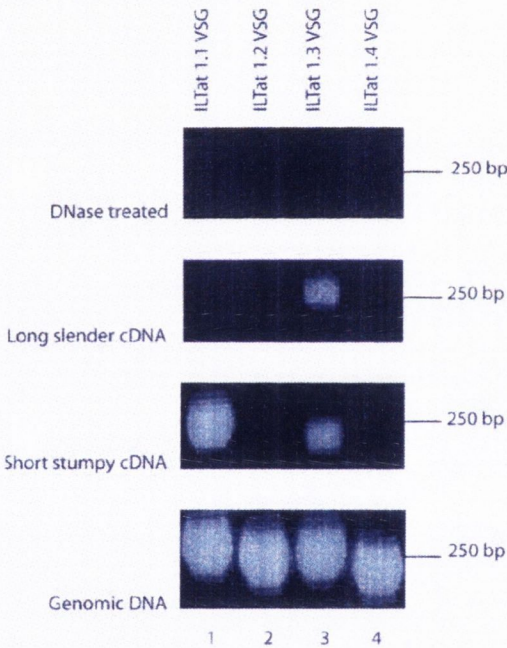


Fig 7.1. Agarose gel depicting fragments of cDNA amplified with the ILTat VSG primers. The 1 % agarose gel depicts amplification of cDNA with primers for the ILTat 1.1 VSG (lane 1), ILTat 1.2 VSG (lane 2), ILTat 1.3 VSG (lane 3), and ILTat 1.4 VSGs (lane 4). The figure shows there is amplification of long slender cDNA ILTat 1.1 VSG (lane 1, 2nd panel), long slender ILTat 1.3 VSG (lane 3, 2nd panel) and short stumpy ILTat 1.3 VSG (lane 3, 3rd panel). Note that no amplification was detected in DNase I treated genomic DNA, and products from all VSGs were amplified in the untreated genomic DNA.

7.3 Interpretation of the ILTat VSG Results

ILTat 1.1 VSG was predominantly detected in long slender cells, but ILTat 1.3 VSG was also detected; whilst in short stumpy cells only ILTat 1.3 VSG was detected. This shows that although ILTat 1.1 was the predominant VSG expressed in these long slender cells, it was not detected in short stumpy cells. A recent paper showed that the system conferring a selective RNA processing/elongation to a single gene is lost following differentiation of bloodstream forms from long slender into short stumpy forms (Amiguet-Vercher *et al.* 2004), specifically resulting in loss of VSG mRNA in short stumpy forms. The authors attributed the loss of VSG mRNA to interference from the differentiation process, such that RNA elongation and process factors were no longer recruited to active expression sites.

Although ILTat 1.1 VSG detection is lost in short stumpy cells, ILTat 1.3 VSG was detected in both long slender and short stumpy cells. This may be due to possibility (2, section 7.1) that cells expressing the ILTat 1.3 mRNA are all long slender cells and represent a minor population that has switched or possibility (3, section 7.1) that there is a low level of expression of this VSG in the majority of ILTat 1.1 cells but is under different control. If it is the former, a minor population of ILTat 1.3 VSG expressing cells in a major population of ILTat 1.1 VSG expressing cells, then these ILTat 1.3 VSG expressing cells are somehow insensitive to the SIF/T-GIF factor (the differentiation trigger) and can escape its effects. If it is the latter, then there may be a different mechanism by which VSG gene switching occurs. Further analyses are required to explain what is occurring in these short stumpy cells and why any ILTat 1.3 VSG can be detected at all. The use of specific antibodies against each

VSG would be required to resolve this question (bearing in mind that the detection of mRNA doesn't necessarily mean that the protein product will be produced).

In both of the above experiment, further investigation of ILTat VSG expression, and the preparation of TbPP69 receptor/transporter (Chapter 3, Section 3.3), the *T. b. brucei* ILTat 1.1 clone was used to derive mRNA and surface proteins respectively. Though the cells used to make these isolations were from the same clone, ILTat 1.1, they differed from one another in that they represented two different batch preparations of the same clone.

All of the above points represent interesting possibilities, but in the course of this work they were not followed up as the materials, reagents and time required were not available.

References

- Abergel C, Chavrier P, Claverie JM (1998) Triple association of CDC25-, Dbl- and Sec7-related domains in mammalian guanine-nucleotide-exchange factors. *Trends Biochem Sci* 23(12): 472-473.
- Alfalah M, Jacob R, Preuss U, Zimmer KP, Naim H et al. (1999) O-linked glycans mediate apical sorting of human intestinal sucrase-isomaltase through association with lipid rafts. *Curr Biol* 9(11): 593-596.
- Altschuler Y, Liu S, Katz L, Tang K, Hardy S et al. (1999) ADP-ribosylation factor 6 and endocytosis at the apical surface of Madin-Darby canine kidney cells. *J Cell Biol* 147(1): 7-12.
- Ames, B. N. (1966) Assay of Inorganic Phosphate, Total Phosphate and Phosphatases. In: *Methods in Enzymology*, Vol. VIII: Complex Carbohydrates. E. Neufeld and V. Ginsburg, eds. (Academic Press, New York, NY), pp. 115-118.
- Amiguet-Vercher A, Perez-Morga D, Pays A, Poelvoorde P, Van Xong H et al. (2004) Loss of the mono-allelic control of the VSG expression sites during the development of *Trypanosoma brucei* in the bloodstream. *Mol Microbiol* 51(6): 1577-1588.
- Antonny B, Beraud-Dufour S, Chardin P, Chabre M (1997) N-terminal hydrophobic residues of the G-protein ADP-ribosylation factor-1 insert into membrane phospholipids upon GDP to GTP exchange. *Biochemistry* 36(15): 4675-4684.
- Aoe T, Cukierman E, Lee A, Cassel D, Peters PJ et al. (1997) The KDEL receptor, ERD2, regulates intracellular traffic by recruiting a GTPase-activating protein for ARF1. *Embo J* 16(24): 7305-7316.
- Balch WE, Kahn RA, Schwaninger R (1992) ADP-ribosylation factor is required for vesicular trafficking between the endoplasmic reticulum and the cis-Golgi compartment. *J Biol Chem* 267(18): 13053-13061.
- Barr FA, Preisinger C, Kopajtich R, Korner R (2001) Golgi matrix proteins interact with p24 cargo receptors and aid their efficient retention in the Golgi apparatus. *J Cell Biol* 155(6): 885-891.
- Barry JD, Turner CM (1991) The dynamics of antigenic variation and growth of African trypanosomes. *Parasitol Today* 7(8): 207-211.
- Beck JT, Ullman B (1991) Biopterin conversion to reduced folates by *Leishmania donovani* promastigotes. *Mol Biochem Parasitol* 49(1): 21-28.
- Beemiller P, Hoppe AD, Swanson JA (2006) A phosphatidylinositol-3-kinase-dependent signal transition regulates ARF1 and ARF6 during Fcγ receptor-mediated phagocytosis. *PLoS Biol* 4(6): e162.
- Belden WJ, Barlowe C (1996) Erv25p, a component of COPII-coated vesicles, forms a complex with Emp24p that is required for efficient endoplasmic reticulum to Golgi transport. *J Biol Chem* 271(43): 26939-26946.
- Bendtsen JD, Nielsen H, von Heijne G, Brunak S (2004) Improved prediction of signal peptides: SignalP 3.0. *J Mol Biol* 340(4): 783-795.
- Beraud-Dufour S, Robineau S, Chardin P, Paris S, Chabre M et al. (1998) A glutamic finger in the guanine nucleotide exchange factor ARNO displaces Mg²⁺ and the beta-phosphate to destabilize GDP on ARF1. *Embo J* 17(13): 3651-3659.

- Bernards A (2003) GAPs galore! A survey of putative Ras superfamily GTPase activating proteins in man and *Drosophila*. *Biochim Biophys Acta* 1603(2): 47-82.
- Berriman M, Hall N, Sheader K, Bringaud F, Tiwari B et al. (2002) The architecture of variant surface glycoprotein gene expression sites in *Trypanosoma brucei*. *Mol Biochem Parasitol* 122(2): 131-140.
- Berriman M, Ghedin E, Hertz-Fowler C, Blandin G, Renauld H et al. (2005) The genome of the African trypanosome *Trypanosoma brucei*. *Science* 309(5733): 416-422.
- Betz SF, Schnuchel A, Wang H, Olejniczak ET, Meadows RP et al. (1998) Solution structure of the cytohesin-1 (B2-1) Sec7 domain and its interaction with the GTPase ADP ribosylation factor 1. *Proc Natl Acad Sci U S A* 95(14): 7909-7914.
- Bjellqvist B, Hughes GJ, Pasquali C, Paquet N, Ravier F et al. (1993) The focusing positions of polypeptides in immobilized pH gradients can be predicted from their amino acid sequences. *Electrophoresis* 14(10): 1023-1031.
- Bonifacino JS (2004) The GGA proteins: adaptors on the move. *Nat Rev Mol Cell Biol* 5(1): 23-32.
- Bornancin F, Franco M, Bigay J, Chabre M (1992) Functional modifications of transducin induced by cholera or pertussis-toxin-catalyzed ADP-ribosylation. *Eur J Biochem* 210(1): 33-44.
- Borst P (2002) Antigenic variation and allelic exclusion. *Cell* 109: 5-8.
- Breidbach T, Ngazoa E, Steverding D (2002) *Trypanosoma brucei*: in vitro slender-to-stumpy differentiation of culture-adapted, monomorphic bloodstream forms. *Exp Parasitol* 101(4): 223-230.
- Bremser M, Nickel W, Schweikert M, Ravazzola M, Amherdt M et al. (1999) Coupling of coat assembly and vesicle budding to packaging of putative cargo receptors. *Cell* 96(4): 495-506.
- Brown HA, Gutowski S, Moomaw CR, Slaughter C, Sternweis PC (1993) ADP-ribosylation factor, a small GTP-dependent regulatory protein, stimulates phospholipase D activity. *Cell* 75(6): 1137-1144.
- Cavalier-Smith T (2004) Only six kingdoms of life. *Proc Biol Sci* 271(1545): 1251-1262.
- Chantalat S, Courbeyrette R, Senic-Matuglia F, Jackson CL, Goud B et al. (2003) A novel Golgi membrane protein is a partner of the ARF exchange factors Gea1p and Gea2p. *Mol Biol Cell* 14(6): 2357-2371.
- Chardin P, Paris S, Antonny B, Robineau S, Beraud-Dufour S et al. (1996) A human exchange factor for ARF contains Sec7- and pleckstrin-homology domains. *Nature* 384(6608): 481-484.
- Chen K, Rajewsky N (2007) The evolution of gene regulation by transcription factors and microRNAs. *Nat Rev Genet* 8(2): 93-103.
- Chen ZS, Lee K, Walther S, Raftogianis RB, Kuwano M et al. (2002) Analysis of methotrexate and folate transport by multidrug resistance protein 4 (ABCC4): MRP4 is a component of the methotrexate efflux system. *Cancer Res* 62(11): 3144-3150.
- Cherfils J, Menetrey J, Mathieu M, Le Bras G, Robineau S et al. (1998) Structure of the Sec7 domain of the Arf exchange factor ARNO. *Nature* 392(6671): 101-105.

- Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162(1): 156-159.
- Clark J, Moore L, Krasinskas A, Way J, Battey J et al. (1993) Selective amplification of additional members of the ADP-ribosylation factor (ARF) family: cloning of additional human and *Drosophila* ARF-like genes. *Proc Natl Acad Sci U S A* 90(19): 8952-8956.
- Cockcroft S, Thomas GM, Fensome A, Geny B, Cunningham E et al. (1994) Phospholipase D: a downstream effector of ARF in granulocytes. *Science* 263(5146): 523-526.
- Coppens I, Courtoy PJ (1995) Exogenous and endogenous sources of sterols in the culture-adapted procyclic trypomastigotes of *Trypanosoma brucei*. *Mol Biochem Parasitol* 73(1-2): 179-188.
- Coppens I, Oppendoes FR, Courtoy PJ, Baudhuin P (1987) Receptor-mediated endocytosis in the bloodstream form of *Trypanosoma brucei*. *J Protozool* 34(4): 465-473.
- Coppens I, Baudhuin P, Oppendoes FR, Courtoy PJ (1988) Receptors for the host low density lipoproteins on the hemoflagellate *Trypanosoma brucei*: purification and involvement in the growth of the parasite. *Proc Natl Acad Sci U S A* 85(18): 6753-6757.
- Cukierman E, Huber I, Rotman M, Cassel D (1995) The ARF1 GTPase-activating protein: zinc finger motif and Golgi complex localization. *Science* 270(5244): 1999-2002.
- D'Angelo MA, Sanguineti S, Reece JM, Birnbaumer L, Torres HN et al. (2004) Identification, characterization and subcellular localization of TcPDE1, a novel cAMP-specific phosphodiesterase from *Trypanosoma cruzi*. *Biochem J* 378(Pt 1): 63-72.
- D'Souza-Schorey C, Boshans RL, McDonough M, Stahl PD, Van Aelst L (1997) A role for POR1, a Rac1-interacting protein, in ARF6-mediated cytoskeletal rearrangements. *Embo J* 16(17): 5445-5454.
- D'Souza-Schorey C, van Donselaar E, Hsu VW, Yang C, Stahl PD et al. (1998) ARF6 targets recycling vesicles to the plasma membrane: insights from an ultrastructural investigation. *J Cell Biol* 140(3): 603-616.
- De Greef C, Imberechts H, Matthyssens G, Van Meirvenne N, Hamers R (1989) A gene expressed only in serum-resistant variants of *Trypanosoma brucei* rhodesiense. *Mol Biochem Parasitol* 36(2): 169-176.
- Desquesnes M, Davila AM (2002) Applications of PCR-based tools for detection and identification of animal trypanosomes: a review and perspectives. *Vet Parasitol* 109(3-4): 213-231.
- Doering TL, Schekman R (1996) GPI anchor attachment is required for Gas1p transport from the endoplasmic reticulum in COP II vesicles. *Embo J* 15(1): 182-191.
- Dogic D, de Chasse B, Pick E, Cassel D, Lefkir Y et al. (1999) The ADP-ribosylation factor GTPase-activating protein Glo3p is involved in ER retrieval. *Eur J Cell Biol* 78(5): 305-310.
- Domenicali Pfister D, Burkard G, Morand S, Renggli CK, Roditi I et al. (2006) A Mitogen-activated protein kinase controls differentiation of bloodstream forms of *Trypanosoma brucei*. *Eukaryot Cell* 5(7): 1126-1135.
- Donaldson JG, Lippincott-Schwartz J (2000) Sorting and signaling at the Golgi complex. *Cell* 101(7): 693-696.

- Donaldson JG, Honda A (2005) Localization and function of Arf family GTPases. *Biochem Soc Trans* 33(Pt 4): 639-642.
- Donaldson JG, Honda A, Weigert R (2005) Multiple activities for Arf1 at the Golgi complex. *Biochim Biophys Acta* 1744(3): 364-373.
- Donaldson JG, Kahn RA, Lippincott-Schwartz J, Klausner RD (1991) Binding of ARF and beta-COP to Golgi membranes: possible regulation by a trimeric G protein. *Science* 254(5035): 1197-1199.
- Donaldson JG, Cassel D, Kahn RA, Klausner RD (1992) ADP-ribosylation factor, a small GTP-binding protein, is required for binding of the coatomer protein beta-COP to Golgi membranes. *Proc Natl Acad Sci U S A* 89(14): 6408-6412.
- Donelson JE, Gardner MJ, El-Sayed NM (1999) More surprises from Kinetoplastida. *Proc Natl Acad Sci U S A* 96(6): 2579-2581.
- Donelson JE, Young JR, Dorfman D, Majiwa PA, Williams RO (1982) The ILtat 1.4 surface antigen gene family of *Trypanosoma brucei*. *Nucleic Acids Res* 10(21): 6581-6595.
- Dong X, Stothard P, Forsythe IJ, Wishart DS (2004) PlasMapper: a web server for drawing and auto-annotating plasmid maps. *Nucleic Acids Res* 32(Web Server issue): W660-664.
- Facer CA, Crosskey JM, Clarkson MJ, Jenkins GC (1982) Immune haemolytic anaemia in bovine trypanosomiasis. *J Comp Pathol* 92(3): 393-401.
- Field H, Sherwin T, Smith AC, Gull K, Field MC (2001) Cell-cycle and developmental regulation of TbRAB31 localisation, a GTP-locked Rab protein from *Trypanosoma brucei* *Mol Biochem Parasitol* 10: 329-330
- Field MC (2005) Signalling the genome: the Ras-like small GTPase family of trypanosomatids. *Trends Parasitol* 21(10): 447-450.
- Fielder M, Pirt SJ, Tarpey I, Wilson C, Cunningham P et al. (1995) Molecular mimicry and ankylosing spondylitis: possible role of a novel sequence in pullulanase of *Klebsiella pneumoniae*. *FEBS Lett* 369(2-3): 243-248.
- Figarella K, Rawer M, Uzcategui NL, Kubata BK, Lauber K et al. (2005) Prostaglandin D2 induces programmed cell death in *Trypanosoma brucei* bloodstream form. *Cell Death Differ* 12(4): 335-346.
- Firtel RA, van Haastert PJ, Kimmel AR, Devreotes PN (1989) G protein linked signal transduction pathways in development: dictyostelium as an experimental system. *Cell* 58(2): 235-239.
- Franco M, Chardin P, Chabre M, Paris S (1995) Myristoylation of ADP-ribosylation factor 1 facilitates nucleotide exchange at physiological Mg²⁺ levels. *J Biol Chem* 270(3): 1337-1341.
- Franco M, Peters PJ, Boretto J, van Donselaar E, Neri A et al. (1999) EFA6, a sec7 domain-containing exchange factor for ARF6, coordinates membrane recycling and actin cytoskeleton organization. *Embo J* 18(6): 1480-1491.
- Frank SR, Hatfield JC, Casanova JE (1998) Remodeling of the actin cytoskeleton is coordinately regulated by protein kinase C and the ADP-ribosylation factor nucleotide exchange factor ARNO. *Mol Biol Cell* 9(11): 3133-3146.
- Geiger C, Nagel W, Boehm T, van Kooyk Y, Figdor CG et al. (2000) Cytohesin-1 regulates beta-2 integrin-mediated adhesion through both ARF-GEF function and interaction with LFA-1. *Embo J* 19(11): 2525-2536.
- Goldberg J (1998) Structural basis for activation of ARF GTPase: mechanisms of guanine nucleotide exchange and GTP-myristoyl switching. *Cell* 95(2): 237-248.

- Goldberg J (1999) Structural and functional analysis of the ARF1-ARFGAP complex reveals a role for coatamer in GTP hydrolysis. *Cell* 96(6): 893-902.
- Gong KW, Kunz S, Zoraghi R, Kunz Renggli C, Brun R et al. (2001) cAMP-specific phosphodiesterase TbPDE1 is not essential in *Trypanosoma brucei* in culture or during midgut infection of tsetse flies. *Mol Biochem Parasitol* 116(2): 229-232.
- Gottesdiener KM (1994) A new VSG expression site-associated gene (ESAG) in the promoter region of *Trypanosoma brucei* encodes a protein with 10 potential transmembrane domains. *Mol Biochem Parasitol* 63(1): 143-151.
- Graham SV, Barry JD (1995) Transcriptional regulation of metacyclic variant surface glycoprotein gene expression during the life cycle of *Trypanosoma brucei*. *Mol Cell Biol* 15(11): 5945-5956.
- Grunfelder CG, Engstler M, Weise F, Schwarz H, Stierhof YD et al. (2003) Endocytosis of a glycosylphosphatidylinositol-anchored protein via clathrin-coated vesicles, sorting by default in endosomes, and exocytosis via RAB11-positive carriers. *Mol Biol Cell* 14(5): 2029-2040.
- Gruszyński AE, DeMaster A, Hooper NM, Bangs JD (2003) Surface coat remodeling during differentiation of *Trypanosoma brucei*. *J Biol Chem* 278(27): 24665-24672.
- Gupta R, Birch H, Rapacki K, Brunak S, Hansen JE (1999) O-GLYCBASE version 4.0: a revised database of O-glycosylated proteins. *Nucleic Acids Res* 27(1): 370-372.
- Gut A, Kappeler F, Hyka N, Balda MS, Hauri HP et al. (1998) Carbohydrate-mediated Golgi to cell surface transport and apical targeting of membrane proteins. *Embo J* 17(7): 1919-1929.
- Hager KM, Hajduk SL (1997) Mechanism of resistance of African trypanosomes to cytotoxic human HDL. *Nature* 385(6619): 823-826.
- Hajduk SL, Moore DR, Vasudevacharya J, Siqueira H, Torri AF et al. (1989) Lysis of *Trypanosoma brucei* by a toxic subspecies of human high density lipoprotein. *J Biol Chem* 264(9): 5210-5217.
- Hammarton TC, Mottram JC, Doerig C (2003) The cell cycle of parasitic protozoa: potential for chemotherapeutic exploitation. *Prog Cell Cycle Res* 5: 91-101.
- Harroun TA, Bradshaw JP, Balali-Mood K, Katsaras J (2005) A structural study of the myristoylated N-terminus of ARF1. *Biochim Biophys Acta* 1668(1): 138-144.
- Hatzfeld M (1999) The armadillo family of structural proteins. *Int Rev Cytol* 186: 179-224.
- Hertz-Fowler C, Peacock CS, Wood V, Aslett M, Kerhornou A, Mooney P, Tivey A, Berriman M, Hall N, Rutherford K, Parkhill J, Ivens AC, Rajandream MA, Barrell B (2004) GeneDB: a resource for prokaryotic and eukaryotic organisms. *Nucleic Acids Res* 32: D339-43.
- Honda A, Nogami M, Yokozeki T, Yamazaki M, Nakamura H et al. (1999) Phosphatidylinositol 4-phosphate 5-kinase alpha is a downstream effector of the small G protein ARF6 in membrane ruffle formation. *Cell* 99(5): 521-532.
- Houslay MD, Milligan G (1997) Tailoring cAMP-signalling responses through isoform multiplicity. *Trends Biochem Sci* 22(6): 217-224.
- Huang CF, Buu LM, Yu WL, Lee FJ (1999) Characterization of a novel ADP-ribosylation factor-like protein (yARL3) in *Saccharomyces cerevisiae*. *J Biol Chem* 274(6): 3819-3827.

- Ireland LS, Johnston GC, Drebot MA, Dhillon N, DeMaggio AJ et al. (1994) A member of a novel family of yeast 'zn-finger' proteins mediates the transition from stationary phase to cell proliferation. *Embo J* 13(16): 3812-3821.
- Jackson CL, Casanova JE (2000) Turning on ARF: the Sec7 family of guanine-nucleotide-exchange factors. *Trends Cell Biol* 10(2): 60-67.
- Jackson TR, Brown FD, Nie Z, Miura K, Foroni L et al. (2000) ACAPs are arf6 GTPase-activating proteins that function in the cell periphery. *J Cell Biol* 151(3): 627-638.
- Jacques KM, Nie Z, Stauffer S, Hirsch DS, Chen LX et al. (2002) Arf1 dissociates from the clathrin adaptor GGA prior to being inactivated by Arf GTPase-activating proteins. *J Biol Chem* 277(49): 47235-47241.
- Kabayo JP (2002) Aiming to eliminate tsetse from Africa. *Trends Parasitol* 18(11): 473-475.
- Kabututu Z, Martin SK, Nozaki T, Kawazu S, Okada T et al. (2002) Prostaglandin production from arachidonic acid and evidence for a 9,11-endoperoxide prostaglandin H2 reductase in *Leishmania*. *Int J Parasitol* 32(14): 1693-1700.
- Kahn RA, Gilman AG (1986) The protein cofactor necessary for ADP-ribosylation of Gs by cholera toxin is itself a GTP binding protein. *J Biol Chem* 261(17): 7906-7911.
- Kahn RA, Cherfils J, Elias M, Lovering RC, Munro S et al. (2006) Nomenclature for the human Arf family of GTP-binding proteins: ARF, ARL, and SAR proteins. *J Cell Biol* 172(5): 645-650.
- Kirchhausen T (2000) Three ways to make a vesicle. *Nat Rev Mol Cell Biol* 1(3): 187-198.
- Klarlund JK, Guilherme A, Holik JJ, Virbasius JV, Chawla A et al. (1997) Signaling by phosphoinositide-3,4,5-trisphosphate through proteins containing pleckstrin and Sec7 homology domains. *Science* 275(5308): 1927-1930.
- Kobayashi A, Tizard IR, Woo PT (1976) Studies on the anemia in experimental African trypanosomiasis. II. The pathogenesis of the anemia in calves infected with *Trypanosoma congolense*. *Am J Trop Med Hyg* 25(3): 401-406.
- Krogh A, Larsson B, von Heijne G, Sonnhammer EL (2001) Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. *J Mol Biol* 305(3): 567-580.
- Kronegg J, Buloz D (1999), "Detection/prediction of GPI cleavage site (GPI-anchor) in a protein (DGPI)", Retrieved 2003 from <http://129.194.185.165/dgpi/>
- Kubata BK, Duzenko M, Kabututu Z, Rawer M, Szallies A et al. (2000) Identification of a novel prostaglandin f(2alpha) synthase in *Trypanosoma brucei*. *J Exp Med* 192(9): 1327-1338.
- Kumar S, Tamura K, Nei M (2004) MEGA3: Integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. *Brief Bioinform* 5(2): 150-163.
- Lang PA, Kempe DS, Myssina S, Tanneur V, Birka C et al. (2005) PGE(2) in the regulation of programmed erythrocyte death. *Cell Death Differ* 12(5): 415-428.
- Langreth SG, Balber AE (1975) Protein uptake and digestion in bloodstream and culture forms of *Trypanosoma brucei*. *J Protozool* 22(1): 40-53.
- Lawrence JT, Birnbaum MJ (2001) ADP-ribosylation factor 6 delineates separate pathways used by endothelin 1 and insulin for stimulating glucose uptake in 3T3-L1 adipocytes. *Mol Cell Biol* 21(15): 5276-5285.

- Laxman S, Riechers A, Sadilek M, Schwede F, Beavo JA (2006) Hydrolysis products of cAMP analogs cause transformation of *Trypanosoma brucei* from slender to stumpy-like forms. *Proc Natl Acad Sci U S A* 103(50): 19194-19199.
- Lee MG, Bihain BE, Russell DG, Deckelbaum RJ, Van der Ploeg LH (1990) Characterization of a cDNA encoding a cysteine-rich cell surface protein located in the flagellar pocket of the protozoan *Trypanosoma brucei*. *Mol Cell Biol* 10(9): 4506-4517.
- Lemley C, Yan S, Dole VS, Madhubala R, Cunningham ML et al. (1999) The *Leishmania donovani* LD1 locus gene ORFG encodes a biopterin transporter (BT1). *Mol Biochem Parasitol* 104(1): 93-105.
- Lewis SM, Poon PP, Singer RA, Johnston GC, Spang A (2004) The ArfGAP Glo3 is required for the generation of COPI vesicles. *Mol Biol Cell* 15(9): 4064-4072.
- Li H, Adamik R, Pacheco-Rodriguez G, Moss J, Vaughan M (2003) Protein kinase A-anchoring (AKAP) domains in brefeldin A-inhibited guanine nucleotide-exchange protein 2 (BIG2). *Proc Natl Acad Sci U S A* 100(4): 1627-1632.
- Liniger M, Bodenmuller K, Pays E, Gallati S, Roditi I (2001) Overlapping sense and antisense transcription units in *Trypanosoma brucei*. *Mol Microbiol* 40(4): 869-878.
- Loomis WF (1993) Lateral inhibition and pattern formation in *Dictyostelium*. *Curr Top Dev Biol* 28: 1-46.
- Lu L, Hong W (2003) Interaction of Arl1-GTP with GRIP domains recruits autoantigens Golgin-97 and Golgin-245/p230 onto the Golgi. *Mol Biol Cell* 14(9): 3767-3781.
- Lu L, Horstmann H, Ng C, Hong W (2001) Regulation of Golgi structure and function by ARF-like protein 1 (Arl1). *J Cell Sci* 114(Pt 24): 4543-4555.
- Majoul I, Straub M, Hell SW, Duden R, Soling HD (2001) KDEL-cargo regulates interactions between proteins involved in COPI vesicle traffic: measurements in living cells using FRET. *Dev Cell* 1(1): 139-153.
- Mandiyan V, Andreev J, Schlessinger J, Hubbard SR (1999) Crystal structure of the ARF-GAP domain and ankyrin repeats of PYK2-associated protein beta. *Embo J* 18(24): 6890-6898.
- Marti, G and Seebeck, T (2007) Characterization of cyclic AMP export from *Trypanosoma brucei*. *Kinetoplast Molecular Cell Biology Abstract* 232B.
- Mato JM, Krens FA, van Haastert PJ, Konijn TM (1977a) Unified control of chemotaxis and cAMP mediated cGMP accumulation by cAMP in *Dictyostelium discoideum*. *Biochem Biophys Res Commun* 77(1): 399-402.
- Mato JM, van Haastert PJ, Krens FA, Konijn TM (1977b) An acrasin-like attractant from yeast extract specific for *Dictyostelium lacteum*. *Dev Biol* 57(2): 450-453.
- Mato JM, Krens FA, van Haastert PJ, Konijn TM (1977c) 3':5'-cyclic AMP-dependent 3':5'-cyclic GMP accumulation in *Dictyostelium discoideum*. *Proc Natl Acad Sci U S A* 74(6): 2348-2351.
- Mato JM, Van Haastert PJ, Krens FA, Rhijnsburger EH, Dobbe FC et al. (1977d) Cyclic AMP and folic acid mediated cyclic GMP accumulation in *Dictyostelium discoideum*. *FEBS Lett* 79(2): 331-336.
- Matthews KR, Shiels PG, Graham SV, Cowan C, Barry JD (1990) Duplicative activation mechanisms of two trypanosome telomeric VSG genes with structurally simple 5' flanks. *Nucleic Acids Res* 18(24): 7219-7227.
- McCulloch R (2004) Antigenic variation in African trypanosomes: monitoring progress. *Trends Parasitol* 20(3): 117-121.

- McCulloch R, Rudenko G, Borst P (1997) Gene conversions mediating antigenic variation in *Trypanosoma brucei* can occur in variant surface glycoprotein expression sites lacking 70-base-pair repeat sequences. *Mol Cell Biol* 17(2): 833-843.
- McLafferty, F. W. & Turecek, F. (1993). Interpretation of Mass Spectra. Sausalito, CA: University Science Books.
- Merkle RK, Cummings RD (1988) Asparagine-linked oligosaccharides containing poly-N-acetyllactosamine chains are preferentially bound by immobilized calf heart agglutinin. *J Biol Chem* 263(31): 16143-16149.
- Morgan GW, Allen CL, Jeffries TR, Hollinshead M, Field MC (2001) Developmental and morphological regulation of clathrin-mediated endocytosis in *Trypanosoma brucei*. *J Cell Sci* 114(Pt 14): 2605-2615.
- Morsomme P, Riezman H (2002) The Rab GTPase Ypt1p and tethering factors couple protein sorting at the ER to vesicle targeting to the Golgi apparatus. *Dev Cell* 2(3): 307-317.
- Moss J, Vaughan M (1995) Structure and function of ARF proteins: activators of cholera toxin and critical components of intracellular vesicular transport processes. *J Biol Chem* 270(21): 12327-12330.
- Moss J, Vaughan M (1998) Molecules in the ARF orbit. *J Biol Chem* 273(34): 21431-21434.
- Mossessova E, Gulbis JM, Goldberg J (1998) Structure of the guanine nucleotide exchange factor Sec7 domain of human arno and analysis of the interaction with ARF GTPase. *Cell* 92(3): 415-423.
- Mouratou B, Biou V, Joubert A, Cohen J, Shields DJ et al. (2005) The domain architecture of large guanine nucleotide exchange factors for the small GTP-binding protein Arf. *BMC Genomics* 6(1): 20.
- Mueckler M, Caruso C, Baldwin SA, Panico M, Blench I et al. (1985) Sequence and structure of a human glucose transporter. *Science* 229(4717): 941-945.
- Muniz M, Morsomme P, Riezman H (2001) Protein sorting upon exit from the endoplasmic reticulum. *Cell* 104(2): 313-320.
- Munro S (2005) The Arf-like GTPase Arl1 and its role in membrane traffic. *Biochem Soc Trans* 33(Pt 4): 601-605.
- Murphy, N.B. & Olijhoek, T. (2001) Trypanosome factors controlling population size and differentiation status. In *World Class Parasites*, vol. 1, pp 113-126, Eds. S.J. Black and J.R. Seed, Kluwer Academic Publishers, USA .
- Musmann R, Janssen H, Calafat J, Engstler M, Ansorge I et al. (2003) The expression level determines the surface distribution of the transferrin receptor in *Trypanosoma brucei*. *Mol Microbiol* 47(1): 23-35.
- Myler PJ, Lodes MJ, Merlin G, de Vos T, Stuart KD (1994) An amplified DNA element in *Leishmania* encodes potential integral membrane and nucleotide-binding proteins. *Mol Biochem Parasitol* 66(1): 11-20.
- Myler PJ, Audleman L, deVos T, Hixson G, Kiser P et al. (1999) *Leishmania major* Friedlin chromosome 1 has an unusual distribution of protein-coding genes. *Proc Natl Acad Sci U S A* 96(6): 2902-2906.
- Navarro M, Cross GA, Wirtz E (1999) *Trypanosoma brucei* variant surface glycoprotein regulation involves coupled activation/inactivation and chromatin remodeling of expression sites. *Embo J* 18(8): 2265-2272.
- Neuhoff V, Arold N, Taube D, Ehrhardt W (1988) Improved staining of proteins in polyacrylamide gels including isoelectric focusing gels with clear background

- at nanogram sensitivity using Coomassie Brilliant Blue G-250 and R-250. Electrophoresis 9(6): 255-262.
- Nicholas, K.B., Nicholas H.B. Jr., and Deerfield, D.W. II. (1997) GeneDoc: Analysis and Visualization of Genetic Variation, EMBNET News 4:14.
- Nie Z, Hirsch DS, Randazzo PA (2003a) Arf and its many interactors. Curr Opin Cell Biol 15(4): 396-404.
- Nie Z, Boehm M, Boja ES, Vass WC, Bonifacino JS et al. (2003b) Specific regulation of the adaptor protein complex AP-3 by the Arf GAP AGAP1. Dev Cell 5(3): 513-521.
- Nolan DP, Geuskens M, Pays E (1999) N-linked glycans containing linear poly-N-acetyllactosamine as sorting signals in endocytosis in *Trypanosoma brucei*. Curr Biol 9(20): 1169-1172.
- Notredame C, Higgins DG, Heringa J (2000) T-Coffee: A novel method for fast and accurate multiple sequence alignment. J Mol Biol 302(1): 205-217.
- O'Beirne C, Lowry CM, Voorheis HP (1998) Both IgM and IgG anti-VSG antibodies initiate a cycle of aggregation-disaggregation of bloodstream forms of *Trypanosoma brucei* without damage to the parasite. Mol Biochem Parasitol 91(1): 165-193.
- Oberholzer M, Marti G, Baresic M, Kunz S, Hemphill A et al. (2007) The *Trypanosoma brucei* cAMP phosphodiesterases TbrPDEB1 and TbrPDEB2: flagellar enzymes that are essential for parasite virulence. Faseb J 21(3): 720-731.
- Ooi CE, Dell'Angelica EC, Bonifacino JS (1998) ADP-Ribosylation factor 1 (ARF1) regulates recruitment of the AP-3 adaptor complex to membranes. J Cell Biol 142(2): 391-402.
- Ouellette M, Drummelsmith J, El-Fadili A, Kundig C, Richard D et al. (2002) Pterin transport and metabolism in *Leishmania* and related trypanosomatid parasites. Int J Parasitol 32(4): 385-398.
- Overath P, Engstler M (2004) Endocytosis, membrane recycling and sorting of GPI-anchored proteins: *Trypanosoma brucei* as a model system. Mol Microbiol 53(3): 735-744.
- Overath P, Steverding D, Chaudhri M, Stierhof YD, Ziegelbauer K (1994) Structure and function of GPI-anchored surface proteins of *Trypanosoma brucei*. Braz J Med Biol Res 27(2): 343-347.
- Paindavoine P, Rolin S, Van Assel S, Geuskens M, Jauniaux JC et al. (1992) A gene from the variant surface glycoprotein expression site encodes one of several transmembrane adenylate cyclases located on the flagellum of *Trypanosoma brucei*. Mol Cell Biol 12(3): 1218-1225.
- Pal A, Hall BS, Jeffries TR, Field MC (2003) Rab5 and Rab11 mediate transferrin and anti-variant surface glycoprotein antibody recycling in *Trypanosoma brucei*. Biochem J 374(Pt 2): 443-451.
- Paris S, Beraud-Dufour S, Robineau S, Bigay J, Antonny B et al. (1997) Role of protein-phospholipid interactions in the activation of ARF1 by the guanine nucleotide exchange factor Arno. J Biol Chem 272(35): 22221-22226.
- Peyroche A, Paris S, Jackson CL (1996) Nucleotide exchange on ARF mediated by yeast Gea1 protein. Nature 384(6608): 479-481.
- Peyroche A, Courbeyrette R, Rambourg A, Jackson CL (2001) The ARF exchange factors Gea1p and Gea2p regulate Golgi structure and function in yeast. J Cell Sci 114(Pt 12): 2241-2253.

- Peyroche A, Antonny B, Robineau S, Acker J, Cherfils J et al. (1999) Brefeldin A acts to stabilize an abortive ARF-GDP-Sec7 domain protein complex: involvement of specific residues of the Sec7 domain. *Mol Cell* 3(3): 275-285.
- Poon PP, Nothwehr SF, Singer RA, Johnston GC (2001) The Gcs1 and Age2 ArfGAP proteins provide overlapping essential function for transport from the yeast trans-Golgi network. *J Cell Biol* 155(7): 1239-1250.
- Poon PP, Cassel D, Spang A, Rotman M, Pick E et al. (1999) Retrograde transport from the yeast Golgi is mediated by two ARF GAP proteins with overlapping function. *Embo J* 18(3): 555-564.
- Poon PP, Wang X, Rotman M, Huber I, Cukierman E et al. (1996) *Saccharomyces cerevisiae* Gcs1 is an ADP-ribosylation factor GTPase-activating protein. *Proc Natl Acad Sci U S A* 93(19): 10074-10077.
- Porter-Kelley JM, Gerald NJ, Engel JC, Ghedin E, Dwyer DM (2004) LdARF1 in trafficking and structural maintenance of the trans-Golgi cisternal network in the protozoan pathogen *Leishmania donovani*. *Traffic* 5(11): 868-883.
- Price HP, Goulding D, Smith DF (2005a) ARL1 has an essential role in *Trypanosoma brucei*. *Biochem Soc Trans* 33(Pt 4): 643-645.
- Price HP, Stark M, Smith DF (2007) *Trypanosoma brucei* ARF1 Plays a Central Role in Endocytosis and Golgi-Lysosome Trafficking. *Mol Biol Cell*. 18(3): 864-873.
- Price HP, Panethymitaki C, Goulding D, Smith DF (2005b) Functional analysis of TbARL1, an N-myristoylated Golgi protein essential for viability in bloodstream trypanosomes. *J Cell Sci* 118(Pt 4): 831-841.
- Puertollano R, Randazzo PA, Presley JF, Hartnell LM, Bonifacino JS (2001) The GGAs promote ARF-dependent recruitment of clathrin to the TGN. *Cell* 105(1): 93-102.
- Randazzo PA, Hirsch DS (2004) Arf GAPs: multifunctional proteins that regulate membrane traffic and actin remodelling. *Cell Signal* 16(4): 401-413.
- Randazzo PA, Andrade J, Miura K, Brown MT, Long YQ et al. (2000) The Arf GTPase-activating protein ASAP1 regulates the actin cytoskeleton. *Proc Natl Acad Sci U S A* 97(8): 4011-4016.
- Reiber K, Reeves EP, Neville CM, Winkler R, Gebhardt P et al. (2005) The expression of selected non-ribosomal peptide synthetases in *Aspergillus fumigatus* is controlled by the availability of free iron. *FEMS Microbiol Lett* 248(1): 83-91.
- Renault L, Christova P, Guibert B, Pasqualato S, Cherfils J (2002) Mechanism of domain closure of Sec7 domains and role in BFA sensitivity. *Biochemistry* 41(11): 3605-3612.
- Rice-Ficht AC, Chen KK, Donelson JE (1982) Point mutations during generation of expression-linked extra copy of trypanosome surface glycoprotein gene. *Nature* 298(5875): 676-679.
- Rodriguez A, Martinez I, Chung A, Berlot CH, Andrews NW (1999) cAMP regulates Ca²⁺-dependent exocytosis of lysosomes and lysosome-mediated cell invasion by trypanosomes. *J Biol Chem* 274(24): 16754-16759.
- Rojo M, Pepperkok R, Emery G, Kellner R, Stang E et al. (1997) Involvement of the transmembrane protein p23 in biosynthetic protein transport. *J Cell Biol* 139(5): 1119-1135.
- Roepstorff, P. (1997). Mass spectrometry in protein studies from genome to function. *Current Opinion in Biotechnology* 8, 6-13.

- Rosenwald AG, Rhodes MA, Van Valkenburgh H, Palanivel V, Chapman G et al. (2002) ARL1 and membrane traffic in *Saccharomyces cerevisiae*. *Yeast* 19(12): 1039-1056.
- Sambrook J, Fritsch EF, Maniatis, T (1989) *Molecular Cloning - A Laboratory Manual*, 2nd Edition. Cold Spring Harbour Laboratory Press, New York.
- Santy LC, Casanova JE (2001) Activation of ARF6 by ARNO stimulates epithelial cell migration through downstream activation of both Rac1 and phospholipase D. *J Cell Biol* 154(3): 599-610.
- Scheiffele P, Peranen J, Simons K (1995) N-glycans as apical sorting signals in epithelial cells. *Nature* 378(6552): 96-98.
- Schimmoller F, Singer-Kruger B, Schroder S, Kruger U, Barlowe C et al. (1995) The absence of Emp24p, a component of ER-derived COPII-coated vesicles, causes a defect in transport of selected proteins to the Golgi. *Embo J* 14(7): 1329-1339.
- Seyfang A, Mecke D, Duszenko M (1990) Degradation, recycling, and shedding of *Trypanosoma brucei* variant surface glycoprotein. *J Protozool* 37(6): 546-552.
- Simpson AG, Lukes J, Roger AJ: The evolutionary history of kinetoplasts and their kinetoplasts (2002) *Mol Biol Evol* 19: 2071-83.
- Sohn K, Orci L, Ravazzola M, Amherdt M, Bremser M et al. (1996) A major transmembrane protein of Golgi-derived COPI-coated vesicles involved in coatamer binding. *J Cell Biol* 135(5): 1239-1248.
- Sprang S (2001) GEFs: master regulators of G-protein activation. *Trends Biochem Sci* 26(4): 266-267.
- Sprang SR, Coleman DE (1998) Invasion of the nucleotide snatchers: structural insights into the mechanism of G protein GEFs. *Cell* 95(2): 155-158.
- Stamnes MA, Rothman JE (1993) The binding of AP-1 clathrin adaptor particles to Golgi membranes requires ADP-ribosylation factor, a small GTP-binding protein. *Cell* 73(5): 999-1005.
- Stamnes MA, Craighead MW, Hoe MH, Lampen N, Geromanos S et al. (1995) An integral membrane component of coatamer-coated transport vesicles defines a family of proteins involved in budding. *Proc Natl Acad Sci U S A* 92(17): 8011-8015.
- Steverding D, Stierhof YD, Fuchs H, Tauber R, Overath P (1995) Transferrin-binding protein complex is the receptor for transferrin uptake in *Trypanosoma brucei*. *J Cell Biol* 131(5): 1173-1182.
- Studier FW, Moffatt BA (1986) Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. *J Mol Biol* 189(1): 113-130.
- Szafer E, Rotman M, Cassel D (2001) Regulation of GTP hydrolysis on ADP-ribosylation factor-1 at the Golgi membrane. *J Biol Chem* 276(51): 47834-47839.
- Takeuchi M, Ueda T, Yahara N, Nakano A (2002) Arf1 GTPase plays roles in the protein traffic between the endoplasmic reticulum and the Golgi apparatus in tobacco and Arabidopsis cultured cells. *Plant J* 31(4): 499-515.
- Tamkun JW, Kahn RA, Kissinger M, Brizuela BJ, Rulka C et al. (1991) The arflike gene encodes an essential GTP-binding protein in *Drosophila*. *Proc Natl Acad Sci U S A* 88(8): 3120-3124.
- Tamura G, Ando K, Suzuki S, Takatsuki A, Arima K (1968) Antiviral activity of brefeldin A and verrucarins. *J Antibiot (Tokyo)* 21(2): 160-161.

- Taylor TC, Kahn RA, Melancon P (1992) Two distinct members of the ADP-ribosylation factor family of GTP-binding proteins regulate cell-free intra-Golgi transport. *Cell* 70(1): 69-79.
- Theibert A, Devreotes PN (1986) Surface receptor-mediated activation of adenylate cyclase in *Dictyostelium*. Regulation by guanine nucleotides in wild-type cells and aggregation deficient mutants. *J Biol Chem* 261(32): 15121-15125.
- Turner CM, Barry JD, Vickerman K (1988) Loss of variable antigen during transformation of *Trypanosoma brucei rhodesiense* from bloodstream to procyclic forms in the tsetse fly. *Parasitol Res* 74(6): 507-511.
- Untalan PM, Guerrero FD, Haines LR and Pearson TW (2005) Proteome of abundantly expressed proteins from unfed larvae of the cattle tick, *Boophilus microplus*. *Insect Biochem. Mol. Biol.* 35: 141-151.
- Van Haastert PJ, Snaar-Jagalska BE, Janssens PM (1987) The regulation of adenylate cyclase by guanine nucleotides in *Dictyostelium discoideum* membranes. *Eur J Biochem* 162(2): 251-258.
- Van Haastert PJ, De Vries MJ, Penning LC, Roovers E, Van der Kaay J et al. (1989) Chemoattractant and guanosine 5'-[gamma-thio]triphosphate induce the accumulation of inositol 1,4,5-trisphosphate in *Dictyostelium* cells that are labelled with [3H]inositol by electroporation. *Biochem J* 258(2): 577-586.
- Vandromme M, Carnac G, Gauthier-Rouviere C, Fesquet D, Lamb N et al. (1994) Nuclear import of the myogenic factor MyoD requires cAMP-dependent protein kinase activity but not the direct phosphorylation of MyoD. *J Cell Sci* 107 (Pt 2): 613-620.
- Vanhamme L, Paturiaux-Hanocq F, Poelvoorde P, Nolan DP, Lins L et al. (2003) Apolipoprotein L-I is the trypanosome lytic factor of human serum. *Nature* 422(6927): 83-87.
- Vassella E, Boshart M (1996) High molecular mass agarose matrix supports growth of bloodstream forms of pleomorphic *Trypanosoma brucei* strains in axenic culture. *Mol Biochem Parasitol* 82(1): 91-105.
- Vassella E, Reuner B, Yutzy B, Boshart M (1997) Differentiation of African trypanosomes is controlled by a density sensing mechanism which signals cell cycle arrest via the cAMP pathway. *J Cell Sci* 110 (Pt 21): 2661-2671.
- Vassella E, Den Abbeele JV, Butikofer P, Renggli CK, Furger A et al. (2000) A major surface glycoprotein of *trypanosoma brucei* is expressed transiently during development and can be regulated post-transcriptionally by glycerol or hypoxia. *Genes Dev* 14(5): 615-626.
- Vickerman K (1965) Polymorphism and mitochondrial activity in sleeping sickness trypanosomes. *Nature* 208(12): 762-766.
- Vickerman K (1985) Developmental cycles and biology of pathogenic trypanosomes. *Br Med Bull* 41(2): 105-114.
- Vickerman K, Tetley L, Hendry KA, Turner CM (1988) Biology of African trypanosomes in the tsetse fly. *Biol Cell* 64(2): 109-119.
- Welburn SC, Fevre EM, Coleman PG, Odiit M, Maudlin I (2001) Sleeping sickness: a tale of two diseases. *Trends Parasitol* 17(1): 19-24.
- Wheeler DL, Barrett T, Benson DA, Bryant SH, Canese K, Chetvernin V, Church DM, DiCuccio M, Edgar R, Federhen S, Geer LY, Kapustin Y, Khovayko O, Landsman D, Lipman DJ, Madden TL, Maglott DR, Ostell J, Miller V, Pruitt KD, Schuler GD, Sequeira E, Sherry ST, Sirotkin K, Souvorov A, Starchenko G, Tatusov RL, Tatusova TA, Wagner L, Yaschenko E (2007) Database

- resources of the National Center for Biotechnology Information. *Nucleic Acids Res* 35: D5-12.
- Wijers DJ, Willett KC (1960) Factors that may influence the infection rate of *Glossina palpalis* with *Trypanosoma gambiense*. II. The number and morphology of the trypano-somes present in the blood of the host at the time of the infected feed. *Ann Trop Med Parasitol* 54: 341-350.
- Williams RO, Young JR, Majiwa PA, Doyle JJ, Shapiro SZ (1980) Analyses of variable antigen gene rearrangements in *Trypanosoma brucei*. *Am J Trop Med Hyg* 29(5 Suppl): 1037-1042.
- Wirtz E, Leal S, Ochatt C, Cross GA (1999) A tightly regulated inducible expression system for conditional gene knock-outs and dominant-negative genetics in *Trypanosoma brucei*. *Mol Biochem Parasitol* 99(1): 89-101.
- Xong HV, Vanhamme L, Chamekh M, Chimfwembe CE, Van Den Abbeele J et al. (1998) A VSG expression site-associated gene confers resistance to human serum in *Trypanosoma rhodesiense*. *Cell* 95(6): 839-846.
- Yang CZ, Mueckler M (1999) ADP-ribosylation factor 6 (ARF6) defines two insulin-regulated secretory pathways in adipocytes. *J Biol Chem* 274(36): 25297-25300.
- Zakai HA, Chance ML, Bates PA (1998) In vitro stimulation of metacyclogenesis in *Leishmania braziliensis*, *L. donovani*, *L. major* and *L. mexicana*. *Parasitology* 116 (Pt 4): 305-309.
- Zakrzewska E, Perron M, Laroche A, Pallotta D (2003) A role for GEA1 and GEA2 in the organization of the actin cytoskeleton in *Saccharomyces cerevisiae*. *Genetics* 165(3): 985-995.
- Zeng C, Kouprina N, Zhu B, Cairo A, Hoek M et al. (2001) Large-insert BAC/YAC libraries for selective re-isolation of genomic regions by homologous recombination in yeast. *Genomics* 77(1-2): 27-34.
- Zhang CJ, Bowzard JB, Anido A, Kahn RA. (2003) Four ARF GAPs in *Saccharomyces cerevisiae* have both overlapping and distinct functions. *Yeast* 20(4):315-30
- Zhang Q, Cox D, Tseng CC, Donaldson JG, Greenberg S (1998) A requirement for ARF6 in Fcγ receptor-mediated phagocytosis in macrophages. *J Biol Chem* 273(32): 19977-19981.
- Zhao ZS, Khan S, O'Brien PJ (1997) The prevention of ferric nitrilotriacetate-induced nephro- and hepatotoxicity by methylenedioxybenzene antioxidants. *Chem Biol Interact* 108(1-2): 107-118.
- Ziegelbauer K, Quinten M, Schwarz H, Pearson TW, Overath P (1990) Synchronous differentiation of *Trypanosoma brucei* from bloodstream to procyclic forms in vitro. *Eur J Biochem* 192(2): 373-378.
- Zoraghi R, Seebeck T (2002) The cAMP-specific phosphodiesterase TbPDE2C is an essential enzyme in bloodstream form *Trypanosoma brucei*. *Proc Natl Acad Sci U S A* 99(7): 4343-4348.
- Zoraghi R, Kunz S, Gong K, Seebeck T (2001) Characterization of TbPDE2A, a novel cyclic nucleotide-specific phosphodiesterase from the protozoan parasite *Trypanosoma brucei*. *J Biol Chem* 276(15): 11559-11566.