Regulation of Cell Differentiation in *Dictyostelium*: The Role of Calcium and Calmodulin

by

Yekaterina Poloz

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy

Cell and Systems Biology

University of Toronto

© Copyright by Yekaterina Poloz 2012
Regulation of Cell Differentiation in *Dictyostelium*: The Role of Calcium and Calmodulin

Yekaterina Poloz
Doctor of Philosophy
Cell and Systems Biology
University of Toronto
2012

Abstract

*Dictyostelium* is a well established model for the study of differentiation and morphogenesis. It has previously been shown that Ca\(^{2+}\) and its primary sensor calmodulin (CaM) have roles in cell differentiation and morphogenesis in *Dictyostelium* and higher eukaryotes. Here I further elucidated the role of Ca\(^{2+}\) and CaM in cell differentiation in *Dictyostelium*. No previous work existed on the regulation of CaM-binding proteins (CaMBPs) or their binding partners by developmental morphogens. First, I gained insight into the developmental role of nucleomorphin (NumA1), a novel CaMBP, as well as its binding partners Ca\(^{2+}\)-binding protein 4a (CBP4a) and puromycin-sensitive aminopeptidase A (PsaA). I showed that NumA1 and CBP4a expression is co-regulated by differentiation-inducing factor-1 (DIF-1), a stalk cell morphogen. Both proteins likely have a role in prestalk-O cell differentiation. On the other hand, I showed that PsaA expression is regulated by cAMP and PsaA regulates spore cell differentiation. Thus, NumA1 likely differentially regulates stalk and spore cell differentiation by interacting with CBP4a and PsaA, respectively. I also used *Dictyostelium* as a model to gain insight into the mechanism of action of colchicine, a microtubule disrupting agent that has been shown to affect differentiation and morphogenesis in many organisms. I identified that colchicine affects cell motility, disrupts morphogenesis, inhibits spore cell differentiation and induces stalk cell differentiation through a
Ca\textsuperscript{2+} and CaM-dependent signal transduction pathway. It specifically induced differentiation of 
ecm\textit{B} expressing stalk cells, independent of DIF-1 production. Lastly, I analyzed for the first 
time the role of Ca\textsuperscript{2+} and CaM in 
ecm\textit{B} expression \textit{in vivo}. I showed that Ca\textsuperscript{2+} and CaM regulate 
ecm\textit{B} expression in intact and regenerating slugs and that Ca\textsuperscript{2+} and CaM also regulate cell 
differentiation, motility and slug shape. In conclusion, Ca\textsuperscript{2+} and CaM play integral roles in cell 
motility, cell differentiation and morphogenesis in \textit{Dictyostelium}. 
I would like to extend my most sincere gratitude to my supervisor and friend, Dr. Danton H. O’Day. I started in Dan’s lab as an undergraduate student, first in a Work and Study position and later completing my 4th year Forensic Science Internship. He encouraged me to stay in the lab for a graduate degree and here I am, completing my PhD thesis. Dan’s insight, guidance, encouragement, patience and friendship helped make me into the person and scientist that I am today. Thank you for everything you have done for me. I am forever greatful.

To my friends and lab mates, Andrew Catalano, Robert Huber, Ina Nikolaeva, Aldona Budniak and Andres Suarez, thank you for your friendship, help, advice and humour. You have made everyday special.

I would also like to thank the members of my supervisory committee, Dr. Angela B. Lange and Dr. Steven M. Short. Their constructive criticism and words of encouragement were invaluable to my success.

To my family and friends, who have no idea what I do in the lab but support my endeavours anyway. Thank you for your love and understanding. I am blessed to have you on my support team.

A special thank you to my fiancé, Serge. Thank you for loving me and taking care of me. You have always believed in me, encouraged me and comforted me. I could not have made it to this point without you.

Thank you to the Government of Ontario and the Natural Sciences and Engineering Research Council of Canada for funding support.
Table of Contents

Abstract .............................................................................................................................................. ii
Acknowledgments ........................................................................................................................ iv
Table of Contents .......................................................................................................................... v
List of Figures ................................................................................................................................ xii
List of Appendices ....................................................................................................................... xvi
List of Abbreviations .................................................................................................................... xvii
Chapter 1 Introduction ................................................................................................................... 1
  1.1 Dictyostelium as a model organism ....................................................................................... 1
  1.2 The life cycle of D. discoideum ............................................................................................. 2
  1.3 Cell differentiation in D. discoideum ..................................................................................... 2
    1.3.1 Mounds and slugs ........................................................................................................ 2
    1.3.2 Fruiting bodies ............................................................................................................. 4
  1.4 Cell movements in D. discoideum ......................................................................................... 4
    1.4.1 Aggregation and mound formation .............................................................................. 4
    1.4.2 Slugs ............................................................................................................................ 6
    1.4.3 Fruiting bodies ............................................................................................................. 7
  1.5 Signal transduction pathways associated with cell movement ........................................... 8
  1.6 Factors that affect differentiation and morphogenesis in D. discoideum ......................... 10
    1.6.1 cAMP ........................................................................................................................ 12
    1.6.2 DIF-1 ........................................................................................................................ 14
    1.6.3 Ammonia ...................................................................................................................... 15
  1.7 The role of Ca\(^{2+}\) in D. discoideum differentiation and morphogenesis ....................... 17
  1.8 CaM and CaM binding proteins (CaMBPs) as Ca\(^{2+}\) effectors .................................... 18
  1.9 NumA1, a novel CaMBP .................................................................................................... 19
Chapter 1

1.10 NumA1 binding partners................................................................. 20
  1.10.1 CBP4a .................................................................................. 20
  1.10.2 PsaA ................................................................................... 20
1.11 Colchicine as a tool to study cell differentiation and morphogenesis in *D. discoideum* .. 21
1.12 Thesis goals...................................................................................... 22
  1.12.1 Goal 1 .................................................................................... 22
  1.12.2 Goal 2 .................................................................................... 22
  1.12.3 Goal 3 .................................................................................... 22
1.13 Approach......................................................................................... 23
1.14 References ...................................................................................... 24

Chapter 2 The developmental regulation of nucleomorphin and Ca\(^{2+}\)-binding protein 4 a expression........................................................................................................ 43

2.1 Abstract.............................................................................................. 43
2.2 Introduction......................................................................................... 44
2.3 Materials and Methods...................................................................... 46
  2.3.1 Materials .................................................................................. 46
  2.3.2 Cell culture ............................................................................... 46
  2.3.3 Cell numbers and viability .......................................................... 47
  2.3.4 Acid phosphatase assay ............................................................. 47
  2.3.5 Polyclonal antibody production .................................................... 47
  2.3.6 SDS-PAGE and western blotting ............................................... 48
  2.3.7 Densitometry and statistical analysis .......................................... 48
2.4 Results............................................................................................... 49
  2.4.1 Anti-NumA1 antibody production and verification of specificity .......... 49
  2.4.2 The effect of extracellular signaling molecules on NumA1 protein expression... 49
  2.4.3 The effect of extracellular signaling molecules on acid phosphatase activity….. 50
2.4.4 Cell number and viability studies ................................................................. 52
2.4.5 The effect of extracellular signaling molecules on CBP4a protein expression .... 54
2.5 Discussion ........................................................................................................ 55
2.6 Acknowledgements ......................................................................................... 58
2.7 References ....................................................................................................... 58

Chapter 3 Puromycin-sensitive aminopeptidase A regulates cell differentiation in Dictyostelium

3.1 Abstract ........................................................................................................... 62
3.2 Introduction ..................................................................................................... 63
3.3 Materials and Methods .................................................................................. 64
   3.3.1 Chemicals, strains and culture conditions ................................................. 64
   3.3.2 Northern blotting .................................................................................... 64
   3.3.3 Western blotting ..................................................................................... 65
   3.3.4 Immunolocalizations ............................................................................. 65
   3.3.5 Fluorometric aminopeptidase assay ......................................................... 66
   3.3.6 Viability staining .................................................................................... 66
   3.3.7 Synchronized development of cells ......................................................... 66
   3.3.8 Statistical analysis .................................................................................. 67
3.4 Results ............................................................................................................. 67
   3.4.1 PsaA is differentially expressed throughout growth and development ........ 67
   3.4.2 Production of PsaA overexpressing strains ............................................. 67
   3.4.3 PsaA is distributed throughout the nucleoplasm and cytoplasm in developing cells68
   3.4.4 Overexpression of PsaA-GFP affects spore cell differentiation ............... 71
   3.4.5 PsaA expression is differentially regulated by developmental morphogens .... 71
   3.4.6 Cells overexpressing PsaA-GFP or PsaAΔNLS2-GFP have a sorting defect ...... 73
   3.4.7 PsaA and PsaAΔNLS2 overexpressing cells preferentially differentiate into stalk or spore cells, respectively .......................................................... 73
3.4.8 BME affects spore cell differentiation ........................................... 75
3.4.9 Evidence that BME interacts with PsaA ........................................... 77
3.4.10 BME is not cytotoxic to D. discoideum cells ................................ 80
3.4.11 PsaA is a puromycin-sensitive aminopeptidase and BME inhibits its activity ..... 80
3.5 Discussion ....................................................................................... 83
3.6 Acknowledgments .......................................................................... 88
3.7 References ...................................................................................... 88

Chapter 4 Colchicine affects cell motility, pattern formation and stalk cell differentiation in Dictyostelium by altering Ca^{2+} signaling .................................................. 91
4.1 Abstract ......................................................................................... 91
4.2 Introduction .................................................................................... 92
4.3 Materials and Methods .................................................................. 94
  4.3.1 D. discoideum strains and culture conditions ............................... 94
  4.3.2 Synchronized development of D. discoideum cells ....................... 94
  4.3.3 Microscopy ................................................................................ 95
  4.3.4 Monolayer cell differentiation assay ........................................... 95
  4.3.5 DimB translocation experiments ............................................... 95
  4.3.6 Immunolocalization of tubulin in colchicine-treated cells .............. 95
  4.3.7 Northern blotting ..................................................................... 96
  4.3.8 Histochemical staining for β-galactosidase activity ...................... 97
  4.3.9 Random cell motility assay ....................................................... 97
  4.3.10 Western blotting .................................................................... 97
  4.3.11 Viability staining .................................................................... 98
  4.3.12 Statistical analysis .................................................................. 98
4.4 Results ........................................................................................... 98
  4.4.1 Colchicine disrupts morphogenesis, induces stalk cell differentiation and represses spore cell differentiation .................................................. 98
4.4.2 Colchicine does not induce stalk cell differentiation or repress spore cell differentiation in vitro comparable to DIF-1 .................................................. 101
4.4.3 Colchicine’s action does not depend on DIF-1 production and does not involve the DIF-1 dependent transcription factor DimB ............................................. 101
4.4.4 Colchicine’s developmental effects are independent of microtubule depolymerization ........................................................................................................ 102
4.4.5 Colchicine induces ecmB gene expression and the posterior localization of ecmB-expressing cells .................................................................................. 103
4.4.6 Colchicine enhances random cell motility but not chemotaxis .................. 106
4.4.7 Colchicine enhances random cell motility through a Ca^{2+}-mediated signaling pathway ........................................................................................................ 106
4.4.8 Colchicine does not affect the expression or cytoskeleton-association of actin or myosin ........................................................................................................... 110
4.4.9 Colchicine’s developmental effect is rescued through alterations of Ca^{2+} or antagonism of CaM ......................................................................................... 110

4.5 Discussion ........................................................................................................ 113
4.6 Acknowledgements ........................................................................................ 118
4.7 References ........................................................................................................ 118

Chapter 5 Ca^{2+} signaling regulates ecmB expression patterns, cell differentiation and slug regeneration in Dictyostelium ......................................................................... 127

5.1 Abstract ........................................................................................................... 127
5.2 Introduction ..................................................................................................... 128

5.3 Materials and Methods .................................................................................. 130
5.3.1 Chemicals, strains and culture conditions ................................................. 130
5.3.2 Transformation of D. discoideum ................................................................ 130
5.3.3 Synchronized development and slug manipulation .................................... 131
5.3.4 Histochemical staining for β-galactosidase activity .................................. 131
5.3.5 Microscopy and image analysis ................................................................. 131
5.3.6 Statistical analysis ..................................................................................... 132

5.4 Results ............................................................................................................ 132
5.4.1 Spatial and temporal ecmB expression patterns in bisected slugs .......................... 132

5.4.2 Agents that modulate Ca\textsuperscript{2+} levels or antagonize CaM affect regeneration of ecmB expression in isolated slug backs ................................................................. 133

5.4.3 Agents that modulate Ca\textsuperscript{2+} levels or antagonize CaM activity affect regeneration of ecmB expression in isolated slug tips ................................................................. 137

5.4.4 Agents that modulate Ca\textsuperscript{2+} levels or antagonize CaM affect ecmB expression in uncut slugs .................................................................................................................. 138

5.4.5 Agents that modulate Ca\textsuperscript{2+} levels or antagonize CaM inhibit cell movement in isolated slug backs .............................................................................................................. 140

5.4.6 Agents that decrease Ca\textsuperscript{2+} levels or antagonize CaM activity inhibit prestalk zone regeneration in isolated slug backs ................................................................................. 141

5.4.7 Agents that modulate Ca\textsuperscript{2+} levels or antagonize CaM affect the regeneration of shape in isolated slug tips and backs ......................................................... 141

5.4.8 Agents that affect Ca\textsuperscript{2+} levels affect migration of regenerating slug tips ....... 144

5.5 Discussion ................................................................................................................. 145

5.6 Acknowledgements .................................................................................................. 151

5.7 References .............................................................................................................. 151

Chapter 6 Summary ....................................................................................................... 157

6.1 Summary of goals achieved and hypotheses verified .............................................. 157

6.1.1 Analyze the developmental roles of NumA1 and its binding partners CBP4a and PsaA .................................................................................................................. 157

6.1.2 Analyze colchicine’s mechanism of action in disrupting morphogenesis and inducing stalk cell differentiation ................................................................. 158

6.1.3 Analyze the role of Ca\textsuperscript{2+} and CaM in ecmB expression, cell differentiation and morphogenesis in vivo, in intact and regenerating slugs ........................................................................... 159

6.2 Significance ............................................................................................................. 160

6.3 Future studies .......................................................................................................... 162

6.3.1 Further elucidation of Ca\textsuperscript{2+} and CaM-dependent signaling pathways .......... 162

6.3.2 Development of colchicine as a tool to study cell differentiation ...................... 162

6.3.3 The role of Ca\textsuperscript{2+} and CaM in regeneration .............................................. 163
6.4 References ................................................................................................................. 163

Appendix I The effect of colchicine on expression of developmentally regulated proteins ..... 165
A.1 Introduction and Results ......................................................................................... 165
A.2 References .............................................................................................................. 167

Copyright Acknowledgements ...................................................................................... 169
List of Figures

Figure 1.1. The 24 h developmental cycle of *D. discoideum* .................................................. 3

Figure 1.2. Differentiation and morphogenesis of *D. discoideum* ........................................... 5

Figure 1.3. A summary diagram of some molecules involved in cell motility and differentiation in *D. discoideum* ........................................................................................................................................ 24

Figure 2.1. Some extracellular signaling molecules involved in *D. discoideum* cellular differentiation ........................................................................................................................................... 45

Figure 2.2. Specificity and affinity of polyclonal anti-NumA1 antibody ........................................ 50

Figure 2.3. Effects of extracellular signaling molecules on NumA1 protein expression ............. 51

Figure 2.4. Effects of extracellular signaling molecules on acid phosphatase activity ............... 52

Figure 2.5. Effect of extracellular signaling molecules on cell viability and number ............... 53

Figure 2.6. Effect of extracellular signaling molecules on CBP4a protein expression .......... 54

Figure 3.1. PsaA protein and mRNA expression during *D. discoideum* development ............. 68

Figure 3.2. Western blot showing PsaA-GFP fusion protein expression .................................... 69

Figure 3.3. The effect of overexpression of PsaA on development of *D. discoideum* ........... 70

Figure 3.4. Localization of PsaA in slugs .................................................................................... 72

Figure 3.5. Localization of PsaA in fruiting bodies .................................................................... 74

Figure 3.6. The effect of overexpression of PsaA on spore cell differentiation in *D. discoideum* ........................................................................................................................................ 75
Figure 3.7. The effect of extracellular signaling molecules on PsaA expression..............76

Figure 3.8. The effect of overexpression of PsaA on cell sorting in D. discoideum.............77

Figure 3.9. The effect of overexpression of PsaA on cell sorting in D. discoideum as observed in fruiting bodies........................................................................................................78

Figure 3.10. The effect of BME on D. discoideum AX3 and PsaAΔNLS2-GFP development and spore cell differentiation........................................................................................................79

Figure 3.11. The effect of BME on development of PsaA-GFP overexpressing cells.........81

Figure 3.12. The effect of BME on detection of PsaA via immunostaining.....................82

Figure 3.13. The effect of BME on cell viability in D. discoideum..................................83

Figure 3.14. Aminopeptidase activity in PsaA-GFP overexpressing cell lysates and the inhibition of this activity by different agents..................................................................................84

Figure 4.1. The effect of colchicine (Col), nocodazole (Noc) and vinblastine (Vin) on D. discoideum development.................................................................99

Figure 4.2. The effect of colchicine (Col), nocodazole (Noc) and vinblastine (Vin) on cell differentiation................................................................................................................100

Figure 4.3. Nuclear translocation of DimB-GFP in DIF-1 and colchicine treated cells........102

Figure 4.4. The effect of colchicine (Col), nocodazole (Noc) and vinblastine (Vin) on microtubular networks in cells.................................................................104

Figure 4.5. The effect of colchicine on developmental gene expression patterns.............105

Figure 4.6. The effect of colchicine on spatial gene expression patterns in the developmental structures of D. discoideum after treatment for 24 h.........................................................107
Figure 4.7. The effect of colchicine (C), nocodazole (N) and vinblastine (V) on *D. discoideum* cell motility.................................................................108

Figure 4.8. The effect of agents and mutant strains on colchicine (C)-enhanced random cell motility in *D. discoideum*.................................................................109

Figure 4.9. The effect of colchicine on the expression and cytoskeletal association of actin and myosin in *D. discoideum*.................................................................111

Figure 4.10. Developmental effects of agents on (A-D) 10 mM and (E-F) 50 mM colchicine treated cells.................................................................112

Figure 4.11. The effect of colchicine on development of PLC null mutant strain............114

Figure 5.1. Neutral red staining and bisection of slugs.............................................133

Figure 5.2. Pattern of *ecmB* expression in regenerating slug tips and backs..............134

Figure 5.3. Pattern of *ecmB* expression in bisected slug backs after 6 h of regeneration....135

Figure 5.4. The effect of Ca$^{2+}$ and CaM signal modulation on *ecmB* expression in bisected slug backs after 6 h of regeneration.........................................................136

Figure 5.5. Pattern of *ecmB* expression in bisected slug tips after 8 h of regeneration......139

Figure 5.6. The effect of Ca$^{2+}$ and CaM signal modulation on *ecmB* expression in intact slugs after 4 h of treatment.................................................................140

Figure 5.7. Pattern of *ecmB* expression in bisected slug backs after 1 h of regeneration....142

Figure 5.8. The effect of Ca$^{2+}$ and CaM signal modulation on prestalk zone regeneration in bisected slug backs, measured at 6 h post bisection.......................................................143

Figure 5.9. The effect of Ca$^{2+}$ and CaM signal modulation on the re-establishment of slug shape in bisected slug backs (A) and tips (B) after 6 and 8 h of regeneration, respectively.........144
Figure 5.10. The effect of Ca\(^{2+}\) and CaM signal modulation on the migration of bisected slug backs (A) and tips (B) after 6 and 2 h of regeneration, respectively…………………………145

Figure 6.1. Summary of new insight for the role of Ca\(^{2+}\) and CaM in D. discoideum development……………………………………………………………….161

Figure A.1. Effect of 10 mM colchicine (Col) on developmental expression of NumA1 (A), CBP4a (B), PsA (C), Cdk5 (D) and CyrA (E)…………………………………………………………166
List of Appendices

Appendix 1. The effect of colchicine on expression of developmentally regulated proteins…..165
# List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACA</td>
<td>adenyl cyclase A</td>
</tr>
<tr>
<td>ALC</td>
<td>anterior-like cell</td>
</tr>
<tr>
<td>Amt</td>
<td>ammonium transporter</td>
</tr>
<tr>
<td>BAPTA-AM</td>
<td>1,2-Bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetrakis (acetoxymethyl ester)</td>
</tr>
<tr>
<td>BME</td>
<td>bestatin methyl ester</td>
</tr>
<tr>
<td>BRCT</td>
<td>breast cancer carboxy-terminus</td>
</tr>
<tr>
<td>bZIP</td>
<td>basic leucine zipper</td>
</tr>
<tr>
<td>CaM</td>
<td>calmodulin</td>
</tr>
<tr>
<td>cAMP</td>
<td>adenosine 3’,5’-monophosphate</td>
</tr>
<tr>
<td>CaMBD</td>
<td>calmodulin-binding domain</td>
</tr>
<tr>
<td>CaMBP</td>
<td>calmodulin-binding protein</td>
</tr>
<tr>
<td>cAR</td>
<td>cAMP receptor</td>
</tr>
<tr>
<td>CBP</td>
<td>Ca²⁺-binding protein</td>
</tr>
<tr>
<td>Cdk</td>
<td>cyclin dependent kinase</td>
</tr>
<tr>
<td>Col</td>
<td>colchicine</td>
</tr>
<tr>
<td>CRAC</td>
<td>cytosolic regulator of adenylate cyclase</td>
</tr>
<tr>
<td>DAPI</td>
<td>4′,6′-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DIF-1</td>
<td>differentiation inducing factor-1</td>
</tr>
<tr>
<td>DSHB</td>
<td>Developmental Studies Hybridoma Bank</td>
</tr>
<tr>
<td>Ecm</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>ethylene glycol tetraacetic acid</td>
</tr>
<tr>
<td>EPC</td>
<td>EthylN-phenylcarbamate</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>FDA</td>
<td>fluorescein diacetate</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>gal</td>
<td>galactosidase</td>
</tr>
<tr>
<td>GBF</td>
<td>G-box binding factor</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>---------------------------------</td>
</tr>
<tr>
<td>GS</td>
<td>glutamine synthetase</td>
</tr>
<tr>
<td>GSK</td>
<td>glycogen synthase kinase</td>
</tr>
<tr>
<td>Hsp</td>
<td>heat shock protein</td>
</tr>
<tr>
<td>ile</td>
<td>isoleucine</td>
</tr>
<tr>
<td>KLH</td>
<td>keyhole limpet hemocyanin</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>MTOC</td>
<td>microtubule-organizing center</td>
</tr>
<tr>
<td>MybE</td>
<td>Myb domain protein E</td>
</tr>
<tr>
<td>NES</td>
<td>nuclear export signal</td>
</tr>
<tr>
<td>NLS</td>
<td>nuclear localization signal</td>
</tr>
<tr>
<td>Noc</td>
<td>nocodazole</td>
</tr>
<tr>
<td>NoLS</td>
<td>nucleolar localization signals</td>
</tr>
<tr>
<td>NumA</td>
<td>nucleomorphin</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PI</td>
<td>propidium iodide</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphatidylinositol-3 kinase</td>
</tr>
<tr>
<td>PIP₃</td>
<td>phosphatidylinositol-3,4,5-triphosphate</td>
</tr>
<tr>
<td>PKA</td>
<td>protein kinase A</td>
</tr>
<tr>
<td>PLA</td>
<td>phospholypase A</td>
</tr>
<tr>
<td>PLC</td>
<td>phospholypase C</td>
</tr>
<tr>
<td>Psa</td>
<td>puromycin-sensitive aminopeptidase</td>
</tr>
<tr>
<td>Pst</td>
<td>prestalk</td>
</tr>
<tr>
<td>PVDF</td>
<td>polyvinylidene fluoride</td>
</tr>
<tr>
<td>STAT</td>
<td>signal transducer and activator of transcription</td>
</tr>
<tr>
<td>tub</td>
<td>tubulin</td>
</tr>
<tr>
<td>ubi</td>
<td>ubiquitin</td>
</tr>
<tr>
<td>Vin</td>
<td>vinblastine</td>
</tr>
</tbody>
</table>
Chapter 1

Introduction

1.1  *Dictyostelium* as a model organism

*Dictyostelium* is a simple but fascinating eukaryote that has an enormous multidisciplinary following (reviewed in Nagano, 2000). It is easy and relatively inexpensive to culture. A short doubling time (~ 8 hours) allows the generation of a large number of cells in a short period of time. The genome of this amebozoan is fully sequenced and amenable to manipulation. DictyBase provides a wealth of research support and houses the annotated genome. Different strains and plasmids are readily available from the central repository known as the Dicty Stock Center while gene expression data from microarray and RNA-seq experiments can be accessed through DictyExpress. *Dictyostelium* is more closely related to animals than plants and shares more genes with animals than yeast (reviewed in Williams, 2010). Thus there are many advantages of using *Dictyostelium* as a model organism for the study of basic cellular events including cell division, motility, biomembrane fusion, phagocytosis, signal transduction, differentiation and morphogenesis (reviewed in Koonce, 2000; Koonce and Graf, 2010).

*Dictyostelium* is a particularly useful model organism for the analysis of cell differentiation. Upon starvation, it switches from a unicellular existence to multicellular development. Thus its cell division and cell differentiation stages are temporally separated. It takes only 24 hours to complete multicellular asexual development. There are 2 basic cell types, stalk and spore cells, that are spatially separated in the multicellular structure and can be easily visualized and manipulated. All these attributes have brought *Dictyostelium* to the forefront of biological research.

*Dictyostelium* has also been popularized as a biomedical model for studying problems that are relevant to human health. For example, the analysis of *Dictyostelium* chemotaxis and the associated signaling pathways has contributed the most to the understanding of neutrophil motility, tumour metastasis and axon pathfinding. *Dictyostelium* has also been used to identify drug targets of anti-tumour, psychoactive and neuroprotective drugs (reviewed in Williams et al.,
2006, 2010). This thesis focuses on aspects of cell differentiation and morphogenesis during asexual multicellular development of *Dictyostelium discoideum*.

### 1.2 The life cycle of *D. discoideum*

*D. discoideum* cells propagate in the forest soil as free-living amoebae that phagocytose bacteria as a food source. Once the food source is depleted, the amoebae switch to a social life style where up to 100,000 cells aggregate and co-operate to build a fruiting body ultimately composed of dead stalk cells and dormant but viable spores (Fig. 1.1). Approximately 8 h after starvation ensues, amoebae aggregate to form a mound. The mound then forms a tip by approximately 12 h, which acts as the organizer to direct further development (Spemann, 1938; Raper, 1940; Rubin and Robertson, 1975). The mound extends upwards changing into a finger-like shape, which may proceed directly to fruiting body formation or may fall over and migrate as an intermediate structure, the pseudoplasmodium or slug. The slug migrates along the substratum in search of the optimal culmination conditions, namely high light and heat and low ammonia. When slug ceases movement it transforms into a shape known as the mexican hat and proceeds with culmination. During culmination a series of morphogenetic cell movements and terminal cell differentiation transform the structure into a fruiting body by 24 h post starvation. The life cycle of *D. discoideum* continues when the spore cells germinate to release new amoebae (reviewed in Cotter et al., 1992; Williams, 2006). Alternatively, *D. discoideum* cells can also undergo sexual multicellular development to form macrocysts or single cells can encyst into microcysts depending on environmental conditions (reviewed in Budniak and O’Day, 2011; O’Day and Keszei, 2011). These alternative pathways will not be discussed.

### 1.3 Cell differentiation in *D. discoideum*

#### 1.3.1 Mounds and slugs

The differentiation of amoebae into prestalk or prespore cells begins in the mound. At first, both prestalk and prespore cells appear in a “salt-and-pepper” distribution but subsequently sort giving the subsequent structures a specific pattern and polarity. This pattern and polarity is clearly visible in the slug (Fig. 1.2A). The anterior 20% of the slug is composed of prestalk cells and the posterior 80% of the slug is mainly composed of prespore cells. There are several prestalk cell types and only one prespore cell type that has been identified to date. The different
cell types can be identified by their unique gene expression and movement patterns (Fig. 1.2B). The very tip of the slug is composed of prestalk A cells (pstA) that express the ecmA gene from the proximal part of the promoter, henceforth referred to as ecmA (Jermyn et al., 1989; Early et al., 1993). Just posterior to it are the prestalk O cells (pstO) that express the ecmA gene from the distal part of the promoter, henceforth referred to as ecmO (Early et al., 1993). Prestalk AB (pstAB) cells are in the core of the tip and express both ecmA and ecmB genes (Jermyn et al., 1989; Ceccarelli et al., 1991). The prespore zone has scattered prestalk B (pstB) and anterior like cells (ALCs) that express ecmB or ecmO, respectively (Sternfeld and David, 1981, 1982; Ceccarelli et al., 1991). The ecmA and ecmB genes encode the extracellular matrix proteins that are specifically expressed by prestalk but not prespore cells and make up the slime sheath that coats the slug and the stalk tube of the fruiting body (Jermyn et al., 1987; McRobbie et al.,
Finally, the prespore cells express the *cotB* and *pspA* genes (Grant et al., 1985; Fosnaugh and Loomis, 1993).

### 1.3.2 Fruiting bodies

During culmination pstAB cells are the first to undergo terminal differentiation and they contribute to the inner basal disc and the lower portion of the stalk. PstA cells start expressing the *ecmB* gene and follow pstAB cells, leading to further extension of the stalk (Jermyn and Williams, 1991; Early et al., 1993). PstO cells also contribute to the stalk as pstA cells (Early et al., 1993). The prespore cell mass is raised up the stalk and the prespore cells start expressing the *spiA* gene as they encapsulate into spores (Richardson and Loomis, 1992). The spore mass is supported by the upper and lower cup cells. The upper cup differentiates from pstO cells, pstB cells and ALCs (Sternfeld and David, 1981; Ceccarelli et al., 1991; Early et al., 1993). The lower cup differentiates from pstB cells and ALCs (Sternfeld and David, 1981; Ceccarelli et al., 1991; Early et al., 1993). Finally, a portion of pstB cells and ALCs differentiate into stalk cells of the outer basal disc, the structure that supports the fruiting body on the substratum (Sternfeld and David, 1981; Dormann et al., 1996; Jermyn et al., 1996).

Recent microarray and in situ hybridization studies have revealed a complexity of gene expression patterns of newly identified developmental genes (Maeda et al., 2003; Yamada et al., 2005). This suggests that other cell types may exist that have not been identified to date. In fact, a new cell type, termed pstU has been recently identified that mainly differentiates into upper cup cells during culmination and does not express the *ecmA* or *ecmB* genes (Yamada et al., 2010).

### 1.4 Cell movements in *D. discoideum*

#### 1.4.1 Aggregation and mound formation

Just like in higher eukaryotes *D. discoideum* morphogenesis is largely based on differential cell movement. Development begins when starving amoebae aggregate via chemotaxis towards adenosine 3’,5’-monophosphate (cAMP). A few “pacemaker” amoebae start secreting cAMP first and act as aggregation centers to which other amoebae aggregate through periodic cell movement up the cAMP gradient. The chemotaxing cells also secrete cAMP to relay the signal to cells that are further away. Thus amoebae follow each other in a head to tail fashion and form
Figure 1.2. Differentiation and morphogenesis of *D. discoideum*. A. The pattern of cell types in slugs, culminants and fruiting bodies. B. Specific gene expression patterns of prestalk/stalk and prespore/spore cells. Figure 1 from Poloz and O’Day, 2012.

streams that lead to the aggregation center. As the aggregated cells form a mound they begin to move rotationally around the mound’s vertical axis (Siegert et al., 1994; Rietdorf et al., 1996). As the prestalk and prespore cells form in a “salt-and-pepper” arrangement in the mound they have to sort into their respective positions. *EcmA* expressing pstA and pstO cells sort to the top of the mound and create the tip, *ecmB* expressing pstB cells sort to the base while the prespore cells occupy the main body (Williams et al., 1989; Traynor et al., 1992; Jermyn et al., 1996).

Differential cell adhesion and cAMP mediated chemotaxis have been proposed as possible mechanisms for this differential cell sorting (Sternfeld, 1979; Siegert and Weijer, 1995; Ginger et al., 1998). Prestalk and prespore cells are differentially adhesive and cells with disrupted adhesion molecules show sorting defects (Lam et al., 1981; Dynes et al., 1994; Wong et al.,
2002; Parkinson et al., 2009). More evidence is available for the role of cAMP mediated chemotaxis based on cells’ differential sensitivity to cAMP (Matsukuma and Durston, 1979; Traynor et al., 1992; Early et al., 1995; Rietdorf et al., 1998). Optical density (OD) waves have been detected in mounds and were shown to correspond to cAMP waves (Siegert and Weijer, 1995; Patel et al., 2000). Micro-injection of cAMP pulses into the base of the mounds redirects prestalk cell sorting to the base instead of the tip, while microinjection of cAMP pulses into the side leads to formation of a second tip (Traynor et al., 1992; Rietdorf et al., 1998). Chemotaxis assays with disaggregated mound cells revealed that pstA cells move towards the cAMP source with an average speed of 12 μm/minute, pstO cells 4.5 μm/minute and prespore cells 3.4 μm/minute (pstB and pstAB cells were not analyzed) (Early et al., 1995). Early et al., 1995, have also shown that pstA and pstO cells do not appear in random locations in the mound but rather seem to differentiate according to some positional information as the pstA cells first appear at the periphery of the mound and encircle the pstO cells. Then both cell types sort to form the tip of the mound. Because pstA cells are more sensitive to cAMP they sort faster and end up in the apex of the tip while pstO cells arrive later and end up just posterior to pstA cells. It is still not clear how pstB cells sort to the base of the mound.

1.4.2 Slugs

As the mound extends upwards into a finger and finger falls over to form a slug the pattern and polarity of the structure is maintained. The slug migrates along the substratum leaving a trail of slime sheath behind. The protein-rich sheath contains the cysteine-rich protein CyrA which possesses epidermal growth factor like domains that regulate cell motility within the slug (Suarez et al, 2011). As the slug begins migrating the different cell types have specific movement patterns. PstA and pstO cells rotate around the long axis of the slug. Some pstA cells start expressing the ecmB gene and become pstAB cells. These cells occasionally move towards the posterior of the slug and are discarded into the slime trail (Siegert and Weijer, 1991, 1992; Sternfeld, 1992; Abe et al., 1994). Prespore cells and prestalk cells that are interspersed throughout the prespore zone move forward along the slug’s long axis (Siegert and Weijer, 1991, 1992; Abe et al., 1994). PstA and pstO cells move with an average speed of 23.7 μm/minute, prestalk cells in the prespore zone with an average speed of 16 μm/minute and prespore cells with an average speed of 17.3 μm/minute (Siegert and Weijer, 1992).
Differential sensitivity of the cell types and cAMP-mediated chemotaxis have again been proposed to explain the movement patterns of the different cell types (Dormann et al., 2000). Optical density waves, corresponding to cAMP waves, have been shown to emanate from the tips and to control the movement of slugs in some *D. discoideum* strains (Dormann and Weijer, 2001). If the tip is cut off the OD waves are no longer visible throughout the prespore zone and the prespore zone seizes movement until a new tip is formed (slug regeneration is covered in section 1.6). The cut off tip continues to move forward and maintains its OD waves (Dormann and Weijer, 2001). Further evidence comes from studies on dissociated slugs. When an exogenous cAMP pulse is applied to a reforming slug the prestalk cells sort towards this source of cAMP and reform the tip there (Sternfeld and David, 1981). When an exogenous cAMP pulse is applied to unmanipulated slugs in the prespore zone it induces sorting of ALCs to form a second tip and eventually a new slug that splits away from the original (Sternfeld and David, 1981; Dormann et al., 2002).

### 1.4.3 Fruiting bodies

During culmination a portion of pstB cells and ALCs accumulate at the prestalk/prespore boundary, adjacent to the substratum (Dormann et al., 1996; Jermyn et al., 1996). The prespore cells continue their forward movement over the stationary pstB cells and ALCs and are lifted into the air (Dormann et al., 1996). The pstB cells and ALCs end up at the back of the forming culminant and act as a proposed secondary signaling center that controls morphogenetic cell movement during culmination (Dormann et al., 1996). The rest of pstB cells and ALCs sort to the front of the prespore zone and eventually will make up the upper cup. What mediates this differential movement of pstB cells and ALCs is not known. Meanwhile pstAB cells start synthesizing and entering the stalk tube, which forms in the prestalk zone and extends downward through the prespore zone to the substratum. As mentioned in the previous section pstA and pstO cells follow the pstAB cells in a reverse fountain effect as the stalk elongates. The upper cup acts as a motor that lifts the prespore zone up the forming stalk (Sternfeld, 1998). A portion of the basal pstB cells and ALCs remain and make up the outer basal disc while a portion follow the prespore zone and make up the lower cup that supports the spores on the stalk. Spore mass slides down the stalk in mutants defective in lower cup construction (Saito et al., 2008). Morphogenetic cell movements stop when stalk construction is complete.
1.5 Signal transduction pathways associated with cell movement

Most of the research has focused on the characterization of signal transduction pathways associated with cAMP mediated chemotaxis at the aggregation stage (summary Fig. 1.3). There are four transmembrane cAMP receptors (cARs) in *D. discoideum* that are expressed at different stages of development and in different cell types. They are closely related in structure but have different affinities for cAMP (reviewed in Dormann et al., 2002; Kim et al., 1998). cAR1 is a high affinity receptor that is primarily expressed during aggregation and null mutants of cAR1 do not aggregate (see section 1.6.1 for cAR2-4; Klein et al., 1988). When cells carrying a temperature sensitive null mutant of cAR1 are allowed to aggregate and then shifted to the restrictive temperature at the mound stage the cells in the mound lose their coordination of movement, fail to form tips and eventually disperse (Patel et al., 2000). Cells sense the extracellular cAMP gradient through cARs. The binding of cAMP to cAR1 during aggregation induces signal transduction cascades that result in cell movement up the cAMP gradient, intracellular cAMP synthesis and secretion for signal relay to neighbouring cells, as well as expression of genes associated with aggregation and further development.

Intracellularly, cAR1 is coupled to the heterotrimeric G protein, G2 (Janetopoulos et al., 2001). Upon cAMP binding to cAR1 the cytoplasmic domain of cAR1 is activated and catalyzes the exchange of GDP to GTP on G protein α2 subunit. GTP-bound G protein α2 subunit dissociates from the G protein βγ subunits and both have the ability to bind to and regulate a multitude of effectors (Gilman, 1987). Most evidence has been gathered for the role of G protein βγ subunits in chemotaxis and development. The liberated G protein βγ subunits activate phosphatidylinositol-3 kinase (PI3K) through RasG, which synthesizes phosphatidylinositol-3,4,5-triphosphate (PIP₃), specifically at the leading edge of the cells (Zhou et al., 1998; Funamoto et al., 2002; Sasaki et al., 2004; Xu et al., 2005). The accumulation of PIP₃ induces pseudopod formation likely through Rac-mediated actin polymerization (Huang et al., 2003). cAMP stimulation also induces the translocation of the cytosolic regulator of adenylate cyclase (CRAC) from the cytosol to the cell membrane where together with Pianissimo it activates the membrane-bound adenylate cyclase (ACA) at the trailing edge of the cell (Insall et al., 1994; Lilly and Devreotes, 1994; Chen et al., 1997; Parent et al., 1998). ACA synthesizes cAMP, the majority of which is secreted while the rest activates protein kinase A (PKA) (Firtel, 1995; Wu et al., 1995; Aubry et al., 1997). The secreted cAMP propagates the chemotactic signal to
neighboring cells while PKA regulates further intracellular signal transduction pathways and gene expression associated with cAMP-mediated chemotaxis and development (to be discussed in section 1.6; Mann et al., 1997).

There is a parallel cAR1 and G protein-dependent pathway that plays a major role in the chemotactic response. It is mediated by phospholipase A2 (PLA2) (Chen et al., 2007; van Haastert et al., 2007). The downstream components of this pathway have not been identified to date but a rise in intracellular Ca\(^{2+}\) is involved (van Haastert et al., 2007). Double null mutants of PI3K and PLA2 have a very small chemotactic response (Chen et al., 2007). Other cAR1 and G protein-dependent and independent signaling pathways have been identified but will not be discussed here. PI3K and PLA2 will be discussed further in Chapter 4.

Stimulation of cells with cAMP also leads to an influx and efflux of Ca\(^{2+}\), independent of heterotrimeric G proteins (Wick et al., 1978; Bumann et al., 1984; Abe et al., 1988; Milne and Coukell, 1991; Milne et al., 1995; Yumura et al., 1996; Nebl and Fisher, 1997). Extracellular Ca\(^{2+}\) has also been proposed to induce intracellular signal transduction events by binding to Ca\(^{2+}\) receptors in the cell membrane, independent of an increase in intracellular Ca\(^{2+}\) (Lusche et al., 2009). Chelation of Ca\(^{2+}\) with EGTA inhibits aggregation (Mason et al., 1971; Malchow et al., 1982). The downstream components of the Ca\(^{2+}\) signal transduction pathways have not been identified to date but calmodulin (CaM) (discussed in section 1.8) is involved. The treatment of cells with CaM antagonists inhibits chemotaxis (Gauthier and O’Day, 2001). It is hypothesized that Ca\(^{2+}\) regulates myosin II assembly through regulation of myosin heavy chain kinase (Yumura and Kitanishi-Yomura, 1992). Myosin II regulates cortical tension and therefore inhibits lateral pseudopod extension and induces contraction at the trailing edge of a migrating cell (Yumura and Fukui, 1985; Wessels et al., 1988; Egelhoff et al., 1996). Ca\(^{2+}\) may also regulate the activity of a CaMBD CyrA, which may then regulate cell motility. Recently Ca\(^{2+}\) and CaM signaling has also been shown to play a role in epidermal growth factor like-mediated cell motility in D. discoideum (Huber and O’Day, 2011a). In mammals, Ca\(^{2+}\) plays a role in the migration of axons, smooth muscle cells, and white blood cells (Scherberich et al., 2000; Kindzelskii et al., 2004; Zheng and Poo, 2007; Clark and Petty, 2008; Randolph et al., 2008). The interplay between Ca\(^{2+}\) and cAMP signaling pathways is under active investigation. The role of Ca\(^{2+}\) in cell motility will be discussed further in Chapter 4 and 5. The research on cAMP-mediated chemotaxis in D. discoideum has pioneered the research on mammalian chemotaxis.
associated with immunity, angiogenesis, wound healing, embryogenesis and neuronal pathfinding (Downey, 1994).

1.6 Factors that affect differentiation and morphogenesis in *D. discoideum*

*D. discoideum* development is unique in comparison to other organisms in the sense that multicellularity results from aggregation rather than cell division. In fact during *D. discoideum* development cell division ceases during aggregation but then is reinitiated only in prespore cells at the mound stage. Prespore cells go through one round of mitosis before cell division ceases again (Zada-Hames and Ashworth, 1978; Cappuccinelli et al., 1979; Zimmermann and Weijer, 1993). When starvation ensues the amoebae are already predisposed to differentiate into prestalk or prespore cells based on their position in the cell cycle and cellular levels of Ca\(^{2+}\). Cells that are in S-phase or early G2-phase preferentially differentiate into prestalk cells while cells that are in the later stages of G2 differentiate into prespore cells (Weijer et al., 1984; Zimmermann and Weijer, 1993). Cell cycle position and cell fate have also been shown to correlate with cellular Ca\(^{2+}\) levels. Cells in S-phase and early G2-phase have higher Ca\(^{2+}\) content than cells in late G2-phase while cells with a higher Ca\(^{2+}\) content preferentially differentiate into prestalk cells (Saran et al., 1994; Azhar et al., 1996).

There are two schools of thought about how pattern and polarity are established during *D. discoideum* development and in higher eukaryotes. The first suggests that cells differentiate based on positional information (Wolpert, 1969). That is the specific location of a cell within a multicellular structure provides it with a specific environment by which its fate is established. This specification mainly depends on the presence of a specific concentration of a morphogen (Crick, 1970). One of the most well understood examples of this is seen in the patterning of the leg and antennae in *Drosophila* and the vertebrate limb (reviewed in Wolpert, 2011). Evidence for differentiation based on positional information in *D. discoideum* mainly comes from the fact that pstA cells first appear specifically at the periphery of the mound and encircle the pstO cells, rather than localizing in random positions throughout the mound. Cell fate can also be regulated *in vitro* by addition of specific molecules. Potential morphogens (e.g., ammonia, cyclic AMP, DIF-1) have been identified in these *in vitro* differentiation assays and will be discussed below.
The second school of thought suggests that pattern and polarity are established mainly through differential cell sorting. That is cells differentiate at random positions within a multicellular structure and then sort to establish the specific pattern and polarity. This mechanism has been recently proposed to function in the differentiation of inner cell mass cells of pre-implantation stage mammalian embryos and differentiation of apical ectodermal ridges, the structures that regulate limb formation in vertebrates (Altabef et al., 1997; Dietrich and Hiiragi, 2007; Plusa et al., 2008). Evidence for sorting in *D. discoideum* comes from several works. During mound formation pstA and pstO cells sort to the tip while pstB cells sort to the base (Clow et al., 2000). If pstA or pstO cells of the slug are removed and injected into the prespore zone of the slug they sort back to their proper positions in the tip (Buhl and MacWilliams, 1991). If slugs are mechanically dissociated into single cells and mixed, the prestalk cells and prespore cells sort into their proper positions (Takeuchi, 1969). Cell sorting is also evident when the anterior prestalk cells of the slug are removed and ALCs quickly sort to the anterior of the prespore zone to reform the tip of the slug (Raper, 1940; Sternfeld and David, 1982).

Another concept that deserves attention is the highly regulative nature of *D. discoideum* and the ability of the different cell types to transdifferentiate. The delicate dissection experiments that were performed by Kenneth Raper, the discoverer of *D. discoideum*, have shed light on the highly regulative nature of this organism (Raper, 1940). He cut the slugs into several segments and observed that each segment was able to develop into a properly proportioned fruiting body. These experiments suggest that cell fates can be re-specified, that is the cells can transdifferentiate to reform the proper pattern. The ability of the different cell types to transdifferentiate was later directly demonstrated in dissected slugs and was found to occur in unmanipulated slugs naturally, on a small scale (Francis and O’Day, 1971; Sakai, 1973; Sternfeld and David, 1982; Sternfeld, 1992; Shaulsky and Loomis, 1993; Abe et al., 1994; Detterbeck et al., 1994; Brown and Firtel, 1999).

The earlier studies on slug regeneration were performed by staining the prestalk cells with vital dyes, neutral red or methylene blue, and following their movement in slugs (Francis and O’Day, 1971; Sakai, 1973; Sternfeld and David, 1982; Sternfeld, 1992). In the later experiments cells were specifically labeled by the expression of β-galactosidase under the control of *ecmA* and *pspA* promoters (Harwood et al., 1991; Shaulsky and Loomis, 1993; Detterbeck et al., 1994). It has been observed that during slug migration small groups of pstAB cells move to the posterior
of the slugs and are shed in the slime trail (Sternfeld, 1992). Thus, there is a continuous loss of pstAB cells. To compensate, there is a continuous transdifferentiation process. Prespore cells transdifferentiate into ALCs, which sort to the tip and transdifferentiate sequentially into pstO, pstA and pstAB cells (Harwood et al., 1991; Abe et al., 1994; Deterbeck et al., 1994). This ensures proper proportions of the different cell types in slugs. When the tip of the slug is cut off this transdifferentiation sequence reforms the tip. When the prespore zone is cut off the transdifferentiation sequence likely occurs in reverse (Akiyama and Inouye, 1987). PstA cells transdifferentiate sequentially into pstO cells, ALCs and then prespore cells. Unfortunately, the pattern of ecmB gene expression has not been analyzed to date and it is not known where pstB cells fit into this transdifferentiation pattern. This will be discussed in more detail in Chapter 5.

What controls cell differentiation, formation of pattern and polarity, morphogenesis and the proportioning of the different cell types in *D. discoideum* is under active investigation. Three potential morphogens have been identified – cAMP, differentiation inducing factor 1 (DIF-1) and ammonia (summary Fig. 1.3; Fig. 2.1).

### 1.6.1 cAMP

As previously mentioned cAMP acts as a chemoattractant that directs cell aggregation and likely cell sorting. It also regulates developmental gene expression (summary Fig. 1.3). cAMP specifically induces the expression of prespore specific genes but has a differential effect on prestalk gene expression. It induces the expression of ecmA but represses the expression of ecmB genes (Berks and Kay, 1990). A gradient of cAMP has been detected in slugs, with the highest levels in the anterior prestalk zone, but the concentration-dependent effect of cAMP on cell differentiation has not been demonstrated yet (Garrod and Malkinson, 1973; Brenner, 1977; Dormann and Weijer, 2001).

*In vivo* experiments at the mound stage have revealed that cAMP binding to cAR1 activates two G protein independent signaling pathways that regulate developmental gene expression (Kimmel and Firtel, 1991; Williams, 1991; Schnitzler et al., 1994; Loomis, 1996). The upstream components of these pathways have not been identified but the end product is the activation of two transcription factors, G-box binding factor (GBF) and *D. discoideum* signal transducer and activator of transcription A (Dd-STATa) (Schnitzler et al., 1995; Brown and Firtel, 1999; Kawata et al., 1997; Araki et al., 1998). GBF activates transcription of both prestalk and
prespore specific genes. GBF null mutant cells can not develop past the mound stage and show no cell type specific gene expression (Datta and Firtel, 1988; Hjorth et al., 1989; Schnitzler et al., 1994, 1995). Dd-STATa becomes phosphorylated upon cAMP stimulation, translocates to the nucleus and activates the transcription of *ecmA* gene while repressing the transcription of *ecmB* gene (Harwood et al., 1993; Kawata et al., 1997; Araki et al., 1998; Mohanty et al., 1999). Dd-STATa null strain shows ectopic expression of *ecmB* throughout the whole prestalk zone (Mohanty et al., 1999).

The other cAMP receptors cAR2, cAR3 and cAR4 also have roles in cell differentiation. These receptors differentially control the activity of GSKA, the *D. discoideum* homolog of glycogen synthase kinase-3 (GSK-3). GSKA represses *ecmB* gene expression and induces prespore gene expression. In prestalk cells cAR2 and cAR4 repress the activity of GSKA and in prespore cells cAR4 represses the activity of GSKA while cAR3 induces it (Berks and Kay, 1988, 1990; Harwood et al., 1995; Ginsburg and Kimmel, 1997; Plyte et al., 1999). Thus the activity of GSKA is precisely controlled in different cell types. GSKA null strain shows ectopic expression of *ecmB* throughout the mound and reduced expression of a prespore marker, *pspA* (Harwood et al., 1995). Thus extracellular cAMP likely regulates differentiation of prespore cells and *ecmA* expressing cells (pstA, pstO, ALCs) while repressing differentiation of *ecmB* expressing cells (pstB and pstAB).

As mentioned in section 1.5, cAMP binding to cAR1 also stimulates synthesis of intracellular cAMP via ACA. Intracellular cAMP plays an important role in cell differentiation by binding to and activating PKA. PKA then regulates downstream signal transduction pathways and gene expression that is essential for differentiation of both prestalk and prespore cells, regulation of culmination and terminal differentiation of stalk and spore cells (Kay et al., 1989; Anjard et al., 1998; Harwood et al., 1992a,b; Simon et al., 1992; Mann and Firtel, 1993; Hopper et al., 1993, 1995; Mann et al., 1994; Loomis, 1998). Thus, extracellular and intracellular cAMP regulate prestalk and prespore gene expression, cell differentiation and morphogenesis. In other organisms, cAMP has also been shown to regulate differentiation of endothelial cells, neurons, glia, erythroblasts, steroidogenic cells, germ cells and kidney cells through common signaling pathways (Grdisa and White, 2003; Kimmins et al., 2004; Sarkar and Howard et al., 2006; Miyamoto et al., 2011; Yamamizu and Yamashita, 2011).
1.6.2 DIF-1

DIF-1 is a chlorinated polyketide that was originally identified as a potential morphogen for its ability to induce stalk cell differentiation in *in vitro* assays (Town et al., 1976; Morris et al., 1987). It was later shown to specifically induce *ecmA* and *ecmB* gene expression and to repress prespore specific gene expression (Williams et al., 1987; summary Fig. 1.3). DIF-1 is synthesized from a 12-carbon polyketide with the first step catalyzed by StlB polyketide synthase and the last step catalyzed by DmtA methyltransferase (Kay, 1998; Thompson and Kay, 2000a; Austin et al., 2006). DmtA will be discussed further in Chapter 4. Prespore cells synthesize and secrete DIF-1 which then diffuses into the prestalk zone where it induces prestalk and represses prespore specific gene expression (Kay and Thompson, 2001). In prestalk cells DIF-1 induces its own degradation by inducing the expression of DIF-1 dechlorinase (Kay et al., 1993). This creates a steep gradient of DIF-1 in the slug, with the highest levels in the prespore zone and the lowest in pstO cells. A concentration dependent effect of DIF-1 on cell differentiation has been demonstrated for pstA and pstO cells. A 10 fold higher concentration of DIF-1 is required to induce differentiation of pstA cells than pstO *in vitro* (Early et al., 1995). Whether DIF-1 is a true morphogen and regulates cell differentiation by providing a concentration-dependent positional information is still under investigation.

Surprisingly, StlB null strain slugs contain only 30-40% less prestalk cells and develop into fruiting bodies that contain mature stalk cells (Thompson and Kay, 2000b; Saito et al., 2008). The defect in this strain is a reduction in the outer basal disc and the lower cup, mainly derivatives of *ecmB* expressing pstB cells (Saito et al., 2008). DIF-1 also plays an important role in slug migration as slugs of StlB null strain are often long and thin and break up into fragments (Saito et al., 2008). A DmtA null mutant strain also develops fruiting bodies with reduced outer basal discs and lower cups. DmtA null slugs are also missing pstO cell specific *ecmA* expression (Thompson and Kay, 2000b). It was later demonstrated that this strain accumulates metabolic intermediates of DIF-1, including desmethyl (dM) DIF-1, which has been shown to inhibit pstO differentiation (Saito et al., 2008). Normal development was rescued in both, DmtA and StlB, null mutants with varying concentrations of DIF-1, suggesting that DIF-1 action may not be concentration-dependent afterall (Thompson and Kay, 2000b; Austin et al., 2006). A recently identified upper cup cell type, pstU, also demonstrates that DIF-1 negatively regulates certain prestalk cell types. PstU cell differentiation is inhibited by DIF-1 *in vitro* (Yamada et al., 2010).
The intracellular signal transduction pathways regulated by DIF-1 are largely unknown but an elevation in intracellular Ca\(^{2+}\) is involved (described in section 1.7). Several DIF-1 responsive transcription factors have been identified to date. These include STAT, Myb, basic leucine zipper (bZIP)-type and GATA family proteins (Fukuzawa et al., 2001, 2006; Tsujioka et al., 2007).

DimA and DimB are two members of bZIP transcription factor family (Thompson et al., 2004; Huang et al., 2006). Upon DIF-1 stimulation in vitro they dimerize and accumulate in the nucleus where they regulate DIF-1 dependent induction of prestalk and repression of prespore gene expression (Huang et al., 2006; Yamada et al., 2011). In vivo, DimB is enriched in the nuclei of \textit{ectmB} expressing pstB cells (Yamada et al., 2011). DimA and/or DimB null mutant strains are morphologically similar to DmtA null mutant strain. They form long and thin slugs that break up into fragments and DimA null mutant strain shows defects in pstO differentiation and slug morphology (Thompson et al., 2004; Huang et al., 2006). The fruiting bodies of both strains also have a reduced basal disc while DimB null fruiting bodies have a reduced lower cup (Keller and Thompson, 2008; Yamada et al., 2011). DimB will be discussed further in Chapter 4. The role of other transcription factors will not be discussed here.

Thus DIF-1 has several roles during \textit{D. discoideum} development. Most of the evidence suggests that DIF-1 regulates the differentiation of pstB and pstO cells, but not pstA. DIF-1 has also received attention from the biomedical community for its ability to inhibit proliferation and induce differentiation of leukemic cells (Asahi et al., 1995; Kubohara, 1999).

1.6.3 Ammonia

Ammonia is a byproduct of protein catabolism and it accumulates as development progresses (Hames and Ashworth, 1974). Ammonia has specifically been shown to repress prestalk-specific gene expression and stalk cell differentiation and induce prespore-specific gene expression and spore cell differentiation (summary Fig. 1.3; Schindler and Sussman, 1977; Thadani et al., 1977; Bradbury and Gross, 1989; Riley and Barclay, 1990; Wang et al., 1990; So and Weeks, 1992). High ammonia concentration is also required for spore dormancy (Cotter et al., 1999). Ammonia inhibits cAMP production and secretion thereby affecting cell aggregation, cell sorting and culmination (Schindler and Sussman, 1979; Williams et al., 1984; Feit et al., 1990; Davies et al.,...

Intracellular and extracellular ammonia levels are regulated by its rate of production, assimilation into amino acids, diffusion into the atmosphere and substratum and transport via ammonium transporters (Gregg et al., 1954; Dunbar and Wheldrake, 1995; Follstaedt et al., 2003). Cotter and coworkers have proposed the “source and sink model” for ammonia localization and function in late development (Cotter et al., 1992). It suggests that prestalk cells produce ammonia and prespore cells use it by assimilating it into amino acids. These amino acids then make up the essential proteins needed for spore differentiation and dormancy. In agreement, ammonia concentration is the highest in the anterior prestalk zone and lowest in the prespore zone, with additional high concentration in the rear guard cells (Cotter et al., 1992). Also in agreement, glutamine synthetase (GS) activity is the highest in prespore cells at the time of culmination where this enzyme assimilates ammonia into glutamine residues (Dunbar and Wheldrake, 1995).

There are 5 proteins, AmtA, AmtB, AmtC, RhgA and RhgB, that belong to the family of ammonium transporter/methylammonium permease/rhesus protein (Amt/Mep/Rh). They show spatial and temporal differences in expression during the different stages of development and it has been proposed that they not only transport ammonia in and out of the cells but also act as ammonia sensors and signal transducers. In slugs, AmtA, AmtB and AmtC are expressed by the prespore cells (Follstaedt et al., 2003; Yoshino et al., 2007). AmtC expression is also found in a subpopulation of pstA cells at the very tip of the slug (Follstaedt et al., 2003). During culmination AmtA expression becomes restricted to the ALCs and later on is also found in the cup cells while AmtB and AmtC expression remains confined to the prespore cells (Follstaedt et al., 2003). All three export ammonia out of the cells (Kirsten et al., 2008).

The cells of AmtA null strain have higher intracellular ammonia levels and are more sensitive to the ammonia-mediated inhibition of chemotaxis. They form very small fruiting bodies and their spores are less viable and cannot germinate as readily as the spores of the parental strain (Yoshino et al., 2007). The cells of AmtC null strain develop into “sluggers”, where development is arrested or delayed at the slug stage (Follstaedt et al., 2003). The AmtC null slugs migrate for more than 40 hours. They also do not show ecmB expression in the pstAB zone.
and have a delayed onset and subsequent loss of ecmA expression (Kirsten et al., 2005).
Interestingly, culmination is rescued in AmtC/AmtA double null mutant (Singleton et al., 2006).
Further analysis of the function of Amt and Rhg ammonia transporters is required to better understand this complex regulation of developmental ammonia levels. Thus, ammonia has a complex role in *D. discoideum* development. It regulates aggregation, cell differentiation and morphogenesis.

### 1.7 The role of Ca$^{2+}$ in *D. discoideum* differentiation and morphogenesis

Ca$^{2+}$ plays a major role in differentiation and morphogenesis of many organisms including slime molds, fungi, plants and animals (O'Day, 1990; Hepler, 2005; Webb et al., 2005; Kubohara et al., 2007; Slusarski and Palegri, 2007). For example, in *Xenopus* and *Danio* embryos Ca$^{2+}$ signaling through a non-canonical Wnt/Ca$^{2+}$ pathway has been implicated in dorsal-ventral patterning and the morphogenetic cell movement during gastrulation (reviewed in Slusarski and Pelegri, 2007). A largely unknown Ca$^{2+}$ signaling pathway has also been implicated in the establishment of the left-right axis of *Xenopus*, *Gallus* and *Mus* embryos (reviewed in Slusarski and Pelegri, 2007). There is also evidence for a role of Ca$^{2+}$ in neural induction, somite differentiation and hematopoietic and other stem cell differentiation in numerous vertebrates (reviewed in Webb et al., 2005; Slusarski and Pelegri, 2007).

The use of quin-2/AM, fura-2/AM and the expression of jellyfish aequorin have shown that in *D. discoideum* intracellular Ca$^{2+}$ levels are significantly higher in prestalk cells than in prespore cells (Abe and Maeda, 1989; Saran et al., 1994; Cubitt et al., 1995). DIF-1 has been shown to induce stalk cell differentiation *in vitro* through the elevation of intracellular Ca$^{2+}$ levels (Kubohara and Okamoto, 1994; Schaap et al., 1996; Williams et al., 1987; Kay et al., 1999; Thompson and Kay, 2000a; Kay and Thompson, 2001; Saito et al., 2008; Kubohara et al., 2007). *In vitro* stalk cell differentiation and repression of spore cell differentiation can also be induced through the addition of pharmacological agents that increase intracellular Ca$^{2+}$ levels (Kubohara and Okamoto, 1994; Schaap et al., 1996). Elevation of intracellular Ca$^{2+}$ levels *in vivo* results in long slugs, larger prestalk zones and “stalky” fruiting bodies with reduced spore masses (Baskar et al., 2000). As mentioned previously, Ca$^{2+}$ signaling is also involved in cell motility and therefore has roles in aggregation, cell sorting and culmination (reviewed in Newell et al., 1995;
Cubitt et al., 1998). Spore germination also requires Ca\(^{2+}\) (Lydan and Cotter, 1995). In spite of the importance of Ca\(^{2+}\) in cell differentiation and morphogenesis most downstream components of the Ca\(^{2+}\) signal transduction pathway have not been identified in *D. discoideum* to date (summary Fig. 1.3).

### 1.8 CaM and CaM binding proteins (CaMBPs) as Ca\(^{2+}\) effectors

CaM is the major Ca\(^{2+}\) binding protein (CBP) found in all eukaryotic organisms examined to date. It is small, acidic, ubiquitously expressed and highly conserved (Means and Dedman, 1980; Bazari and Clarke, 1981; Means et al., 1982; Lagace et al., 1983; Means et al., 1991; Liu et al., 1992; Chin and Means, 2000). Null mutations in CaM are lethal (Davis et al., 1986; Takeda and Yamamoto, 1987; Rasmussen et al., 1990). *D. discoideum* has a single CaM gene (*calA*) (Clarke et al., 1980; Liu et al., 1992). Its mRNA is constitutively expressed throughout growth and development (Liu et al., 1992). The protein is 151 amino acids in length and has 19 amino acid differences compared to bovine brain CaM (Marshak et al., 1984; Liu et al., 1992). In fact, *D. discoideum* CaM is functionally interchangeable with bovine CaM (Bazari and Clarke, 1981). In mammals, CaM has been implicated in cell growth, cell division, inflammation, muscle contraction, learning and memory, and differentiation (reviewed in Walsh, 1994; Kahl and Means, 2003; Yamauchi, 2005; Zayzafoon, 2006; Racioppi and Means, 2008).

Ca\(^{2+}\)-free CaM, or Apo-CaM, is composed of two globular domains each containing two EF-hand motifs (Keller et al., 1982; Ulbricht and Soldati, 1999; Symersky et al., 2003). The globular domains are connected by an \(\alpha\)-helix. When Ca\(^{2+}\) binds the EF-hand motifs a conformational change in CaM exposes the \(\alpha\)-helix, which then mediates CaM’s interactions with a diversity of CaMBPs (Meador et al., 1992; Symersky et al., 2003). CaM binding domains (CaMBDs) of Ca\(^{2+}\)-dependent CaMBPs are defined by the presence of hydrophobic amino acids in specific positions, relative to other amino acids (O’Neil and DeGrado, 1990; Chin and Means, 2000). Three classic motifs have been defined: 1-10, 1-14 and 1-16 (Yap et al., 2000; Hoeflich and Ikura, 2002). Variations on these motifs such as 1-5-10 or 1-8-14 have also been identified (Yap et al., 2000). CaMBPs can also interact with CaM in a Ca\(^{2+}\)-independent manner via IQ or IQ-like motifs (Yap et al., 2000; O’Day, 2003).

There are more than four-dozen CaMBPs in *D. discoideum* that are differentially regulated during growth and development (Winckler et al., 1991; Zhu and Clarke, 1992; Lydan and
O’Day, 1993; Lydan et al., 1994; Gauthier and O’Day, 2001; O’Day, 2003). They have roles in cell growth, division, motility, protein translation, spore germination, sexual development, differentiation and morphogenesis (reviewed in Catalano and O’Day, 2008). Of these, calcineurin (CN) and nucleomorphin (NumA) have been characterized the most.

1.9 NumA1, a novel CaMBP

NumA is a novel nuclear and nucleolar Ca\(^{2+}\)-dependent CaMBP in \textit{D. discoideum}. Its gene produces three isoforms: NumA1, NumA2 (formerly NumB) and NumA3 (formerly NumC) (Myre and O’Day, 2004a). NumA1 is the most characterized of the 3 isoforms. It is expressed constitutively throughout growth and development (Myre and O’Day, 2002). The CaMBD of NumA1 has been verified and it has been shown to bind to CaM in a Ca\(^{2+}\)-dependent manner. The CaMBD contains 3 overlapping motifs, the 1-5-10, 1-16 and 1-14 (Myre and O’Day 2002, 2005). NumA1 also possesses an IQ-like motif and may also interact with CaM in a Ca\(^{2+}\)-independent manner (Myre and O’Day, 2002, 2005). It has 4 nuclear/nucleolar localization signals (NLSs/NoLSs), 3 in the N terminus (NLS1,2,4) and 1 in the C terminus (NLS3) (Myre and O’Day, 2002, 2005). Each NLS, coupled to FITC localizes to the nucleus and nucleolus but the function of each in the protein has not been determined yet, though NLS3 was shown to not be required for proper localization (Myre and O’Day, 2005; Catalano and O’Day, 2011). NumA1 also possesses a putative transmembrane domain but it has been shown to be dispensable for NumA1 localization and CaM binding (Myre and O’Day, 2002, 2005).

Nucleolar proteins are known to be involved in ribosomal subunit biogenesis and cell cycle control (reviewed in Visintin and Amon, 2000; Robinson, 2007; Pederson and Tsai, 2009). NumA1 has an acidic DEED domain, characteristic of nucleolar proteins like nucelophosmin, nucleoplasmin, nucleolin and heat shock protein 32 from a variety of organisms (Moerman and Klein, 1998; Ginisty et al., 1999; Prado et al., 2004; Grisendi et al., 2006). Removal of the DEED domain results in multinuclearity (Myre and O’Day, 2002). NumA2 possesses a breast cancer carboxy-terminus (BRCT) domain characteristic of cell cycle check-point proteins (Myre and O’Day, 2004a). Also, NumA1 relocates between the nucleolus, nucleoplasm and the spindle apparatus during mitosis (Catalano and O’Day, 2011). Its nuclear/nucleolar localization, the presence of the DEED and BRCT domains and the relocation pattern during mitosis suggest that NumA may be a cell cycle regulatory protein.
1.10 NumA1 binding partners

A yeast-two hybrid screen has identified Ca\(^{2+}\) binding protein 4 A (CBP4a) and puromycin sensitive aminopeptidase A (PsaA) as two NumA1 binding partners (Myre and O’Day, 2004b; Myre, 2005).

1.10.1 CBP4a

CBP4a is a small hydrophilic protein that contains four EF-hand motifs and interacts with NumA1, through the DEED domain, in a Ca\(^{2+}\)-dependent manner (Dorywalska et al., 2000; Myre and O’Day, 2004b). It belongs to a family of CBP proteins for which no cellular function has been identified to date. It is expressed at low levels during growth and at high levels during slug migration and culmination (Dorywalska et al., 2000). In growing cells it co-localized with NumA1 in nucleoli (Catalano and O’Day, 2011). In situ hybridization experiments have identified that CBP4a is expressed primarily in pstO cells in slugs (Maeda et al., 2003). Considering the role of Ca\(^{2+}\) in stalk cell differentiation and the expression pattern of CBP4a it likely has a role in stalk cell differentiation.

1.10.2 PsaA

PsaA is a homolog of mammalian puromycin-sensitive aminopeptidase (Psa/PSA/NPEPPS). It belongs to the M1 family of Zn\(^{2+}\)-binding aminopeptidases that cleave N-terminal amino acids from peptide substrates (Constam et al., 1995). In COS cells and 3T3 fibroblasts, PSA localizes to the nucleus and the cytoplasm and relocates to the spindle apparatus during mitosis (Constam et al., 1995). The treatment of cells with puromycin and bestatin, inhibitors of PSA, arrests cells in the G2/M phase of the cell cycle (Constam et al., 1995). PAM-1, a PSA ortholog in Caenorhabditis is required for progression through meiosis (Lyczak et al., 2006). Recently, the D. discoideum homolog of cyclin dependent kinase 5 (Cdk5) has been shown to interact with PsaA (Huber and O’Day, 2011b). Cdk5 has previously been shown to be required for optimal growth in D. discoideum and it regulates the cell cycle in other organisms (Sharma et al., 2002; Zhang et al., 2008). Taken together, this data suggests that PsaA, together with NumA1, may regulate the cell cycle in D. discoideum.

PsaA also has developmental roles in other organisms. The overexpression of Drosophila ortholog of PSA, dPsa, results in embryonic lethality (Schulz et al., 2001). Knock-down of
PAM-1 in *Caenorhabditis* also results in embryonic lethality in 30% of embryos and impaired reproduction in the rest (Brooks et al., 2003). Psa deficient mice are infertile and have impaired spermatogenesis (Osada et al., 2001a,b). PSA may also have a role in neurite outgrowth as puromycin inhibits neurite outgrowth and induces apoptosis in mice (Hui and Hui, 2003). The expression of a dominant negative form of Cdk5, a PsaA binding partner, in *D. discoideum* results in impaired aggregation, fruiting body morphogenesis and spore differentiation (Sharma et al., 2002). Thus, PsaA may also have a developmental role in *D. discoideum*.

PSA has been implicated in several diseases. It is the primary aminopeptidase that digests poly-Q repeats released by proteasomes in the brain and is thus implicated in poly-Q disorders including Huntington’s (Bhutani et al., 2007). PSA has also been shown to degrade human tau *in vitro* and to protect against tau-induced neurodegeneration *in vivo* in mice (Karsten et al., 2006; Sengupta et al., 2006). This suggests a central role for PSA in tauopathies, including Alzheimer’s disease. It may also have a neuroprotective role in lateral sclerosis as it has been shown to degrade superoxide dismutase 1 in mice (SOD1; Ren et al., 2011). Additionally, it has roles in antiviral drug activation and MHC class I peptide processing (Stoltze et al., 2000; Tehler et al., 2010). Studying the functions of PSA in lower organisms will provide insights into its roles in normal and diseased cells. Specifically, the analysis of PsaA’s role in cell differentiation in *D. discoideum* will provide insight into PsaA’s role in embryogenesis in higher eukaryotes.

1.11 Colchicine as a tool to study cell differentiation and morphogenesis in *D. discoideum*

Colchicine is a microtubule-depolymerizing agent that affects development of many organisms. It disrupts proper pattern formation in *Hydra*, limb development in *Xenopus* and *Ambystoma*, oral development in *Tetrahymena*, brain and heart development in Gallus, and oogenesis and wing development in *Drosophila* (Diwan, 1966; Corff and Burnett, 1969; Nelsen, 1970; Koch and Spitzer, 1982, 1983; Alberch and Gale, 1983; Drozdovskaya and Rapoport, 1988). O’Day and Durston, (1978), showed that colchicine disrupts *D. discoideum* morphogenesis and pattern formation, inhibits spore cell differentiation and induces stalk cell differentiation. The mode of action of colchicine has never been characterized. It has been hypothesized that colchicine disrupts microtubules and leads to mitotic arrest. However, other microtubule disrupting agents were previously used in *D. discoideum* but did not disrupt morphogenesis or induce stalk cell
differentiation (Kitanishi et al., 1984). Through elucidation of colchicine’s mode of action colchicine could serve as a tool to study differentiation and morphogenesis in *D. discoideum* and higher eukaryotes.

1.12 Thesis goals

1.12.1 Goal 1

The first goal of this thesis is to analyze the developmental roles of NumA1 and its binding partners CBP4a and PsaA (summary Fig. 1.3). Since NumA1 and CBP4a are expressed during development, I hypothesize that they have a role in cell differentiation. CBP4a is expressed specifically in pstO cells, the differentiation of which is regulated by DIF-1. Therefore, CBP4a and its binding partner NumA1 likely have a specific role in pstO cell differentiation and their expression may be regulated by DIF-1. PsaA is another NumA1 binding partner and, in mice, PSA regulates embryogenesis. I hypothesize that it will also have a role in *D. discoideum* cell differentiation. This will provide further insight into the role of CaMBPs in *D. discoideum* development. By studying developmental roles of CaMBDs I will gain insight into the function of Ca$^{2+}$ and CaM in cell differentiation.

1.12.2 Goal 2

The second goal is to analyze colchicine’s mechanism of action in disrupting morphogenesis, and inducing stalk cell differentiation. I hypothesize that colchicine does not act through microtubular disruption as other microtubule disrupting agents do not have colchicine’s effects on *D. discoideum* development. Ca$^{2+}$ induces stalk cell differentiation *in vitro*. Since colchicine induces stalk cell differentiation, I hypothesize that it affects Ca$^{2+}$-signal transduction pathways thereby inducing stalk cell differentiation (summary Fig. 1.3). Elucidation of colchicine’s mechanism of action will also solve a classic developmental problem and will allow the development of colchicine as a tool to study cell differentiation and morphogenesis in *D. discoideum* and other eukaryotes.

1.12.3 Goal 3

The third goal is to provide further insight into the role of Ca$^{2+}$ and CaM in differentiation and morphogenesis by analyzing the role of Ca$^{2+}$ and CaM in *ecmB* gene expression and cell differentiation in *D. discoideum in vivo*, in intact and regenerating slugs. DIF-1 induces *ecmB*
expression and stalk cell differentiation \textit{in vitro} through an unknown Ca$^{2+}$ signal transduction pathway (summary Fig. 1.3). \textit{In vivo}, DIF-1 regulates differentiation of \textit{ecmB} expressing \textit{pstB} cells. Based on this, I hypothesize that Ca$^{2+}$ and CaM play an important role in \textit{ecmB} expression and differentiation of \textit{ecmB} expressing cells \textit{in vivo}. The verification or rejection of these hypotheses will provide unique insight into the role of Ca$^{2+}$ and CaM in cell differentiation in this model amoebozoan.

1.13 Approach

To elucidate the role of NumA1 and its binding partners CBP4a and PsA in development I will analyze the effect of morphogens, cAMP, DIF-1 and ammonia on expression of these proteins (Chapter 2, 3). The regulation of these proteins by morphogens will suggest a developmental role for these proteins. I will then further elucidate the roles of these proteins by analyzing expression, localization and the effect of protein inhibition and overexpression during development (Chapter 3). To elucidate the mechanism of action of colchicine I will use cell type specific reporter expressing strains to analyze how colchicine affects cell differentiation and the formation of pattern (Chapter 4). My goal will be to identify a specific cell type that colchicine affects. I will analyze whether colchicine acts through microtubule disruption by using other microtubule-disrupting agents and monitoring microtubule networks via immunolocalization of tubulin (Chapter 4). I will then analyze which signaling pathways are affected by colchicine by using pharmacological agents and mutant strains (Chapter 4). Finally, I will attempt to rescue the colchicine phenotype by manipulating these signaling pathways (Chapter 4). To gain further insight into the role of Ca$^{2+}$ and CaM in development, I will determine the effect of agents that modulate Ca$^{2+}$ levels or antagonize CaM on the expression of \textit{ecmB} gene in normal and regenerating slugs (Chapter 5). This \textit{in vivo} analysis has never been done before.
Figure 1.3. A summary diagram of some molecules involved in cell motility and differentiation in D. discoideum. A single arrow indicates a direct effect while multiple arrows indicate indirect effects.

1.14 References


Dormann, D., Vasiev, B., & Weijer, C. J. (2002). Becoming multicellular by aggregation; the morphogenesis of the social amoebae *Dicyostelium discoideum*. *Journal of Biological Physics, 28*, 765-780.


Nebl, T., & Fisher, P. R. (1997). Intracellular Ca\textsuperscript{2+} signals in Dictyostelium chemotaxis are mediated exclusively by Ca\textsuperscript{2+} influx. *Journal of Cell Science, 110*, 2845-2853.


Chapter 2

The developmental regulation of nucleomorphin and \( \text{Ca}^{2+} \)-binding protein 4 a expression

This chapter was published as part of a manuscript:


I produced the NumA1 antibody, affinity purified it and verified its specificity. I carried out all experiments and data analysis for this chapter. I wrote all parts of this chapter and made all Figures. Danton H. O’Day initiated this research, offered assistance in experimental design and wrote the published manuscript.

2.1 Abstract

Nucleomorphin (NumA1) is a multi-domain, calmodulin-binding protein (CaMBP) that is implicated in cell cycle regulation in \textit{Dictyostelium}. To gain insight into its regulation, we assessed the effect of the extracellular signaling molecules differentiation inducing factor-1 (DIF-1), cyclic AMP (cAMP) and ammonia on the expression of NumA1 \textit{in vitro}. Starvation, which is a signal for multicellular development, resulted in a reduction in NumA1 protein levels up to 40% of vegetative levels by 8 h. DIF-1, a stalk cell inducing morphogen, counteracted the effects of starvation by increasing NumA1 protein levels up to 150% by 8 h. Treatment of cells with cAMP and/or ammonia, which are involved in spore differentiation, led to decreased NumA1 expression and each of these signaling molecules suppressed the stimulatory effects of DIF-1. Protein expression levels of CBP4a, a calcium-dependent binding partner of NumA1, were regulated in the same manner as NumA1 suggesting this potential co-regulation may be related to their functional relationship. NumA1 is the first CaMBP shown to be regulated by developmental morphogens in \textit{Dictyostelium}, being upregulated by DIF-1 and down-regulated by cAMP and ammonia.
2.2 Introduction

Nucleomorphin (NumA) from *Dictyostelium discoideum* is a nuclear and nucleolar, Ca\(^{2+}\)-dependent, calmodulin-binding protein (CaMBP). The *numA* gene produces 3 isoforms (NumA1-3) one of which possesses a breast cancer carboxy-terminus (BRCT) domain, characteristic of cell cycle check-point proteins (Myre and O’Day, 2002, 2004a). NumA1-3 gene products each possess a highly acidic DEED domain, the removal of which leads to multinuclearity (Myre and O’Day, 2002). During mitosis, NumA1 relocates between the nucleolus, nucleoplasm and the spindle apparatus (Catalano and O’Day, 2011). Its nuclear/nucleolar localization, the presence of the DEED and BRCT domains and the relocation pattern during mitosis suggest that NumA may be a cell cycle regulatory protein. NumA isoforms 1-3 are differentially expressed throughout growth and development but the factors regulating their expression remain to be elucidated (Myre and O’Day, 2002). A yeast two hybrid analysis coupled with co-immunoprecipitation has shown that Ca\(^{2+}\)-binding protein 4a (CBP4a) binds to NumA1 via the DEED domain (Myre and O’Day, 2004b). Since CBP4a is expressed predominantly in pstO cells this suggests that CBP4a and NumA1 expression might be regulated by differentiation inducing factor-1 (DIF-1), a polyketide that induces pstO cell differentiation (Thompson and Kay, 2000; Maeda et al., 2003).

DIF-1 is one of a number of extracellular signaling molecules that have been shown to regulate differentiation and morphogenesis in *D. discoideum* (Fig. 2.1). DIF-1 induces prestalk-specific gene expression and stalk cell differentiation while repressing prespore-specific gene expression and spore cell differentiation *in vitro* (Town et al., 1976; Morris et al., 1987; Williams et al., 1987). During multicellular development DIF-1 specifically regulates differentiation of pstO and pstB cells, as well as slug motility (Thompson and Kay, 2000; Saito et al., 2008). Ammonia and cAMP are two other extracellular signaling molecules that regulate differentiation and morphogenesis. Cyclic AMP regulates differentiation of both prestalk and prespore cells while ammonia regulates differentiation of prespore cells and antagonizes DIF-1 (Fig. 2.1; Schindler and Sussman, 1977; Berks and Kay, 1990; Harwood et al., 1992,1993,1995).

Some of the downstream DIF-1 signaling pathway components have been identified. These include the signal transducer and activator of transcription c (STATc) homolog, Myb domain protein E (MybE), a member of the basic leucine zipper (bZIP) family of transcription factors DimA/B and GATA family proteins (Fukuzawa et al., 2001; Thompson et al., 2004; Fukuzawa et
Figure 2.1. Some extracellular signaling molecules involved in Dictyostelium cellular differentiation. A number of extracellular signaling molecules (morphogens) have been identified in D. discoideum, and shown to serve as regulators of growth and development. The molecules relevant to this study include DIF-1, cAMP and ammonia. Cells within the aggregate are induced to differentiate into prestalk or prespore via the action of these signaling molecules. Prestalk differentiation is controlled by DIF-1 and cAMP, while prespore differentiation is controlled by cAMP and ammonia. Fig. 1 in O’Day et al., 2009.

al., 2006; Huang et al., 2006). DIF-1 induced gene expression in pstO and pstB cells is controlled by an interplay between the above mentioned transcription factors but none of the upstream components have yet been identified.

DIF-1 has been shown to inhibit proliferation and induce differentiation in various cancer cell lines including the human leukemia K562 and myeloid leukemia HL-60 cell lines as well as a murine erythroleukemia B8 cells (Asahi et al., 1995; Kubohara, 1997). In these cells, DIF-1 exerts some of its effects through an increase in intracellular Ca$^{2+}$ levels (Kubohara, 1997). In D. discoideum, in vitro experiments have revealed that DIF-1 regulates gene expression and stalk cell differentiation also through an increase in intracellular Ca$^{2+}$ levels (Kubohara and Okamoto, 1994; Schaap et al., 1996; Kay et al., 1999; Thompson and Kay, 2000; Kay and Thompson, 2001; Saito et al., 2008; Kubohara et al., 2007). However, the intermediate components of this Ca$^{2+}$–mediated signaling pathway, between an increase in intracellular Ca$^{2+}$ and activation of downstream transcription factors, remains unknown.

One of the primary targets of Ca$^{2+}$ signaling is calmodulin (CaM), a small, highly conserved, essential protein that has been shown to regulate chemotaxis, spore germination, gametogenesis and fertilization in Dictyostelium (reviewed in Catalano and O’Day, 2008). Since CaM is an essential protein, one way to learn about its multitude of cellular and developmental functions is
to study its primary targets, CaMBPs. There are more than four-dozen CaMBPs in *D. discoideum* that are differentially regulated during growth and development (Winckler et al., 1991; Zhu and Clarke, 1992; Lydan and O’Day, 1993; Lydan et al., 1994; Gauthier and O’Day, 2001; O’Day, 2003).

Based upon these relationships, we studied the effects of DIF-1, cAMP and ammonia, on the expression of NumA1, to gain insight into the regulation of this protein by developmental signaling molecules. As a control for the effects of these signaling molecules we monitored acid phosphatase activity. Acid phosphatase is an enzyme that is enriched in pstA and pstO cells and has been previously used as a marker for stalk cell differentiation (Tasaka et al., 1986; Maeda et al., 2003). We hypothesized that DIF-1 will induce the expression of NumA1 and CBP4a, as well as enhance the activity of acid phosphatase.

### 2.3 Materials and Methods

#### 2.3.1 Materials

General microbiological media and reagents were purchased from BioShop Canada, VWR Scientific Products and Sigma-Aldrich. Protein molecular weight markers were obtained from SantaCruz Biotechnologies. PVDF membranes were purchased from Pall Corporation. Mouse anti-tubulin antibody was purchased from DSHB. HRP-conjugated goat anti-mouse IgG and goat anti-rabbit IgG antibodies were purchased from DAKO. Adjuvants used for polyclonal antibody production in rabbits were purchased from SantaCruz Biotechnologies. ECL-Plus detection kit was purchased from Amersham. Western blots were analyzed using the Molecular Dynamics™ Storm™ gel and blot system from Molecular Dynamics.

#### 2.3.2 Cell culture

Vegetative cells of *D. discoideum* strain AX3 were grown axenically in HL-5 at 22°C as described elsewhere (Myre and O’Day, 2002). For analysis of protein expression, 2 x 10⁶ axenically grown cells were collected by centrifugation at 1500 rpm for 5 minutes, washed twice with equal volumes of KK₂ buffer and resuspended in fresh KK₂ at a concentration of 5 x 10⁵ cells/ml. Cell numbers were counted in a hemocytometer. The compounds used in the different experiments were added in the same buffer at the indicated concentrations (20 mM NH₄Cl; 1 mM cAMP; or 100 nM DIF-1). Cells were starved in KK₂ at 22°C with shaking at 250 rpm to
reduce cell-cell contact and harvested at 2 and 4 h post-treatment. For certain studies cells were also harvested at 6 and 8 h.

2.3.3 Cell numbers and viability

Cell density was determined at 0 and 4 h in control (KK2) and treated cells by taking an aliquot of culture and counting it in a hemocytometer. Cell viability was monitored by double staining an aliquot of the culture with 10 μg/mL of fluorescein diacetate and 10 μg/mL of propidium iodide. In at least four separate experiments, two hundred and fifty cells were counted in randomly selected fields of view after which the mean and standard deviations were calculated.

2.3.4 Acid phosphatase assay

The acid phosphatase assay was performed according to Gezelius, (1966). In brief, a cell aliquot was taken through 6 freeze/thaw cycles to lyse the cells. The aliquots were centrifuged and protein was quantified using a Bradford assay. Equal amounts of protein (0.1 mg/mL) were added to 0.5 mL of 0.1 M acetate buffer pH 3.6 (0.1 M sodium acetate; 0.1 M acetic acid) with 0.01 M p-nitrophenyl phosphate. The reaction proceeded for 5-20 min and was stopped with 2.0 mL of 0.1 M NaOH. The amount of p-nitrophenol that was produced was quantified with a spectrophotometer at 420 nm and the specific activity was calculated as nmol of p-nitrophenol produced/min/mg of protein.

2.3.5 Polyclonal antibody production

A polyclonal antibody directed against a C-terminal peptide sequence (EHSRQQQHHHHQSSQVNSSK) from nucleomorphin was generated in rabbits. The peptide was synthesized using solid phase synthesis, purified via HPLC and verified via mass spectroscopy. It was then conjugated to KLH (Sigma) via an MBS linker. Peptide synthesis and conjugation was performed by Advanced SynTech Incorporation (Mississauga, Canada). The use of four male New Zealand White rabbits was approved by the University of Toronto Animal Care Committee. Antibody production was carried out according to Production of Polyclonal Antibodies in Rabbits SOP of the University of Toronto. Two rabbits were injected with KLH-peptide while the other two rabbits were injected with KLH only or PBS only (controls). 750 μg of KLH-conjugated peptide in 500 μL PBS mixed with 500 μL Freund’s Complete Adjuvant were injected into rabbits. Three subsequent boost injections 2-3 weeks apart were performed in
the same manner each consisting of 250 µg of KLH-conjugated peptide in 500 µL PBS mixed with 500 µL Freund’s Incomplete Adjuvant. Antibody titer was checked prior to each boost and exsanguination using western blotting, monitoring the appearance of a 40 kDa band corresponding to NumA1. Produced antibody was affinity purified using EpiMAX™ Affinity Purification Kit (Epitomics), aliquoted, and stored at -80 ºC. Specificity was analyzed with a peptide competition assay using western blotting (Fig. 2.2). In addition, the same blots were probed with the secondary antibody only, with pre-immune serum and serum from control rabbits (data not shown). All results indicated that the 40 kDa band was peptide specific and likely the NumA1 isoform. The rabbit anti-CBP4a polyclonal antibody was kindly provided by Andrew Catalano (Dept. of Biology, University of Toronto Mississauga).

### 2.3.6 SDS-PAGE and western blotting

Cells were harvested by centrifugation for 10 seconds at 10000 rpm in a Biofuge Pico (Heraeus), then lysed in lysis buffer (20 mM Tris-HCl pH 6.8; 1% SDS; 1 mM EDTA; 1 mM PMSF; 1 complete protease inhibitor tablet) after which the crude cell lysate was centrifuged for 1 min at 13000 rpm. Protein levels were quantified using the Bradford assay after which 10 µg of protein for each treatment was loaded onto a 12% SDS-PAGE gel. Proteins were subsequently transferred onto a PVDF membrane (Pall corp.). The membrane was blocked in 5% non-fat milk in TTBS overnight at 4 ºC, then probed with affinity purified anti-NumA1 (1:400 dilution) and subsequently goat anti-rabbit (1:16000 dilution), both for 1 h at room temperature and diluted in 5% non-fat milk in TTBS. Without stripping, the same membrane was then re-probed with mouse anti-tubulin (1:600 dilution) and subsequently goat anti-mouse (1:16000 dilution). Proteins were visualized with ECL Plus and STORM Scanner System. Calcium binding protein 4a was analyzed in the same manner with (anti-CBP4a 1:400; goat-anti-rabbit 1:16000).

### 2.3.7 Densitometry and statistical analysis

Band intensity was quantified using the Image Quant 5.2 program according to the instructions manual. In brief, equal sized boxes were placed around each band and a representative background sample (in the same lane but where no bands were visible). Background intensity was subtracted from each band measurement using the “Analyze” tool. The data was normally distributed with equal variances. A two-way ANOVA with Tukey’s Test (when no interaction of two variables was found) was then carried for different treatments and at each time point.
2.4 Results

2.4.1 Anti-NumA1 antibody production and verification of specificity

An anti-nucleomorphin polyclonal antibody was generated in rabbits against a C-terminal peptide. This antibody detected a *D. discoideum* protein that migrates at ~40 kDa (Fig. 2.2A). This corresponds to the calculated MW of the NumA1 isoform (38.7 kDa) (Myre and O’Day, 2002). A second higher molecular weight band is also specifically detected by this antibody which may be another isoform or a post-translationally modified form of NumA1 but this remains to be clarified. The antibody also detected the peptide antigen conjugated to BSA (Fig. 2.2C). Premixing of the peptide and antibody prior to probing abolished binding, revealing the specificity of the antibody for the NumA1 peptide and protein (Fig. 2.2B,D).

2.4.2 The effect of extracellular signaling molecules on NumA1 protein expression

To assess the effects of the signaling molecules on NumA1 protein expression, cells were starved in KK2 buffer in the absence and presence of specific extracellular signaling molecules. NumA1 protein levels increased in control starved cells over the first 2 h before dropping back to vegetative levels (Fig. 2.3A,B). Cells treated with cAMP, cAMP/DIF-1 or cAMP/NH4Cl showed a 30% decrease in NumA1 protein levels within the first 2 h which was maintained over the next 2 h. In contrast, NumA1 protein levels increased over 30% in DIF-1 treated cells by 2 h and this increase was maintained up to 4 h. Cells treated with NH4Cl showed levels of NumA1 expression that were essentially equivalent to vegetative cells, neither increasing nor decreasing within the 4 h period. However, NH4Cl markedly decreased the level of NumA1 protein in DIF-1 co-treated cells both at 2 and 4 h.

To assess the long-term effects of DIF-1 and verify if the enhanced levels of mRNA expression in 4 h cells (O’Day et al., 2009) translated to maintained high levels of NumA1 protein synthesis, NumA1 protein levels were quantified after 6 and 8 h (Fig. 2.3A,B). In DIF-1 treated cells the levels of NumA1 remained high while those in NH4Cl treated cells declined slightly. The short-term negative effects of NH4Cl on DIF-1 induced expression diminished slightly at 6 and 8 h of treatment.
2.4.3 The effect of extracellular signaling molecules on acid phosphatase activity

Acid phosphatase is enriched in stalk cells and has been used as a marker for stalk cell differentiation suggesting that its activity might be induced by DIF-1 or other developmental morphogen (Tasaka et al., 1986; Maeda et al., 2003). Cyclic AMP increased activity of acid phosphatase in starved cells by 2 h with a further increase detected by 4 h (Fig. 2.4). The addition of either DIF-1 or NH₄Cl did not appear to have any effect on this increase. DIF-1 alone
Figure 2.3. Effects of extracellular signaling molecules on NumA1 protein expression. (A) A composite figure (lanes 1-5, 5-10, 11-23) of western blots probed with rabbit anti-NumA1 and mouse anti-tubulin. The positions of protein markers are shown on left, NumA1 and tubulin on the right. The cells were starved in KK2 buffer alone or supplemented with the following compounds as indicated: 1 mM cAMP (pulsed each ½ h); 100 nM DIF-1; and 20 mM NH₄Cl. (B) Quantified band intensities expressed as a percentage of NumA1 levels in vegetative cells (M+S.D.). V = Vegetative (HL-5) cells. This experiment was independently replicated 4 times. A two-way ANOVA with Tukey’s Test was used to analyze significant changes in NumA1 levels. Results for 2 and 8 h are represented. Different treatments were compared at each time point and different time points were compared within each treatment. Groups that do not share a letter are statistically different. a is not different than vegetative levels. p-values<0.05. Fig. 5 from O’Day et al., 2009 with modified statistical analysis.

also increased acid phosphatase activity at 2 and 4 h but to a slightly lesser degree than cAMP treatment. NH₄Cl alone did not enhance acid phosphatase activity and had no evident effect on the DIF-1 induced increase in the enzyme activity.
Figure 2.4. Effects of extracellular signaling molecules on acid phosphatase activity. Cells were treated with cAMP, DIF-1 and/or NH₄Cl for 2 and 4 h and the specific activity (nmol/min/mg of protein) of acid phosphatase was determined using para-nitrophenyl phosphate as detailed in the Materials and Methods (M+S.D.). This experiment was independently replicated 4 times. A two-way ANOVA with Tukey’s Test was used to analyze significant changes in acid phosphatase activity. Different treatments were compared at each time point and different time points were compared within each treatment. Groups that do not share a letter are statistically different. a is not different than vegetative levels. p-values≤0.05. Fig. 6 from O’Day et al., 2009 with modified statistical analysis.

2.4.4 Cell number and viability studies

Control experiments were carried out to ensure our results were not corrupted by non-specific effects of the morphogens on cell number and viability. Over 95% viability was demonstrated through the use of propidium iodide exclusion and fluorescein diacetate hydrolysis (Fig. 2.5A,B). Cell counts were made of all treated and untreated cultures after 4 h. In all cases only slight variations in number compared to the original starting cell concentration were detected (Fig. 2.5C,D).
Figure 2.5. Effect of extracellular signaling molecules on cell viability and number. To determine viability, cells were suspended in a solution of fluorescein diacetate and propidium iodide and images taken using (A) phase contrast and (B) fluorescence microscopy. (C) Quantitative analysis of cell viability determined with fluorescein diacetate (solid bars) and propidium iodide (open bars) after 4 h of treatment. (D) Cell numbers were determined at 4 h. At least 100 cells were counted from 3 independent replicates (M+S.D.). Fig. 7 from O'Day et al., 2009.
Figure 2.6. Effect of extracellular signaling molecules on CBP4a protein expression. (A). Western blot probed with rabbit anti-CBP4a (1:400 dilution) and mouse anti-tubulin (1:600 dilution), followed by goat anti-rabbit and goat anti-mouse IgGs (1:16000 dilution). (B) Quantified band intensities expressed as a percentage of CBP4a levels in vegetative cells (M+SD). V = Vegetative (HL-5) cells. This experiment was independently replicated 4 times. A two-way ANOVA with Tukey’s Test was used to analyze significant changes in CBP4a. Different treatments were compared at each time point and different time points were compared within each treatment. Groups that do not share a letter are statistically different. a is not different from vegetative levels. p-values ≤0.05. Fig. 8 from O’Day et al., 2009 with modified statistical analysis.

### 2.4.5 The effect of extracellular signaling molecules on CBP4a protein expression

CBP4a was shown to be a Ca$^{2+}$-dependent binding partner of NumA1 (Myre and O’Day, 2004b). As a result, any co-regulation events for the two proteins should provide further insight into their functional relationship. Western blotting revealed a strong single band for CBP4a (Fig. 2.6A). During starvation, this protein band decreased steadily in amount over 4 h to reach ~75% of vegetative levels (Fig. 2.6B). In the presence of pulsed cAMP the amount of detected protein decreased more rapidly to become less than 50% of vegetative levels. NH$_4$Cl had an initial
delayed effect on the protein levels which then dropped by 4 h to ~50% of those seen in vegetative cells. In contrast, in the presence of DIF-1, CBP4a protein levels remained at ~80% of vegetative levels over the 4 h. Most combinations failed to alter the results seen with each single morphogen except that in the presence of NH₄Cl, the stimulatory effect of DIF-1 was removed.

2.5 Discussion

NumA1 is the first CaMBP shown to be regulated by developmental signaling molecules in *D. discoideum*. The majority of studies on extracellular signaling molecules in *Dictyostelium* have focused solely on the regulation of gene but not protein expression. From our own experience we have found that mRNA expression profiles do not always follow the protein expression profiles. Clearly, protein expression is the final goal of gene expression regulation and thus the verification that the message is translated into protein is an important step in understanding the effects of extracellular signaling molecules. The data show that NumA1 is repressed by starvation, cAMP and, to a lesser degree, ammonia and suggests the possibility that this particular CaMBP exhibits cAMP and ammonia dual responsiveness. In contrast, this repression of NumA1 expression is inhibited by the morphogen DIF-1, a signaling molecule that regulates stalk cell differentiation in *D. discoideum*.

In *D. discoideum*, the extracellular signaling molecule, DIF-1 mediates slug migration as well as the differentiation pstO and pstB cells (Thompson and Kay, 2000; Saito et al., 2008). It is of interest to note that DIF-1 induces changes in gene and protein expression in shaking cultures starting at 2 h of starvation initiation (O’Day et al., 2009). Most experimental evidence implies that DIF-1 is made largely by prespore cells, which appear at the mound stage of development (Kay and Thompson, 2001). The ability of amoebae to respond to DIF-1 suggests the receptor is present early during starvation and prior to prespore cell differentiation.

In *D. discoideum* and mammalian cells DIF-1 is known to act through an elevation in intracellular Ca²⁺ levels (Kubohara and Okamoto, 1994; Schaap et al., 1996; Kubohara, 1997; Kay et al., 1999; Thompson and Kay, 2000; Kay and Thompson, 2001; Saito et al., 2008; Kubohara et al., 2007). Several DIF-1 responsive transcription factors have been identified but the signaling pathway transducing the Ca²⁺ signal to the activation of these transcription factors is unknown. Since the essential protein CaM is a primary target of Ca²⁺, it is reasonable to assume it might be one of these downstream elements. In support of this, Shimizu et al. (2004)
have shown that the CaMBP phosphodiesterase 1 (PDE1) is one of the downstream targets of DIF-1 action. This supports the importance of CaM in the Ca\(^{2+}\)-mediated functions of this signaling molecule. Dictyostelium also possesses a CaM-dependent PDE along with 4 dozen of other CaMBPs, of which only a small number have been identified.

NumA1 is an interesting CaMBP since it possesses a number of experimentally verified domains including the CaM-binding domain, nuclear localization sequences and an extensive DEED repeat, the removal of which leads to multinuclearity (Myre and O’Day, 2002, 2004a, 2004b, 2005). The cDNA encoding NumA1 was originally isolated from a cDNA expression library from 10-16 h of multicellular development of D. discoideum (Myre and O’Day, 2002). Fittingly, NumA1 is expressed during growth and through development (Myre and O’Day, 2002). So far other cell cycle-linked CaMBPs have been isolated from this same library including phosphoglycerate kinase and thymidine kinase (Myre and O’Day, 2004c; O’Day et al., 2005; Catalano and O’Day, 2008). In this work we have shown that during development NumA1 expression is regulated by DIF-1. Previous work has revealed that the interplay between the different signaling molecules is gene-specific (Williams, 1988). Our findings on NumA1 and CBP4a regulation further support previous work indicating that extracellular cAMP and ammonia act as antagonists of DIF-1 during cell differentiation.

NumA1’s regulation by DIF-1 and its interaction with CBP4a suggest that NumA1 may function during development in the differentiation of pstO cells. Here we have shown that each of the morphogens affected CBP4a and NumA1 levels in a similar way. Thus, cAMP and NH\(_4\)Cl reduced CBP4a levels while DIF-1 maintained them. Similarly, within the time studied, NH\(_4\)Cl suppressed the effects of DIF-1, as it did for NumA1. These data suggest that NumA1 and CBP4a may be co-regulated by the extracellular signaling molecules in D. discoideum and may function together in pstO cell differentiation.

The results presented here further suggest that NumA1 expression is negatively regulated by cAMP and, to a lesser extent, by ammonia. A number of other D. discoideum proteins have been shown to have dual responsiveness to extracellular signaling molecules. Glycogen phosphorylase gene 2 (gp2) expression can be induced by both DIF-1 and cAMP and these inductions inhibited by ammonia (Inouye, 1988; Wang and Schaap, 1989). Studies on two prestalk cell marker genes, ecmA and ecmB, revealed that cAMP enhances the DIF-1-mediated induction of ecmA
expression, but represses the DIF-1-mediated induction of ecmB expression (Kay et al., 1983; Kay and Jermyn, 1983). To date, the effect of extracellular signaling molecules on translation of these proteins remains to be studied. The results here highlight the value of analyzing protein expression to complement the analysis of gene expression. We have shown that NumA1 is a very stable protein as its protein levels did not change to the extent its mRNA levels did. In all cases where a significant reduction in numA1 expression occurred, high levels of NumA1 protein were still maintained over several hours (O’Day et al., 2009). For example, while treatment with NH₄Cl led to an immediate and sustained (greater than 90%) reduction in numA1 RNA levels, protein levels were reduced less (~30%) (O’Day et al., 2009). These results suggest that in D. discoideum, ammonia may function as a signaling molecule that acts as a numA1 transcriptional repressor. Similarly, while treatment with DIF-1 maintained vegetative levels of numA1 RNA expression, NumA1 protein levels increased over 50% within 2 hours (O’Day et al., 2009).

The in vitro approach taken here could facilitate future research both on the regulation of NumA1 and analysis of DIF-1-mediated signaling pathways. We have shown that DIF-1 does not affect cell growth or viability elimination the possibility that the effects of DIF-1 are due to cytotoxicity. DIF-1 also induced acid phosphatase activity, an enzyme linked to stalk cell differentiation (Tasaka et al., 1986). Acid phosphatase activity is also increased in the presence of cAMP alone or combined with DIF-1 suggesting it is regulated in a different way than NumA1. Microarray analysis has shown that acid phosphatase expression is enriched in pstA and pstO cells (Maeda et al., 2003). The induction of NumA1 within hours by DIF-1 is in agreement with its role as a singaling molecule. Hormones, morphogens and other extracellular signaling molecules, acting either via surface or intracellular receptors, typically initiate signaling events within minutes to hours of treatment. Thus while the induction of stalk cell differentiation takes 2 or more days in culture, the early events that drive this differentiation process are likely initiated immediately upon the treatment of cells with these signaling molecules. The simplicity of the method employed here using starved cells will permit the efficient analysis of the early events of DIF-1 action. The data resulting from such work would provide information on the differentiation and morphogenetic functions of DIF-1 that mediate slug migration and stalk cell differentiation.
2.6 Acknowledgements

This research was supported by a grant from the Natural Sciences and Engineering Council of Canada (D.O’D).

2.7 References


Chapter 3

Puromycin-sensitive aminopeptidase A regulates cell differentiation in *Dictyostelium*

Parts of this chapter (Figures 2, 4 and parts of 5) were published:


Parts of this chapter (Figure 1, parts of 5 and 6-13) were accepted for publication:


I carried out all experiments and data analysis for Figures 1-9, 11, parts of 13 and 14. I helped edit the Catalano et al., 2011 manuscript. Andrew Catalano contributed the rest of the data and wrote the manuscript. I wrote the Poloz et al., 2012 manuscript. Danton H. O’Day initiated this research, offered assistance in experimental design and edited both manuscripts. I wrote all parts of this chapter. Andrew Catalano produced anti-PsaA antibody and PsaA-GFP overexpressing strains. He also contributed Figures 10, 12 and parts of 13.

3.1 Abstract

Puromycin sensitive aminopeptidase (PSA) has been shown to be important in mouse embryogenesis and neuronal differentiation. Here we have identified *Dictyostelium* as a simple model system to study developmental roles of PSA. We have shown that a homolog of PSA, PsaA, is differentially expressed throughout *Dictyostelium* development and is distributed throughout the nucleoplasm and cytoplasm of prestalk/stalk and prespore/spore cells. Overexpression of PsaA and/or inhibition with bestatin methyl ester (BME) inhibited normal spore cell differentiation. Fewer prespore cells underwent terminal differentiation and the ones that did were rounded in shape. PsaA expression was also specifically induced by cAMP but repressed by differentiation inducing factor 1 (DIF-1) and ammonia. Using chimeras, we have identified that nuclear versus cytoplasmic localization of PsaA affects the choice between stalk or spore differentiation pathway. Cells that overexpressed PsaA-GFP (primarily nuclear)
differentiated into stalk cells, while cells that overexpressed PsaAΔNLS2-GFP (cytoplasmic) differentiated into spores. Lastly, using a fluorometric aminopeptidase assay we have shown that PsaA cleaves alanine from substrates and that its aminopeptidase activity is inhibited by puromycin, BME and EDTA but induced by ZnCl₂. In conclusion, we have demonstrated that PsaA has a role in Dictyostelium cell differentiation and identified a simple model system for further elucidation of PsaA function.

3.2 Introduction

Puromycin sensitive aminopeptidase (PSA) is an exopeptidase that belongs to the M1 family of Zn²⁺-binding aminopeptidases (Constam et al., 1995). It cleaves amino acids from the N-terminus of oligopeptide chains. It localizes to the nucleus and the cytoplasm in COS cells and Swiss 3T3 fibroblasts (Constam et al., 1995). It is also inhibited by puromycin and bestatin (Constam et al., 1995). PSA is involved in proteolytic events that mediate processes like cell cycle progression in mitosis and meiosis, embryogenesis, neuronal differentiation, establishment of polarity, reproduction, and processing of MHC class I peptides in a variety of organisms (Constam et al., 1995; Hui et al., 1998; Osada et al., 2001a,b; Schulz et al., 2001; Brooks et al., 2003; Sanchez-Moran et al., 2004; Lyczak et al., 2006). It may also have roles in cell signaling and protein trafficking, independent of its enzymatic activity (Peer, 2011). PSA has also been identified as the primary aminopeptidase responsible for the digestion of poly-Q repeats released by proteasomes in neurons and thus is implicated in poly-Q diseases like Huntington’s (Bhutani et al., 2007). It has also been shown to digest neuronal Tau and is also implicated in Alzheimer’s disease and other tauopathies (Kudo et al., 2011).

Two homologs of mammalian PSA have been identified in Dictyostelium discoideum and termed PsaA and PsaB (Catalano et al., 2011). An alignment of D. discoideum PsaA with PSA from other species has revealed the presence of a conserved exopeptidase GAMEN motif, a Zn²⁺-binding domain, one putative nuclear export signal (NES) and at least one nuclear localization signal (NLS2) (Catalano et al., 2011).

In D. discoideum PsaA has been shown to interact with nucleomorphin (NumA1) and cyclin dependent kinase 5 (Cdk5) (Catalano et al., 2011; Huber and O’Day, 2011a). NumA1 is an acidic, nucleolar Ca²⁺-dependent calmodulin-binding protein (CaMBP; Myre and O’Day, 2002). Several pieces of evidence suggest that it regulates cell cycle in D. discoideum (Myre and
O’Day, 2002, 2004a; Catalano and O’Day, 2011). NumA1 is also expressed throughout development (Fig. A.1; Myre and O’Day, 2002). Its expression is regulated by DIF-1 and it interacts with a prestalk O (pstO)-specific protein Ca$^{2+}$-binding protein 4a (CBP4a; Myre and O’Day, 2004b; O’Day et al., 2009). Thus, NumA1 likely plays a role in pstO cell differentiation. Cdk5 is a nucleoplasmic and cytoplasmic protein that regulates spore cell differentiation in D. discoideum (Sharma et al., 2002; Huber and O’Day, 2011b). As these proteins are PsaA’s binding partners, PsaA may also have a role in prestalk and/or prespore cell differentiation in D. discoideum.

Our goal is to identify the role of PsaA in D. discoideum development. D. discoideum is used extensively as a model organism for analysis of basic cellular events like cell growth, cell division, differentiation and morphogenesis. It has also been identified as a biomedical model for many human diseases, including Huntington’s (Myre et al., 2011; Williams, 2010). The study of PsaA in D. discoideum is invaluable for better understanding of the function of this protein in basic cellular events in normal and diseased cells. Our goal was to determine the developmental expression of PsaA, its regulation by extracellular signaling molecules, as well as the effect of its inhibition and overexpression on cell differentiation and morphogenesis in D. discoideum.

3.3 Materials and Methods

3.3.1 Chemicals, strains and culture conditions

All generic chemicals were obtained from BioShop Canada (Burlington, ON, Canada) or Sigma-Aldrich (St. Louis, MO, USA). Bestatin methyl ester was purchased from Santa Cruz Biotechnologies (Santa Cruz, CA, USA). D. discoideum PsaA-GFP, PsaAΔNLS2-GFP, GFP and the parental AX3 strains were all maintained on SM agar plates in association with E. coli B/r at 21 °C in the dark. For the selection of GFP strains 100 μg/mL G418 was added to the SM plates. Alternatively, all strains were maintained axenically in HL-5 medium, shaking at 180 rpm at 21 °C. For selection of GFP strain 10 μg/mL G418 was added to the HL-5 medium. The expression of all GFP constructs is governed by the constitutively active actin-15 promoter.

3.3.2 Northern blotting

Total RNA was isolated from growing and developing cells, separated in a 1% agarose-formaldehyde gel (25 μg/lane), transferred onto positively charged nylon membrane and fixed
onto the membrane via UV-crosslinking according to a described method (see section 4.3.7). Northern blotting was performed according to a previously described method with a few modifications (see section 4.3.7). The following primers were used for PCR amplification of DNA probes: \textit{psaA} F: GGTGAATCATTTAGAAAGGGTC, R: CTAACCGATTCTTGGTGCTGAAAGGGTC, \textit{rnlA} F: AGGGTTACGCGATCGCTAA, R: TTCGCTACCTTAGGACCGTC. DIG-labeling and purification were followed by the prehybridization (2 h) and hybridization (overnight) at 42 °C. The blots were exposed to Bioflex MS Film for 5 min (Clonex Corporation; Markham, ON, Canada) and developed using CB² Developer and Chemblend Fixer (Simon; Baltimore, MA, USA). Blots were scanned and band intensities were quantified using ImageQuant Pro 5.2 (GE Healthcare; Baie d’Urfe, QC, Canada).

3.3.3 Western blotting

For the analysis of developmental levels of \textit{PsaA}, $2 \times 10^6$ growing and developing cells were harvested and lysed with NP-40 lysis buffer. For the analysis of effect of morphogens on \textit{PsaA} expression, growing cells were washed and resuspended in KK$_2$ at a concentration of $2 \times 10^6$ cells/ml. Cell numbers were counted in a hemocytometer. The compounds used in the different experiments were added in the same buffer at the indicated concentrations (20 mM NH$_4$Cl; 1 mM cAMP; or 100 nM DIF-1). Cells were starved in KK$_2$ at 22 °C with shaking at 250 rpm to reduce cell–cell contact and harvested at 2 and 4 h post-treatment. Cells were also lysed with NP-40 lysis buffer. The rest of the procedure was conducted as previously described (O’Day et al., 2009). Samples were separated on 12% SDS-PAGE gel (25 μg/lane), transferred onto PVDF membrane and probed with rabbit anti-\textit{PsaA} (1:500; Catalano et al., 2011), mouse anti-α-tubulin (1:1000; 12G10; Developmental Studies Hybridoma Bank; Iowa, IA, USA) or mouse anti-GFP (1:600; Santa Cruz Biotechnologies). Membranes were developed with the Amersham™ ECL Plus Western Blotting Detection System (GE Healthcare, Buckinghamshire, UK) and scanned using a Storm 860 Phosphorimager/Fluorimager (Molecular Dynamics Inc., Sunnyvale, CA, USA). Blots were analyzed and the band intensities were quantified using ImageQuant Pro 5.2.

3.3.4 Immunolocalizations

The immunolocalization of \textit{PsaA} and tubulin was performed according to a previously described protocol (Catalano et al., 2011). Cells were fixed in -80 °C methanol, blocked and incubated with
anti-PsaA (1:20) followed by either Alexa Fluor® 555 or 488 secondary antibodies (1:40; Invitrogen; Burlington, ON, Canada). Cells were then incubated with anti-α-tubulin (1:100; Developmental Studies Hybridoma Bank) followed by Alexa Fluor® 555 or 488 secondary antibody (1:100; Invitrogen). Cover slips with cells were mounted using Prolong Gold Antifade (Invitrogen) containing DAPI. Slides were viewed and cell images were captured using the Nikon Eclipse 50i microscope with the mounted Nikon Digital Sight DS-Ri1 camera.

3.3.5 Fluorometric aminopeptidase assay

Cells were harvested, washed and lysed in NP-40 lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% NP-40). Total cell protein was quantified using the Bradford assay and 1 mg/mL protein aliquots were made. 10 μL of total cell protein was mixed with 290 μL of substrate solution (0.1 mg/mL DTT, 0.1 mg/mL albumin and 1mM alanine-β-naphthylamide) (Alba et al., 1989). Fluorometric measurements (340 nm excitation, 400 nm emission) were made after 15 and 30 minutes. The slope of the line between the 15 and 30 min measurements was used to represent aminopeptidase activity. Total cell protein was preincubated with bestatin, amastatin, puromycin, EDTA and/or ZnCl₂ for 20 min before the fluorometric aminopeptidase assay.

3.3.6 Viability staining

Cells were harvested after treatment with the different concentrations of BME for 24 h and were stained with 20 μg/mL fluorescein diacetate and propidium iodide for 5 min. Cells were deposited onto slides and viewed using the Nikon Eclipse 50i microscope with the mounted Nikon Digital Sight DS-Ri1 camera. At least 100 cells were counted for each treatment.

3.3.7 Synchronized development of cells

Cells were harvested, washed and plated at 2x10⁷ cell/mL onto black membrane filters (Millipore; Billerica, MA, USA) as detailed elsewhere (Fey et al., 2007). Filter pads were soaked in ddH₂O ± BME. Development was allowed to proceed for 24 h. Membrane filters were propped at a 90-degree angle for side-view photography. The structures formed by 24 h were stained with 0.01% calcofluor (Ciba-Geigy, now Novartis; Basel, Switzerland) before being viewed and photographed. A Nikon Eclipse 50i microscope with the mounted Nikon Digital Sight DS-Ri1 camera (Nikon; Melville, NY, USA) was used to view and capture all images. Slug
fluorescence scans were done using the Profile tool of NIS Elements 3.2Br software. All slug lengths were standardized to 1 in Excel (Microsoft Office) and average fluorescence intensity values were calculated for each 0.1 of the slug’s length. Spore shapes were measured using the Measurements tool of NIS Elements 3.2Br software.

3.3.8 Statistical analysis

All statistical analyses were performed using a one-way ANOVA followed by Tukey’s Test in Minitab15 (Minitab; State College, PA, USA).

3.4 Results

3.4.1 PsaA is differentially expressed throughout growth and development

Western and northern blots were used to analyze expression levels of PsaA in growing and developing cells (Fig. 3.1). PsaA protein levels were the highest in growing cells, decreased to 20 h of development and increased slightly by 24 h of development (Fig. 3.1A,C). PsaA mRNA levels followed a similar trend. PsaA mRNA levels decreased from levels found in growing cells to 16 h of development and increased again by 24 h of development (Fig. 3.1B,D). The mitochondrial large subunit rRNA, RnlA, was used as a developmental loading control, a standard in the field, and its levels did not change significantly throughout development. Based on the pattern of PsaA expression, it likely plays important roles during late development.

3.4.2 Production of PsaA overexpressing strains

PsaA-GFP, PsaAΔNLS2-GFP and GFP strains were produced to analyze the effect of PsaA overexpression on D. discoideum development (Catalano et al., 2011). Expression of GFP fusion proteins was verified by western blotting (Fig. 3.2). Anti-PsaA detected endogenous PsaA and PsaA-GFP in the lysate of cells expressing PsaA-GFP however only endogenous PsaA was detected in cells expressing GFP alone and untransformed cells. Anti-GFP detected GFP and PsaA-GFP in the lysate of cells transformed with the plasmid coding for either GFP or PsaA-GFP respectively. GFP was not detected in the lysate of untransformed cells. These results confirm that GFP and PsaA-GFP were properly expressed. Expression of PsaAΔNLS2-GFP was verified in the same way (Catalano et al., 2011). This also further verified the specificity of anti-PsaA antibody. The intensity of PsaA-GFP bands relative to endogenous PsaA suggests that the
Figure 3.1. PsaA protein and mRNA expression during *D. discoideum* development. (A) A representative western blot showing PsaA (98 kDa) protein levels in growing (0 h of development) and developing cells. α-tubulin (α-tub; 55 kDa) protein levels were used as a loading control. (B) A representative northern blot showing PsaA (2.58 Kb) mRNA levels in growing and developing cells. RnlA (2.8 Kb) mRNA levels were used as a loading control. (C,D) A quantification of band intensities of PsaA protein (C) and mRNA levels (D) from (A) and (B) respectively (M+S.D.). A one-way ANOVA with Tukey’s Test was used to analyze significant changes in protein and mRNA levels in developing cells. Data points that do not share a letter are significantly different. p-values ≤ 0.05. This experiment was independently replicated 4 times.

fusion protein was overexpressed (Fig. 3.2).

3.4.3 PsaA is distributed throughout the nucleoplasm and cytoplasm in developing cells

PsaA-GFP, PsaAΔNLS2-GFP and GFP expressing cells developed normally, forming slugs by 16 h and fruiting bodies by 24 h (Fig. 3.3A-D). In slugs, PsaA-GFP distributed mainly throughout the nucleoplasm and to a lesser extent the cytoplasm in both prestalk and prespore cells (Fig. 3.4A). PsaAΔNLS2-GFP distributed mainly throughout the cytoplasm in both prestalk and prespore cells (Fig. 3.4B). This suggests that NLS2 is necessary for proper nuclear
Figure 3.2. Western blot showing PsaA-GFP fusion protein expression. Whole cell lysates of untransformed (AX3) cells or cells expressing GFP or PsaA-GFP were probed with anti-PsaA or anti-GFP. A representative western blot is shown. This experiment was independently replicated 2 times. Part of Fig. 2 from Catalano et al., 2011.

Localization of PsaA. GFP alone was found evenly distributed throughout both prestalk and prespore cells (Fig. 3.4C). The same pattern of localization of both GFP fusion proteins and GFP alone was observed in mature stalk and spore cells of fruiting bodies (Fig. 3.5A-C). Due to extensive vacuolization of stalk cells most of the cytoplasm and the nucleus were found at cell periphery. To confirm nucleoplasmic and cytoplasmic distribution of PsaA, slugs were fixed and immunostained with anti-PsaA antibody, followed by a fluorophore-conjugated secondary antibody (Fig. 3.4D). Nucleoplasmic and cytoplasmic distribution of PsaA was confirmed (Fig. 3.4E). PsaA also appeared to localize to microtubule organizing centers (MTOCs) and microtubules in both prestalk and prespore cells.
Figure 3.3. The effect of overexpression of PsaA on development of *D. discoideum*. Light microscopy images of parental AX3 cells (A) and cells overexpressing PsaA-GFP (B), PsaAΔNLS2-GFP (C) or GFP (D) developing on filters for up to 24 h. Hours 12-20 are top view images and hour 24 is a side view image. This experiment was independently replicated 5 times. Mounds (12 h), slugs (16 h), culminants (20 h) and fruiting bodies (24 h) are clearly visible.
3.4.4 Overexpression of PsaA-GFP affects spore cell differentiation

The fruiting bodies formed by PsaA-GFP, PsaAΔNLS2-GFP and GFP overexpressing cells were stained with calcofluor for the examination of morphology of stalk and spore cells (Fig. 3.5A-C). Calcofluor is a fluorescent cellulose specific stain that allows the visualization of cellulose walls of stalk and spore cells that form during terminal differentiation. Spore differentiation was inhibited by the overexpression of PsaA-GFP but not PsaAΔNLS2-GFP or GFP alone. 38.4±31.4% of cells in the spore masses of fruiting bodies formed by PsaA-GFP overexpressing cells were spores (Fig. 3.6A). Other cells appeared non-vacuolated and did not have a cellulose wall, thus they were likely prespore cells that did not undergo terminal differentiation into spores. In comparison, spores comprised 97.7±1.2 %, 97.8±2.1 % and 99±1.0 % of cells in the spore masses of fruiting bodies formed by the parental AX3 cells and cells overexpressing PsaAΔNLS2-GFP or GFP, respectively (Fig. 3.6A). Stalk cell morphology (basal disc and stalk proper) was not affected by the overexpression.

PsaA-GFP overexpressing spores that did terminally differentiate were rounded. The shape was calculated by dividing spore length by the width. Spores formed by PsaA-GFP overexpressing cells were rounder than spores formed by the parental AX3 cells (Fig. 3.6B). Overexpression of PsaAΔNLS2-GFP or GFP did not affect spore shape. Thus, spore cell differentiation is affected by overexpression of PsaA in the nucleus but not the cytoplasm.

3.4.5 PsaA expression is differentially regulated by developmental morphogens

AX3 cells were treated for up to 4 h with cAMP, NH₄Cl, DIF-1 or any two in combination. Western blots of whole cell extracts from treated and untreated cells were probed with anti-PsaA (Fig. 3.7A). The blots were subsequently probed with anti-α-tubulin. Tubulin was used as a loading control. Treatment of cells with cAMP for 4 h induced PsaA expression to 120.32±9.7% of untreated control (Fig. 3.7B). Co-treatment of cells with cAMP and DIF-1 inhibited this increase in expression slightly. Treatment of cells with DIF-1 alone inhibited PsaA expression to 81.34±6.5% of untreated control. Co-treatment of cells with cAMP and NH₄Cl inhibited PsaA expression to 82.94±9.5% of untreated control. A similar level of inhibition was observed in cells treated with NH₄Cl alone. Co-treatment of cells with DIF-1 and NH₄Cl did not affect PsaA
Figure 3.4. Localization of PsaA in slugs. Intact or mechanically disrupted slugs were fixed and stained. PsaA-GFP (A), PsaAΔNLS2-GFP (B) and GFP (C) expressing slug cells stained with DAPI (blue). n=nucleus, c=cytoplasm. D. Whole slugs immunostained with anti-PsaA antibody followed by Alexa-555 conjugated secondary antibody (red). Slugs were then immunostained with anti-α-tubulin, followed by Alexa-488 conjugated secondary antibody (green). Lastly, slugs were stained with DAPI (blue). Tips of slugs point left. E. Immunolocalization of PsaA (red) and α-tubulin (green) in individual slug cells. At least 100 immunostained or GFP fusion protein-expressing cells were viewed in 3 independent replicates. At least 10 slugs were immunostained and viewed in 3 independent replicates. Part of Fig. 4 from Catalano et al., 2011.

expression levels significantly. Thus morphogens differentially regulate PsaA expression. PsaA expression is induced by cAMP but repressed by DIF-1 or NH₄Cl.
3.4.6 Cells overexpressing PsaA-GFP or PsaAΔNLS2-GFP have a sorting defect

To further analyze the effect of PsaA overexpression, the overexpressing cells were mixed 20% to 80% with parental AX3 cells. Other mixing proportions (1:9-9:1) were also examined and provided consistent results but the sorting defect was most clearly visible when the overexpressing cells were mixed in at 20%. Development was followed for 24 h to see if overexpressing cells show sorting defects and thus differentiation defects. In chimeric slugs (16 h of development) PsaA-GFP overexpressing cells sorted to both the anterior of slugs, specifically the posterior of prestalk zone, and to the posterior of slugs, the posterior portion of prespore zone (Fig. 3.8A,B). PsaAΔNLS2-GFP overexpressing cells localized primarily to the prespore zone (Fig. 3.8A,B). GFP overexpressing cells did not sort preferentially into a specific zone of the slug but rather were dispersed throughout the whole slug (Fig. 3.8A,B). These sorting patterns were quantified by analyzing fluorescence levels along the length of slugs using the NIS Profile tool, a technique that could be of tremendous benefit to the field.

3.4.7 PsaA and PsaAΔNLS2 overexpressing cells preferentially differentiate into stalk or spore cells, respectively

The sorting defects of PsaA overexpressing cells in chimeras were further analyzed at 24 h of development, in fruiting bodies. PsaA-GFP overexpressing cells were found mainly in the lower and upper cup, the stalk and the basal disc, but not the spore mass of chimeric fruiting bodies (Fig. 3.9A,B). PsaAΔNLS2-GFP overexpressing cells were mainly found in the spore mass (Fig. 3.9A,B). GFP overexpressing cells were found throughout the fruiting body, in the basal disc, stalk, spore mass and cup areas (Fig. 3.9A,B). The number of fluorescent spores (expressing the different GFP constructs) was counted in respect to all spores in the spore mass to quantify the tendency of the overexpressing cells to differentiate into spores. PsaA-GFP overexpressing cells comprised 0.3±0.6 % of spores in the spore mass, PsaAΔNLS2-GFP comprised 43.95±5.5 % and GFP comprised 21.1±3.4 % (Fig. 3.9C). The expected percentage of fluorescent spores if the cells did not show a differentiation preference is 20 %. GFP expressing cells therefore did not show a tendency to differentiate into stalk or spores but the nuclear versus the cytoplasmic overexpression of PsaA resulted in a bias in the differentiation pathway.
Figure 3.5. Localization of PsaA in fruiting bodies. PsaA-GFP, PsaAΔNLS2-GFP and GFP overexpressing cells were developed to the fruiting body stage and stained with calcofluor (blue). Localization of PsaA-GFP (A), PsaAΔNLS2-GFP (B) and GFP (C) in spores, stalk cells and basal disc cells of fruiting bodies. At least 10 fruiting bodies (at least 100 spores in each) were stained and viewed in 4 independent replicates. Part of Fig. 4 from Catalano et al., 2011.
3.4.8 BME affects spore cell differentiation

Bestatin methyl ester (BME) is an inhibitor of Zn$^{2+}$-binding aminopeptidases of which PsaA and PsaB are the only two that have been identified in *D. discoideum* (Sekine et al., 2001). The parental (AX3) and GFP fusion protein overexpressing cells were allowed to develop on filters soaked in 600 or 900 µM BME. AX3 and PsaAΔNLS2-GFP overexpressing cells developed normally and formed fruiting bodies by 24 h (Fig. 3.10A,C). Fruiting bodies were stained with calcofluor to analyze the morphology of formed stalk and spore cells. In both strains, 600 µM BME resulted in abnormal spore shape (Fig. 3.10B,D,E). The spores were rounded in 600 µM BME treated AX3 and PsaAΔNLS2-GFP strains. 900 µM BME inhibited terminal spore cell differentiation in both strains as none, or very few (and round) spores were present in treated AX3 and PsaAΔNLS2-GFP strains, respectively (Fig. 3.10B,D).

The effect of BME on PsaA-GFP overexpressing cells was more severe than the effect of
The effect of extracellular signaling molecules on PsaA expression. Cells were starved (in KK2) for 2-4 h in the presence or absence of cAMP, DIF-1, NH₄Cl or any two in combination. A. Western blot showing PsaA and α-tubulin expression in treated cells. B. The quantification of band intensities from western blot in (A) (M+S.D.). A two-way ANOVA with Tukey’s Test was used to analyze changes in band intensities. Different treatments were compared at each time point and different time points were compared within each treatment. Groups that do not share a letter are statistically different. p-value ≤ 0.05. This experiment was independently replicated at least 4 times.

overexpression alone. PsaA-GFP overexpressing cells treated with 600 and 900 µM BME developed abnormally and no fruiting bodies were formed by 24 h. At both concentrations of BME, cells aggregated to form large and irregular shaped mounds that often broke apart later on
in the development (Fig. 3.11A,B). At 600 µM BME some of the mounds extended to form fingers (Fig. 3.11A). Some of the fingers fell over and resembled slugs but did not migrate on filters. By 24 h some standing fingers were observed. After staining with calcofluor it was evident that all cells of the mounds and fingers were terminally differentiated stalk cells (Fig. 3.11C). At 900 µM BME the development was completely arrested at the mound stage (Fig. 3.11B). All cells of the mounds were terminally differentiated stalk cells (Fig. 3.11D). No spores were formed in 600 or 900 µM BME treated structures.

### 3.4.9 Evidence that BME interacts with PsaA

PsaA was not detected by anti-PsaA in the nucleoplasm of BME-treated (600 µM) cells (Fig. 3.12A,B). This suggests that either PsaA is absent from the nucleoplasm in these cells or that
Figure 3.9. The effect of overexpression of PsaA on cell sorting in *D. discoideum* as observed in fruiting bodies. A. Fruiting bodies composed of 20% of cells overexpressing PsaA-GFP, PsaAΔNLS2-GFP or GFP alone and 80% of parental AX3 cells. Phase images with GFP fluorescence overlay are shown. B. Calcofluor (blue) stained spores, stalk and basal disc cells of fruiting bodies in (A). Arrows in the first panel point to small, partially vacuolated, fluorescent cells that are likely cup cells. C. Quantification of the number of fluorescent spore in (B) as a percentage of total number of spores (M+S.D.). A one-way ANOVA with Tukey’s Test was used to analyze significant changes in the number of fluorescent spores. Data points that do not share a letter are statistically different, p-value ≤ 0.05. At least 10 fruiting bodies from 3 independent replicates were viewed. At least 100 spores/fruiting body were counted.
Figure 3.10. The effect of BME on *D. discoideum* AX3 and PsaAΔNLS2-GFP development and spore cell differentiation. A. Fruiting bodies formed by 24 h by untreated AX3 cells and AX3 cells treated with 600 or 900 μM BME. B. Calcofluor stained spores from fruiting bodies in (A). C. Fruiting bodies formed by 24 h by untreated PsaAΔNLS2-GFP overexpressing cells and PsaAΔNLS2-GFP overexpressing cells treated with 600 or 900 μM BME. D. Calcofluor stained spores from fruiting bodies in (C). E. The quantification of spore shape (spore length divided by the width) in untreated AX3 cells, AX3 cells treated with 600 μM BME or PsaAΔNLS2-GFP overexpressing cells treated with 600 μM BME (M+S.D.). A one-way ANOVA with Tukey’s Test was used to analyze significant changes in the shape of spores. Data points that do not share a letter are statistically different. p-value ≤ 0.05. This experiment was independently replicated 3 times. At least 100 spores from 4 fruiting bodies were measured each time.
BME-binding blocks anti-PsaA from detecting PsaA. The presence of PsaA-GFP in the nucleoplasm of BME-treated (600 µM) cells confirms that the latter is true and strongly implies an interaction between BME and PsaA (Fig. 3.12C,D). This suggests, that BME is a specific inhibitor of PsaA in *D. discoideum*.

### 3.4.10 BME is not cytotoxic to *D. discoideum* cells

In order to determine if BME was cytotoxic, BME-treated cells were double stained with fluorescein diacetate (FDA) and propidium iodide (PI). FDA is cleaved in live cells and fluoresces green. PI is a fluorescent nuclear stain that can only enter cells with compromised cell membrane permeability, and thus dying or dead (Fig. 3.13B). Cultures treated with either 300 µM or 600 µM BME contained 99.14±1.22 % or 95.78±3.25 % live cells respectively (Fig. 3.13A). As well, cells were observed in all stages of the cell cycle in cultures treated with 300, or 600 µM BME (Fig. 3.13C). Thus BME is not cytotoxic to cells therefore its effect on spore cell differentiation is likely directly due to its interaction with PsaA.

### 3.4.11 PsaA is a puromycin-sensitive aminopeptidase and BME inhibits its activity

A fluorometric assay with alanine-β-naphthylamide as a substrate was used to analyze aminopeptidase activity in whole cell lysates of PsaA-GFP, GFP and AX3 cells. Aminopeptidase activity was detected, as measured by the liberation of naphthylamide after cleavage of alanine. Lysates of cells overexpressing PsaA-GFP had 229.97±41.3% of aminopeptidase activity of lysates of parental AX3 cells (Fig. 3.14A). Lysates of cells overexpressing GFP alone had 95.33±6.2% of aminopeptidase activity of lysates of AX3 cells (Fig. 3.14A). Thus, cells overexpressing GFP have the same alanine aminopeptidase activity as AX3 cells while cells overexpressing PsaA-GFP have ~2.3 fold greater activity. This suggests that PsaA is an alanine aminopeptidase that is overexpressed in PsaA-GFP strain. Puromycin inhibited aminopeptidase activity in lysates of PsaA-GFP cells by 39.39±3.1% of untreated control (Fig. 3.14B). In lysates of GFP overexpressing cells promycin inhibited activity by 17.68±1.9% of control. Thus, lysates of PsaA-GFP overexpressing cells are more sensitive to the inhibition of aminopeptidase activity by puromycin than lysates of GFP overexpressing cells. Same difference in the sensitivity to inhibition was seen after treatment with BME and EDTA. BME inhibited aminopeptidase activity in lysates of PsaA-GFP and GFP overexpressing cells by 69.39±10.5% and 39.93±18.7%
Figure 3.11. The effect of BME on development of PsaA-GFP overexpressing cells. A developmental sequence of PsaA-GFP overexpressing cells treated with 600 (A) or 900 (B) μM BME and allowed to develop on filters for up to 24 h. The 24 h image in (A) is a side view, the rest of the images are top views. C-D. Calcofluor stained cells of the structures formed on 600 (C) and 900 (D) μM BME, at 24 h. These correspond to structures seen at 24 h in (A) and (B), respectively. Solid arrow points to finger and empty arrows point to mounds. Last panel in (C) and (D) shows the stalk cells that comprise these structures. This experiment was independently replicated 4 times. At least 10 finger and mound-like structures were stained and viewed each time.
Figure 3.12. The effect of BME on detection of PsaA via immunostaining. Untreated AX3 cells (A) or cells treated with 600 µm BME (B) were fixed and probed with anti-PsaA (red) and anti-α-tubulin (green) and counterstained with DAPI (blue). Nuclear PsaA was not detected in cells treated with BME (arrow). Nuclear localization of PsaA-GFP in untreated cells (C) and 600 µm BME (D) treated cells. The same procedure as in (A) and (B) was used. Anti-PsaA (green), anti-α-tubulin (red) and DAPI (blue). At least 100 cells were viewed from 3 independent replicates. n=nucleus, c=cytoplasm.

of control, respectively. EDTA inhibited aminopeptidase activity in these lysates by 43.00±4.7% and 14.90±19.0% of control, respectively. Amastatin inhibited aminopeptidase activity in lysates of both PsaA-GFP and GFP overexpressing cells by 37.24±3.6% and 47.01±12.2% of control, respectively. Thus, both lysates were inhibited by amastatin to the same degree. ZnCl₂ partially rescued the inhibition of aminopeptidase activity by EDTA in PsaA-GFP expressing cells.
Figure 3.13. The effect of BME on cell viability in *D. discoideum*. A. Percent live cells in BME-treated cultures after 24 h (M+S.D.). All cells were double stained with FDA and PI. B. Representative cells showing FDA and PI staining, respectively. C. The presence of cells in all mitotic stages in BME treated cultures. Tubulin (green) was immunolocalized and the cells were counterstained with DAPI (blue). Representative cells from 600 μM BME treated cultures are shown. At least 100 cells were counted from 3 independent replicates. c=cytoplasm; n=nucleus.

Treatment of lysates of PsaA-GFP expressing cells with EDTA and ZnCl$_2$ resulted in a 17.68% rescue in aminopeptidase activity compared to treatment with EDTA alone. Both also rescued and even induced aminopeptidase activity in lysates of GFP expressing cells by 27.24±16.0% of untreated control. Treatment of lysates of PsaA-GFP and GFP overexpressing cells with ZnCl$_2$ induced aminopeptidase activity by 85.18±19.4% and 49.35±18.0% of untreated control, respectively. Thus, lysates of PsaA-GFP overexpressing cells are more sensitive to ZnCl$_2$ than lysates of GFP overexpressing cells. This provides strong evidence that PsaA is a Zn$^{2+}$-binding puromycin sensitive aminopeptidase that is specifically inhibited by BME.

3.5 Discussion

Here we have gained insight into the role of PsaA, a NumA1 and Cdk5 binding partner, during development. PsaA is a nucleoplasmic and cytoplasmic protein whose expression is developmentally regulated. When PsaA-GFP is overexpressed it inhibits normal spore cell
differentiation. We have also identified that nuclear and cytoplasmic PsaA likely play different roles in stalk and spore cell differentiation. In chimeric organisms, when cells overexpressed mainly nuclear PsaA-GFP they differentiated into stalk cells. On the other hand, when cells overexpressed a form of PsaA that cannot properly enter the nucleus (PsaAΔNLS2-GFP) and instead accumulates in the cytoplasm cells differentiated into spores. Treatment of cells with
BME, an inhibitor of Zn\(^{2+}\)-binding aminopeptidases inhibited normal spore cell differentiation. Interestingly, the developmental effect of BME was more severe in PsaA-GFP overexpressing cells than in the parental AX3, where development was arrested and cells differentiated only into stalk cells and no spores were ever formed. Lastly, we have specifically shown that PsaA can cleave alanine from substrates and that its activity is inhibited by puromycin, BME and EDTA but induced by ZnCl\(_2\). Thus PsaA is a functional enzyme in *D. discoideum* that shares attributes with PsaA in other species.

We have shown that PsaA is distributed throughout the nucleoplasm and cytoplasm in both pre stalk/stalk and prespore/spore cells. Similarly, PSA localizes to the nucleoplasm and cytoplasm in COS cells, 3T3 fibroblasts and rat sciatic nerves (Constam et al., 1995; Yamamoto et al., 2002). PsaA’s binding partner Cdk5 is also a nucleoplasmic and cytoplasmic protein (Huber and O’Day, 2011a). Nucleoplasm is likely where PsaA interacts with its other binding partner, NumA1 (Catalano and O’Day, 2011; Catalano et al., 2011). Immunodetection of PsaA at MTOCs and microtubules also suggests that PsaA localizes to MTOCs and microtubules in cells.

PSA has previously been shown to localize to the centrosome in dividing COS cells and 3T3 fibroblasts (Constam et al., 1995). However the immunolocalization of PsaA to the MTOC was not supported by PsaA-GFP expression data. PsaA-GFP localized to the nucleoplasm and cytoplasm but not MTOCs or microtubules. Furthermore, proteomic analysis of MTOCs in *D. discoideum* did not detect the presence of PsaA (Reinders et al., 2006). Thus, the association of PsaA with MTOCs and microtubules remains in question.

Overexpression of PsaA in PsaA-GFP and PsaAΔNLS2-GFP strains has been verified by western blotting, demonstrating the expression of large amounts of fusion proteins in comparison to native PsaA. The aminopeptidase assay has demonstrated that *D. discoideum* cells have an alanyl-aminopeptidase(s) that is sensitive to puromycin, BME, amastatin and EDTA, all attributes of puromycin-sensitive aminopeptidase. This aminopeptidase(s) also requires ZnCl\(_2\) for its activity. PsaA-GFP overexpressing cells have more aminopeptidase activity than cells overexpressing GFP alone and this activity is more sensitive to puromycin, BME, EDTA and ZnCl\(_2\). This provides strong evidence that both endogenous and expressed PsaA are functional Zn\(^{2+}\)-binding puromycin sensitive aminopeptidases that can cleave alanine from substrates. In keeping with this, BME specifically inhibits both endogenous and expressed PsaA activity. PSA in COS cells and human liver cytosol has previously been shown to cleave alanine (Constam et
Alanine specific aminopeptidase had previously been detected in developing *D. discoideum* cells (North, 1982).

BME is a cell permeable analog of bestatin where the C-terminal COOH group has been replaced with COOCH$_3$ (Sekine et al., 2001). BME specifically inhibits Zn$^{2+}$-binding aminopeptidases by binding to the Zn$^{2+}$-binding domain within the substrate-binding domain (Umezawa et al., 1976; Burley et al., 1991; Taylor, 1993). Nine aminopeptidases have been identified in *D. discoideum* but only PsaA and PsaB contain the conserved Zn$^{2+}$-binding domain that conforms to the consensus sequence HExxH$_{18}$E and the more stringent sequence abXHEbbHbc (Rawlings and Barrett, 1995; Catalano et al., 2011). The fact that anti-PsaA was unable to detect PsaA when cells were treated with BME but the fluorescence from PsaA-GFP was still visible suggests that BME does not decrease the amount of nuclear PsaA but rather interacts with PsaA, blocking antibody binding. BME also inhibited aminopeptidase activity more in PsaA-GFP than GFP overexpressing cells. This provides another piece of evidence that BME specifically interacts with and inhibits PsaA.

This suggests that the effect of BME on spore cell differentiation was the result of PsaA and/or PsaB inhibition. Since treatment of cells with BME inhibited proper spore cell differentiation PsaA and/or PsaB likely have a role in spore cell differentiation. PsaA expression profile is in agreement with a role for PsaA during late development, when spore cells are undergoing terminal differentiation, as the levels of PsaA increase at that point. Several developmentally regulated aminopeptidases have been shown to exist in *D. discoideum* (Firtel and Blackenbury, 1972; North, 1982; Chan et al., 1985). However, PsaA is the first that has been characterized to any extent (Catalano et al., 2011; this study). Further support for the role of PsaA in spore cell differentiation comes from the fact that PsaA expression was specifically induced by cAMP but repressed by DIF-1 and ammonia.

Overexpression of PsaA-GFP had the same effect on spore cell differentiation as the treatment of cells with BME. The effect of BME was also more severe in PsaA-GFP strain than the parental AX3 such that morphogenesis was inhibited and cells differentiated only into stalk cells and no spores were ever formed. Inhibition is usually rescued by overexpression, but not in this case. Immunolocalization and aminopeptidase assay both suggest that BME specifically inhibits PsaA. This suggests that BME inhibits PsaA in a specific conformation. For example, BME may inhibit
cytoplasmic but not nuclear PsaA. BME may also inhibit PsaB but the role of PsaB in *D. discoideum* development is not known. Additionally, any agents that affect PsaA activity may affect proper spore cell differentiation, suggesting that PsaA levels are tightly regulated during development. Alternatively, although the aminopeptidase assay suggests that PsaA is enzymatically active it is still possible that PsaA-GFP acts as a dominant negative agent, antagonizing native PsaA function. If this is true, then nuclear PsaA is essential for proper spore cell differentiation. Future analysis of the developmental phenotype of PsaA knockout cells will clarify this issue.

Differential sorting of cells expressing PsaA-GFP or PsaAΔNLS2-GFP also suggests that nuclear and cytoplasmic PsaA play different roles in cell differentiation. High levels of nuclear PsaA-GFP inhibit spore cell differentiation. Fittingly, in chimeras PsaA-GFP expressing cells do not sort to the prespore zone and do not differentiate into spores. Others have shown that mutants with compromised ability to form functional spores also do not sort to the prespore zone or differentiate into spores in chimeras with parental cells (Niswonger and O’Halloran, 1997). On the other hand, cells that overexpress PsaAΔNLS2-GFP, preferentially sort to the prespore zone and differentiate into spores. It is of interest to note that 43.95±5.5 % of spores expressed PsaAΔNLS2-GFP when only 20% of these cells were mixed with 80% parental AX3 cells. About 80% of cells in each aggregate are expected to differentiate into spores, so if all PsaAΔNLS2-GFP expressing cells differentiated into spores then they should have comprised 25% of spores in the spore mass. This phenomenon could be explained by the observation that slugs containing PsaAΔNLS2-GFP expressing cells were significantly smaller than control slugs (data not shown). One explanation is that PsaAΔNLS2-GFP expression has a nonautonomous effect on cells, altering cell aggregation abilities. This remains to be elucidated in detail in future studies. Thus, overexpression of nuclear PsaA (PsaA-GFP) drives cells to the stalk cell pathway while overexpression of cytoplasmic PsaA (PsaAΔNLS2-GFP) drives cells to the spore cell pathway. Cell fractionation into nuclear and cytoplasmic fragments and analysis of PsaA in both will help elucidate the relationship between nuclear and cytoplasmic PsaA in cell differentiation.

Further support for the role of PsaA in cell differentiation comes from the observation that effects of Cdk5 inhibition strongly resemble the effects of PsaA overexpression or the treatment of cells with BME. PsaA is a binding partner of Cdk5 (Huber and O’Day, 2011b). *D. discoideum* cells expressing a dominant negative form of Cdk5 produce 80% less spores than the wild type
(Sharma et al., 2002). Treatment of cells with roscovitine, an inhibitor of Cdks, also inhibits *D. discoideum* spore cell differentiation (Huber and O’Day, 2011b). This suggests that PsaA and Cdk5 may be functionally linked during development. Both likely regulate spore cell differentiation. Further work is required to elucidate the role of NumA1 and PsaA interaction during development. NumA1 is expressed in both prestalk and prespore cells (Poloz and O’Day, unpublished). Thus, NumA1 interacting with PsaA may regulate spore cell differentiation while NumA1 interacting with CBP4a likely regulates stalk cell differentiation.

In conclusion, *D. discoideum* provides a simple model system for the analysis of PsaA role in cell function and development. Further elucidation of PsaA function will provide invaluable insight into cell fate choice and cell differentiation in *D. discoideum* as well as the function of PSA in mammalian cells.

### 3.6 Acknowledgments

This work was supported by the Natural Sciences and Engineering Research Council of Canada (DHO’D; A6807).

### 3.7 References


aminopeptidases and aminopeptidases in Dictyostelium discoideum. Biochemical and
Biophysical Research Communications, 127, 962-968.

Puromycin-sensitive aminopeptidase. Sequence analysis, expression, and functional


acid metabolizing enzymes in the cellular slime mold Dictyostelium discoideum.

and its binding to puromycin-sensitive aminopeptidase in Dictyostelium discoideum.

cell proliferation, multicellular development, and Cdk5 nuclear translocation in

synaptosomes. Its identification, purification, and characterization. Journal of Biological
Chemistry, 273, 31053-31060.

Kudo, L. C., Parfenova, L., Ren, G., Vi, N., Hui, M., Ma, Z., Lau, K., Gray, M., Bardag-Gorce,
aminopeptidase (PSA/NPEPPS) impedes development of neuropathology in
HPSA/TAUP301L double-transgenic mice. Human Molecular Genetics, 20, 1820-1833.

Lyczak, R., Zweier, L., Group, T., Murrow, M. A., Synder, C., Kulovitz, L., Beatty, A., Smith,
K., & Bowerman, B. (2006). The puromycin-sensitive aminopeptidase PAM-1 is required
for meiotic exit and anteroposterior polarity in the one-cell Caenorhabditis elegans

Myre, M. A., Lumsden, A. L., Thompson, M. N., Wasco, W., MacDonald, M. E., & Gusella, J.
F. (2011). Deficiency of huntingtin has pleiotropic effects in the social amoeba

Myre, M. A., & O'Day, D. H. (2004a). Dictyostelium nucleomorphin is a member of the BRCT-
domain family of cell cycle checkpoint proteins. Biochimica et Biophysica Acta -
General Subjects, 1675, 192-197.

nucleomorphin, a BRCT-domain protein that regulates nuclear number. Biochemical and
Biophysical Research Communications, 322, 665-671.

binding protein from Dictyostelium that regulates nuclear number. Journal of Biological
Chemistry, 277, 19735-19744.


Chapter 4

Colchicine affects cell motility, pattern formation and stalk cell differentiation in *Dictyostelium* by altering Ca\(^{2+}\) signaling

This chapter was accepted for publication as is:


I carried out all experiments and data analysis for this chapter. I made all Figures and wrote the chapter. Danton H. O’Day offered assistance in experimental design and edited the manuscript.

4.1 Abstract

Previous work, verified here, showed that colchicine affects *Dictyostelium* pattern formation, disrupts morphogenesis, inhibits spore differentiation and induces terminal stalk cell differentiation. Here we show that colchicine specifically induces *ecmB* expression and enhances accumulation of *ecmB*-expressing cells at the posterior end of multicellular structures. Colchicine did not induce a nuclear translocation of DimB, a DIF-1 responsive transcription factor *in vitro*. It also induced terminal stalk cell differentiation in a mutant strain that does not produce DIF-1 (*dmtA\(^{-}\)*) and after the treatment of cells with DIF-1 synthesis inhibitor cerulenin (100 µM). This suggests that colchicine induces the differentiation of *ecmB*-expressing cells independent of DIF-1 production and likely through a signaling pathway that is distinct from the one that is utilized by DIF-1. Depending on concentration, colchicine enhanced random cell motility, but not chemotaxis, by 3-5 fold (10-50 mM colchicine, respectively) through a Ca\(^{2+}\)-mediated signaling pathway involving phospholipase C, calmodulin and heterotrimeric G proteins. Colchicine’s effects were not due to microtubule depolymerization as other microtubule-depolymerizing agents did not have these effects. Finally normal morphogenesis and stalk and spore cell differentiation of cells treated with 10 mM colchicine were rescued through chelation of Ca\(^{2+}\) by BAPTA-AM and EDTA and calmodulin antagonism by W-7 but not PLC inhibition by U-73122. Morphogenesis or spore cell differentiation of cells treated with 50 mM colchicine could not be rescued by the above treatments but terminal stalk cell differentiation was inhibited by BAPTA-AM, EDTA and W-7, but not U-73122. Thus colchicine disrupts morphogenesis and
induces stalk cell differentiation through a Ca$^{2+}$-mediated signaling pathway involving specific changes in gene expression and cell motility.

### 4.2 Introduction

Colchicine is a microtubule-depolymerizing agent that at high doses has major developmental effects in many organisms. It inhibits proper pattern formation in *Hydra* (Corff and Burnett, 1969). In *Xenopus* and *Ambystoma*, colchicine inhibits normal limb development (Alberch and Gale, 1983). In *Tetrahymena*, it arrests or reverses oral development (Nelsen, 1970). Colchicine treatment of developing *Gallus* embryos has been shown to result in abnormal development of the brain and the heart (Diwan, 1966). It also inhibits oogenesis and wing development in *Drosophila* (Koch and Spitzer, 1982, 1983; Drozdovskaya and Rapoport, 1988). O’Day and Durston, (1978), showed that colchicine affects *Dictyostelium* pattern formation, disrupts morphogenesis, inhibits spore differentiation and induces terminal stalk cell differentiation. In spite of this, the mode of action of colchicine has not been characterized. It has been suggested, but not directly demonstrated, that colchicine affects development through microtubule-depolymerization and mitotic arrest. Because of its significant developmental effects, the elucidation of colchicine’s mode of action could provide invaluable insight into the developmental regulation in many organisms.

Here we analyze this classic developmental problem using *Dictyostelium discoideum* as a model system. The developmental program of *D. discoideum* has recently been reviewed (Williams, 2006; Schaap, 2011). On the road to fruiting body formation, starved amoebae aggregate to form a multicellular slug or pseudoplasmodium within which the pattern of differentiation of prestalk and prespore cells becomes evident (Fig. 1.2A). The anterior 1/5th of the slug, the tip, is composed of prestalk cells, while the prespore cells are located in the posterior 4/5th of the slug. There are different types of prestalk cells, characterized by their unique gene expression patterns (Fig. 1.2A,B). Prestalk A cells occupy the very tip of the slug and express *ecmA* gene from the proximal part of the promoter (Jermyn et al., 1989; Early et al., 1993). Prestalk O cells are just posterior and express *ecmA* from the distal part of the promoter (Early et al., 1993). Prestalk AB cells are in the core of the tip and express *ecmA* and *ecmB* genes (Jermyn et al., 1989; Ceccarelli et al., 1991). The prespore zone has scattered prestalk B, anterior like cells (ALCs) and prestalk U cells. Prestalk B cells express the *ecmB* gene, ALCs express *ecmA* and/or *ecmB* genes and
prestalk U cells express neither (Sternfeld and David, 1981; Ceccarelli et al., 1991; Yamada et al., 2010). Finally, the prespore cells express the cotB gene (Fosnaugh and Loomis, 1993).

A sequence of morphogenetic cell movements transforms the slug into a culminant. Prestalk AB cells vacuolate and die first as they synthesize and then enter the forming stalk tube and contribute to the inner basal disc and lower portion of the stalk (Raper and Fennel, 1952; Jermyn and Williams, 1991). Prestalk A cells start expressing the ecmB gene and follow prestalk AB cells, leading to further extension of the stalk (Jermyn and Williams, 1991; Early et al., 1993). Prestalk O cells also contribute to the stalk as prestalk A cells (Early et al., 1993). The prespore cell mass moves up the stalk and the prespore cells start expressing the spiA gene as they encapsulate into spores (Richardson and Loomis, 1992). The spore mass is supported by the upper and lower cup cells. The upper cup differentiates from prestalk O cells, prestalk B, prestalk U cells and ALCs (Sternfeld and David, 1981; Ceccarelli et al., 1991; Early et al., 1993; Yamada et al., 2010). The lower cup differentiates from prestalk B cells and ALCs (Sternfeld and David, 1981; Ceccarelli et al., 1991; Early et al., 1993). Finally, a portion of prestalk B cells and ALCs differentiate into stalk cells of the outer basal disc, the structure that supports the fruiting body on the substratum (Sternfeld and David, 1981; Dormann et al., 1996; Jermyn et al., 1996). The regulation of these different cell types is under active study.

Calcium (Ca\(^{2+}\)) plays a major role in differentiation and morphogenesis of many organisms including slime molds, fungi, plants and animals (Paranjape et al., 1990; Hepler, 2005; Kubohara et al., 2007; Cao and Chen, 2009). In *D. discoideum* intracellular Ca\(^{2+}\) levels are significantly higher in prestalk versus prespore cells and Ca\(^{2+}\) has been shown to play a major role in stalk cell differentiation (Maeda and Maeda, 1973; Abe and Maeda, 1989; Kubohara and Okamoto, 1994; Saran et al., 1994; Cubitt et al., 1995; Schaap et al., 1996; Cubitt et al., 1998; Verkerke-van Wijk et al., 1998; Baskar et al., 2000; Kubohara et al., 2007). Differentiation inducing factor-1 (DIF-1) is a morphogen that induces prestalk and stalk cell differentiation through the elevation of intracellular Ca\(^{2+}\) levels (Williams et al., 1987; Kay et al., 1999; Thompson and Kay, 2000; Kay and Thompson, 2001; Saito et al., 2008; Kubohara et al., 2007). Most downstream components of the Ca\(^{2+}\) signal transduction pathway have not been identified in *D. discoideum*. Calmodulin (CaM) is an essential, ubiquitously expressed, highly conserved, primary Ca\(^{2+}\) sensor of all eukaryotic cells and it is likely the major target of this Ca\(^{2+}\) signaling, operating through a diversity of calmodulin binding proteins (Catalano and O’Day, 2008).
Differential cell motility is also a major force driving differentiation and morphogenesis in *D. discoideum* and many other organisms (Firtel and Meili, 2000; Dormann et al., 2000). Numerous individual cell tracking studies have shown that *D. discoideum* pre stalk cells are more motile than prespore cells (Siegert and Weijer, 1991; Siegert and Weijer, 1992; Abe et al., 1994; Dormann et al., 1996). Thus there is a complex interplay between cell signaling and cell motility that regulates cell differentiation and morphogenesis in *D. discoideum*.

Our goal is to identify the mechanism of action of colchicine. We hypothesize that colchicine may induce stalk cell differentiation by affecting Ca\(^{2+}\)-mediated signaling pathways.

### 4.3 Materials and Methods

#### 4.3.1 *D. discoideum* strains and culture conditions

*D. discoideum* AX3, HM18, V12M2, PLC null (deposited by Peter van Haastert), G protein G2 null (deposited by Peter Devreotes), DmtA null (deposited by Rob Kay), TL-6 (*ecmA::lacZ*), TL-86 (*ecmO::lacZ*), TL-7 (*ecmB::lacZ*) and TL-1 (*cotB::lacZ*) strains were acquired from the *Dictyostelium* stock center (www.Dictybase.org). All *lacZ* expressing cells were deposited by Bill Loomis. The *dimB*/*[dimB]:dimB:GFP* strain was a kind gift from Jeff Williams. Cells were grown on SM agar in association with *E.coli* B/r at 21 °C in the dark. Vegetative cells were harvested and washed 4 times with KK2 phosphate buffer before all developmental studies. All generic chemicals were obtained from BioShop Canada (Burlington, ON, Canada) or Sigma-Aldrich (St.Louis, MO, USA). Colchicine and vinblastine were obtained from Sigma-Aldrich, while nocodazole was from Biomol (Plymouth, MA, USA).

#### 4.3.2 Synchronized development of *D. discoideum* cells

Vegetative cells (2x10^7) were plated onto black membrane filters (25 mm diameter; Millipore; Billerica, MA, USA) as detailed elsewhere (Fey et al., 2007). Filter pads were soaked in KK2 buffer with or without an added agent. Development was allowed to proceed for 24-48 h. Membrane filters were propped at a 90° angle for side-view photography. To visualize stalk cell differentiation, structures were stained with 0.01% calcofluor (Ciba-Geigy, now Novartis; Basel, Switzerland) before being viewed and photographed. For rescue experiments cells were allowed to develop on filters up to the slug stage and then transferred onto new filters soaked with colchicine with or without an added agent.
4.3.3 Microscopy

Nikon Eclipse 50i microscope with the mounted Nikon Digital Sight DS-Ri1 camera (Nikon; Melville, NY, USA) was used for viewing and image capture and analysis for all experiments, except cell motility analysis. For the analysis of cell motility, cell spot diameters were viewed and photographed using Zeiss Axiovert 100 microscope equipped with Sony 950 digital camera (Carl Zeiss Canada Ltd.; Toronto, ON, Canada).

4.3.4 Monolayer cell differentiation assay

The monolayer cell differentiation assay was performed according to the previously described protocol (Kubohara et al., 2007). In brief, vegetative cells were plated at 2x10^5 cells/well in multi (12)-well plates at 21 °C with 0.5 mL of a salt solution (2 mM NaCl, 10 mM KCl, 1 mM CaCl2, 1 mM MgSO4 (only for HM18), 50 μg/mL ampicilin, 100 μg/mL streptomycin sulfate, 10 mM MES-KOH pH 6.2) with added 5 mM cAMP. Colchicine was then added at 10 or 50 mM, or DIF-1 at 100 nM. Stalk and spore cell differentiation was scored after 48 h based on the percentage of cells that were vacuolated stalk cells versus refractive elliptical spores. This experiment was independently replicated 4 times and at least 100 cells were counted each time.

4.3.5 DimB translocation experiments

DimB-GFP expressing cells were starved for 4 h in KK2, transferred onto cover slips, stimulated with 100nM DIF-1, 10 mM colchicine or 50 mM colchicine for 10 min, fixed in -80 °C methanol for 40 min, washed with PBS and mounted using ProLong Gold antifade with DAPI (Invitrogen; Carlsbad, CA, USA). At least 100 cells were viewed.

4.3.6 Immunolocalization of tubulin in colchicine-treated cells

Vegetative cells (2x10^6 cells/mL) were incubated in KK2 buffer with or without an added agent for 4 h at 180 rpm at 21 °C. Immunolocalization was performed as detailed elsewhere (Hagedorn et al., 2006). In brief, cover slips with cells were submerged in -80 °C methanol for 40 min, washed with PBS, incubated in the blocking solution (0.2% w/v gelatin, 0.1% v/v Triton® X-100) for 30 min and then probed with the primary and the secondary antibodies. Mouse anti-tubulin antibody (12G10; DSHB, Iowa City, IA, USA) and goat Alexa 488 conjugated anti-mouse antibody (Santa Cruz Biotechnology; Santa Cruz, CA, USA) were both used at 1:100, diluted in the blocking buffer. Coverslips were mounted using ProLong Gold antifade with
DAPI. Cells with/without microtubular disruption were viewed, photographed and counted. NIS Elements 3.2Br software (Nikon; Melville, NY, USA) was used to quantify the cytoplasmic fluorescence intensities and the microtubular lengths/numbers. Immunolocalization was performed on treated cells from 3 independent replicates and at least 100 cells were viewed and counted each time. Analysis of fluorescence levels and microtubule lengths was performed on at least 5 representative cells from each treatment.

### 4.3.7 Northern blotting

Total RNA was isolated from cells of different developmental stages using the RNeasy Mini kit (Qiagen; Mississauga, ON, Canada). RNA samples were separated in a 1% agarose-formaldehyde gel and RNA was transferred onto positively charged nylon membranes (Roche; Mississauga, ON, Canada) using the capillary transfer method, overnight in 20xSSC. RNA was fixed onto the membrane via UV-crosslinking at 20000 μJ/cm². Northern blotting was performed using the DIG-high prime DNA labeling and detection starter kit II (Roche) according to the manual, with a few alterations. DNA probes were made via PCR, followed by asymmetric PCR and random prime labeling with digoxigenin-11-dUTP. PCR was carried out using the following conditions: 5 min at 95 °C, followed by 40 cycles of 20 seconds at 94 °C, 30 seconds at 58-60 °C and 40 seconds at 72 °C, followed by 10 min at 72 °C. The following primers were used: *CotB* F:GTTAGAGGTCCCCAAGCTG, R:GGATCGACCACCAATGA; *ecmB* F:CTCCAATTGCTTTGCTTGACG, R:CATTGATTGGGTTAGGC; *ecmA* F:TCAATTCTCTCAATGTGATTCAG, R:CAACCTTTACCTCCTGTACCAC; *spiA* F:GCTGCTGATTAGTTTACAAT, R:ACGAGACTTGATGTATTCG; *rnlA* F:AGGGTTACGCGATCGCTAA, R:TTCGCTACCTTAGGACCGT. The asymmetric PCR was performed with 100-fold excess of the forward primers using the following conditions: 5 min at 95 °C, followed by 20 cycles of 20 seconds at 95 °C, 30 seconds at 56-58 °C and 2 min at 72 °C, followed by 10 min at 72 °C. Asymmetric PCR products were purified using QIAquick PCR purification kit (Qiagen). DIG-labeling and purification were followed by the prehybridization (2 h) and hybridization (overnight) at 42 °C. The blots were exposed to Bioflex MSI Film for 5-30 min (Clonex Corporation; Markham, ON, Canada) and developed using CB² Developer and Chemblend Fixer (Simon; Baltimore, MA, USA). Blots were scanned and band intensities were quantified with ImageJ 1.41o (NIH). This experiment was independently replicated 3 times.
4.3.8  **Histochemical staining for β-galactosidase activity**

A previously described protocol was used with no alterations (Dingermann et al., 1989). The structures were counterstained with 100 μg/mL eosin in Z buffer. The structures were viewed and photographed and the intensity of staining was analyzed in gray scale images of 10 mM colchicine-treated slugs using NIS Elements 3.2Br software. The staining intensity was quantified in at least 10 treated and untreated slugs from 3 independent replicates.

4.3.9  **Random cell motility assay**

AX3 cells were starved for 2 h and spotted onto agar plates according to the previously described radial motility bioassay protocol (Browning and O’Day, 1995). Colchicine and other agents were added to agar at the indicated concentrations (10 μM cAMP was added for chemotaxis). The following agents were used: Ly-294002 (50 μM; EMD Biosciences; Gibbstown, NJ, USA), quinacrine (20 μM; EMD Biosciences), U-73122 (10 μM; Alexis Biochemicals, now Enzo Life Sciences; Plymouth Meeting, PA, USA), W-7 (50 μM; Biomol, now Enzo Life Sciences), Ethylenediaminetetraacetic acid (EDTA; 1-5 mM; Bioshop), cadmium chloride (CdCl₂; 100 μM; Sigma-Aldrich), TMB-8 (150 μM; Sigma-Aldrich), and cytochalasin D (1 μM; Sigma-Aldrich). Cell spots were photographed at 0 and 4 h and measured using the Northern Eclipse image analysis program (Impex Imaging; Leeds, UK). The motility experiments were independently replicated 5 times and 4 cell spots were examined per each treatment each time.

4.3.10  **Western blotting**

Cells were treated for 4 h and 2x10^7 cells were lysed with 1 mL lysis buffer (100 mM Pipes pH 6.8, 2.5 mM EGTA, 1 mM MgCl₂, 0.5% Triton and a complete protease inhibitor tablet from Roche) and incubated on ice for 5 min. Actomyosin cytoskeletons were spun down at 13,000 rpm for 5 min and washed with 0.5 mL of lysis buffer followed by 0.5 mL of wash buffer (lysis buffer without Triton). For total cell protein, cells were lysed with RIPA buffer. The rest of the procedure was carried out as previously described (O’Day et al., 2009). Protein content was quantified using the Bradford assay (Bradford, 1976). Samples were separated on 12% SDS-PAGE gel (25 μg/lane) and proteins were transferred onto a PVDF membrane (Pall Corporation; Port Washington, NY, USA). The membrane was blocked in 5% milk overnight at 4 °C. All washes were performed in TTBS 3 times for 10 min. Membranes were probed with mouse anti-actin (1:1000; Santa Cruz Biotechnology; Santa Cruz, CA, USA) or mouse anti-myosin II heavy
chain (1:100; Developmental Studies Hybridoma Bank; Iowa, IA, USA) for 1 h at room temperature. Membranes were then probed with HRP conjugated goat anti-mouse antibody (1:16,000; Santa Cruz Biotechnology) for 45 min at room temperature. Membranes were developed with Amersham ECL Plus Western Blotting Detection System (GE Healthcare Canada Inc.; Mississauga, ON, Canada) and visualized with the STORM 860 Phosphor/Fluorimager (Molecular Dynamics Inc., Sunnyvale, CA, USA). Blots were analyzed and the band intensities were quantified using ImageJ 1.41o (NIH). This experiment was independently replicated 3 times.

4.3.11 Viability staining

Cells (control and treated for 4 h with an indicated agent) were stained with 20 μg/mL fluorescein diacetate and propidium iodide for 5 min. Cells were deposited onto slides, viewed and photographed. The number of cells that fluoresced green versus red was counted. This experiment was independently replicated 3 times and at least 100 cells were counted each time.

4.3.12 Statistical analysis

A Student’s T-Test or a one-way ANOVA with Tukey Test in Minitab 15 (Minitab; State College, PA, USA) were used to analyze statistical differences in data.

4.4 Results

4.4.1 Colchicine disrupts morphogenesis, induces stalk cell differentiation and represses spore cell differentiation

Colchicine treated *D. discoideum* cells aggregated normally but development arrested at the slug stage when treated with 10 mM colchicine or the tipped mound-like stage when treated with 50 mM colchicine. (Fig. 4.1). Treatment with nocodazole or vinblastine, other microtubule-depolymerizing agents, did not affect differentiation or morphogenesis as they resulted in fruiting body formation with morphologically normal stalk and spore cells (Fig. 4.1; 4.2B). In addition, instead of developing cells on colchicine or other microtubule depolymerizing agents at time 0 h, untreated slugs were transferred onto pads soaked in these agents at 16 h. When treated with 10 mM colchicine they arrested at the slug stage but when treated with 50 mM colchicine the slugs broke up into several cell masses, reformed the tips but did not develop any further. Treatment with nocodazole or vinblastine did not affect differentiation or morphogenesis as
normal fruiting bodies formed by 24 h (data not shown). Thus, the same effect of colchicine was observed if cells were exposed to it from beginning of development or during the slug stage. In both cases, by 24-48 h of colchicine treatment the slugs and tipped mound-like structures accumulated terminally differentiated, vacuolated stalk cells at the posterior end (Fig. 4.2A). By 48 h of treatment with 50 mM colchicine almost all cells of the tipped mound-like structures were vacuolated stalk cells. No terminal spore cell differentiation was observed. Terminal stalk cell differentiation does not normally occur in untreated structures at this scale until culmination. Staining with PI and FD showed that colchicine does not cause these developmental effects by affecting cell viability (data not shown). These results suggest that colchicine disrupts morphogenesis and specifically induces stalk cell differentiation while inhibiting spore cell differentiation.
Figure 4.2. The effect of colchicine (Col), nocodazole (Noc) and vinblastine (Vin) on cell differentiation. A-B. Calcofluor staining of treated structures developed on filters for 24 h. A. The accumulation of terminally differentiated stalk cells at the posterior end of colchicine-treated structures. The second panel of each image indicates a magnified area of the previous image. Solid arrows point to slugs while open arrows point to tipped mound-like structures. B. Normal morphology of spore, stalk and basal disc cells in control fruiting bodies and in fruiting bodies that form after treatment with nocodazole or vinblastine. C. The effect of colchicine and DIF-1 on cell differentiation in monolayer assays. Control cells (black bars), 10 mM colchicine (dark grey bars), 50 mM colchicine (light grey bars) and 100 nM DIF-1 (white bars). The bars represent the % of all cells examined that differentiated into stalk or spore cells (M+S.D.). A one-way ANOVA with Tukey’s Test was used to analyze significant differences in cell differentiation patterns. Data points that do not share a letter are significantly different (stalk and spores were analyzed separately). p≤0.05. At least 100 cells were counted in each experiment and the experiment was independently replicated 4 times. Figure 3 from Poloz and O’Day, 2012.
4.4.2 Colchicine does not induce stalk cell differentiation or repress spore cell differentiation \textit{in vitro} comparable to DIF-1

We used a wild type strain, V12M2, and a derived sporogenous mutant strain, HM18, to analyze the effect of colchicine on \textit{in vitro} cell differentiation in monolayers. Colchicine did not induce stalk cell differentiation or suppress spore cell differentiation in V12M2 strain (Fig. 4.2C). In HM18 strain, colchicine induced stalk cell differentiation by 14.41\% and repressed spore cell differentiation by 16.51\% (Fig. 4.2C). This result was not comparable to the effect of DIF-1 on both of these strains. DIF-1 induced stalk cell differentiation in HM18 strain by 73.42\% and repressed spore cell differentiation by 76.64\% (Fig. 4.2C). DIF-1 also induced stalk cell differentiation in V12M2 strain by 18.32\% (Fig. 4.2C). Wild type AX3 cells could be used in the future to avoid the high basal stalk differentiation phenotype of V12M2 cells. This suggests that colchicine requires in vivo multicellular conditions or at least cell-cell interactions to induce stalk cell differentiation to the extent seen in colchicine treated slugs and tipped-mound like structures. This also suggests that DIF-1 and colchicine may act through distinct signaling pathways.

4.4.3 Colchicine’s action does not depend on DIF-1 production and does not involve the DIF-1 dependent transcription factor DimB

Colchicine (at 10 and 50 mM) disrupted morphogenesis and induced stalk cell differentiation in a mutant strain that does not produce DIF-1 (\textit{dmtA}). Colchicine also disrupted morphogenesis and induced stalk cell differentiation in cells developed in the presence of 100 \textmu M cerulenin (data not shown; Kay, 1998; Thompson and Kay, 2000). Cerulenin has previously been shown to affect fatty acid synthesis, including DIF-1, during development (Chance et al., 1976). Colchicine (at 10 and 50 mM) was also not able to induce a nuclear translocation of DimB-GFP, a DIF-1 responsive transcription factor that specifically translocates to the nucleus after treatment with 100 nM DIF-1 \textit{in vitro}. It also localizes mainly to the nucleus in DIF-1 dependent prestalk B cells (Fig. 4.3; Huang et al., 2005; Yamada et al., 2011). This again suggests that colchicine and DIF-1 act through distinct signaling pathways. Alternatively, colchicine may act through a different branch of the DIF-1 signaling pathway, possibly affecting the activity of other transcription factors such as MybE or GtaC (Fukuzawa et al., 2006; Tsujioka et al., 2007; Keller and Thompson, 2008; Saito et al., 2008).
Figure 4.3. Nuclear translocation of DimB-GFP in DIF-1 and colchicine treated cells. Cells were starved for 4 h and not treated (Contr.), or treated with 100 nM DIF-1, 10 mM colchicine (Col) or 50 mM colchicine (Col). Representative cells are shown. This experiment was independently replicated 3 times and at least 100 treated and untreated cells were viewed each time. Figure 4 from Poloz and O’Day, 2012.

4.4.4 Colchicine’s developmental effects are independent of microtubule depolymerization

Tubulin immunolocalization was performed to further analyze whether colchicine disrupted morphogenesis and induced stalk cell differentiation through a microtubule-independent mechanism. Colchicine, nocodazole and vinblastine disrupted microtubules after 4 h of treatment (Fig. 4.4A-F). They reduced the number of microtubules radiating away from the microtubule-
organizing centre from 16.33±0.33 (control) to 8.33±3.38 (10 mM colchicine), 10.39±1.68 (50 mM colchicine), 10.12±1.20 (30 µM nocodazole) and 5.5±0.15 (100 µM vinblastine) (Fig. 4.4C; examined at 1 plane of focus, at MTOC location; Brito et al., 2005). Microtubule organizing centers remained intact after all treatments regardless of the drug concentration used (Fig. 4.4A; Koonce and Khodjakov, 2002). The length of the remaining microtubules was reduced from 7.30±0.64 µm (control) to 1.67±0.30 µm (10 mM colchicine), 1.58±0.22 µm (50 mM colchicine), 2.32±0.21 µm (30 µM nocodazole) and 1.51±0.22 µm (100 µM vinblastine).

Cytoplasmic fluorescence levels were measured to quantify the depolymerization of microtubules into tubulin dimers that dispersed throughout the cytosol. Colchicine enhanced cytoplasmic fluorescence to 561.43±93.73% (10 mM colchicine), 429.54±28.44% (50 mM colchicine), 266.82±65.61% (30 µM nocodazole) and 475.82±111.36% (100 µM vinblastine) of control (Fig. 4.4E). Lastly, microtubules were disrupted in 29.50±0.66% of cells (10 mM colchicine), 92.86±1.68% of cells (50 mM colchicine), 100.00±0.01% of cells (30 µM nocodazole) and 99.34±0.66% of cells (100 µM vinblastine), compared to 3.42±0.16% of cells in control (Fig. 4.4F). Microtubules were also disrupted after 4 h in slugs that were transferred onto filters soaked in colchicine, nocodazole or vinblastine (Fig. 4.4B). For the rest of the experiment, concentrations of nocodazole and vinblastine were chosen based on the minimal concentrations at which microtubular disruption occurred. Only colchicine arrested morphogenesis and induced stalk cell differentiation, therefore its effect is independent of microtubule-depolymerization.

4.4.5 Colchicine induces ecmB gene expression and the posterior localization of ecmB-expressing cells

Northern blotting revealed that colchicine specifically induces ecmB gene expression, but represses ecmA gene expression (Fig. 4.5A,B). Colchicine also repressed cotB gene expression, a prespore cell marker, and spiA gene expression, a terminal spore cell differentiation marker, suggesting that colchicine represses spore cell differentiation (Fig. 4.5C,D). The mitochondrial large subunit rRNA, rnlA, levels were used as a developmental loading control, a standard in the field, and its levels did not change significantly (Fig. 4.5E). At 16 h all RNAs were expressed at a comparable level in colchicine treated and untreated cells but by 24 h colchicine had its effect on the gene expression patterns. To analyze colchicine-induced changes in spatial expression patterns of ecmA, ecmB and cotB genes the regulatory regions of these genes were used to
Figure 4.4. The effect of colchicine (Col), nocodazole (Noc) and vinblastine (Vin) on microtubular networks in cells. Immunolocalization of tubulin (green) in colchicine, nocodazole or vinblastine treated (A) cells or (B) slugs. DNA was stained with DAPI (blue). C. Quantification of the number of microtubules, (D) the length of the microtubules, (E) the level of cytoplasmic fluorescence and (F) the number of cells with disrupted microtubules (M+S.D.). C-E. measurements were made at 1 plane of focus/cell (at MTOC location). This experiment was independently replicated 4 times and at least 100 cells were viewed each time. Microtubular quantifications were performed on at least 5 representative cells/replicate. A one-way ANOVA with Tukey’s Test was used to analyze significant changes in microtubular parameters. Data points that do not share a letter are statistically different. $p \leq 0.05$. Figure 5 from Poloz and O’Day, 2012.
Figure 4.5. The effect of colchicine on developmental gene expression patterns. Quantifications of band intensities from northern blots, demonstrating the expression patterns of ecmA (A), ecmB (B), cotB (C), spiA (D) and rnlA (E) RNAs in untreated (light grey bars) and colchicine treated (dark grey bars) cells over the developmental time course (M+S.D.). Only hours 8-24 are shown as the developmental RNAs were not detected earlier, though rnlA was expressed from 0-24 h at constant levels. A Student’s T-test was used to analyze significant changes in RNA levels in colchicine treated versus untreated cells at each time point. *p-values ≤0.05. This experiment was independently replicated 3 times. Figure 6 from Poloz and O’Day, 2012.
control the synthesis of β-galactosidase (β-gal). Staining for β-gal activity revealed that after 24 h of 10 mM colchicine treatment the number of ecmB-β-gal-expressing cells appeared to increase and expression shifted into the prespore zone (TL-7 strain; Fig. 4.6A,B). This shift corresponded with the accumulation of terminally differentiated stalk cells at the posterior of colchicine treated structures. EcmA-β-gal-expressing cells were identified using two constructs, one where lacZ expression was driven by the distal part of ecmA promoter, ecmO, and the other where lacZ expression was driven by the proximal part of ecmA promoter, ecmA (TL-6/TL-86 strains; Chae et al., 2002). By 24 h of treatment ecmA-β-gal-expressing cells shifted towards the posterior of the structures while ecmO-β-gal-expressing cells expanded towards the tip and slightly the rear (Fig. 4.6A,B). CotB-β-gal-expressing cells shifted into the prestalk region by 24 h (Fig. 4.6A,B). After treatment with 50 mM colchicine, the tipped mound-like structures also accumulated ecmB-β-gal-expressing cells at the posterior end (Fig. 4.6A). There was a general reduction in the number of ecmA, ecmO and cotB-β-gal-expressing cells (Fig. 4.6A). Thus colchicine induces the posterior accumulation of ecmB expressing cells and changes the spatial expression patterns of ecmA and cotB genes. The posterior accumulation of ecmB-β-gal-expressing cells corresponds to the posterior accumulation of terminally differentiated stalk cells in these structures.

4.4.6 Colchicine enhances random cell motility but not chemotaxis

Colchicine enhanced random cell motility as cells migrated 312.4±67.2% more (10 mM colchicine) and 495.0±144.9% more (50 mM colchicine) than the control (Fig. 4.7A). Vinblastine inhibited random cell motility up to 16.3±3.7% of control, while nocodazole had no significant effect (Fig. 4.7A). Colchicine did not affect cell chemotaxis, while nocodazole and vinblastine had an inhibitory effect (Fig. 4.7B). Colchicine therefore specifically enhanced random cell motility and this effect was not due to microtubule-depolymerization.

4.4.7 Colchicine enhances random cell motility through a Ca\(^{2+}\)-mediated signaling pathway

Motility of D. discoideum cells in early development is mediated in part by two parallel pathways, phosphoinositide-3-kinase (PI3K) and phospholipase A2 (PLA2) (Funamoto et al., 2001; Chen et al., 2007; Swayne et al., 2010). LY294002, an inhibitor of PI3K, and quinacrine,
Figure 4.6. The effect of colchicine on spatial gene expression patterns in the developmental structures of *D. discoideum* after treatment for 24 h. A. Slugs and tipped mound-like structures stained with X-gal for β-gal activity. β-gal synthesis was controlled by the regulatory regions of developmental genes: *ecmA*, *ecmB* and *cotB*. Slug tips are towards the left. Tipped mound-like structure tips are towards the top. B. Quantification of the staining intensity of control (grey squares; not treated with colchicine) and 10 mM colchicine-treated (black circles) slugs (at least 10/replicate) (M+S.D.). The smaller the white intensity value the more intense the blue staining was. This experiment was independently replicated 3 times. Figure 7 from Poloz and O’Day, 2012.

an inhibitor of PLA2, or both inhibited colchicine-enhanced motility by a maximum of 34.3±8.2% (Fig. 4.8A; Vigo et al., 1980; Vlahos et al., 1994). This suggests that colchicine acts through a signaling pathway other than PI3K or PLA2. U-73122, an inhibitor of PLC, inhibited colchicine-enhanced motility by 96.3±3.0% (Fig. 4.8A; Bleasdale et al., 1989). W-7, an antagonist of CaM, inhibited colchicine-enhanced motility by 47.2±13.5% (Fig. 4.8A; Hidaka et al., 1981). Furthermore, treatment of cells with TMB-8, an antagonist of intracellular Ca²⁺, cadmium chloride, a cell membrane Ca²⁺ channel blocker, and EDTA, a chelator of extracellular
Figure 4.7. The effect of colchicine (C), nocodazole (N) and vinblastine (V) on *D. discoideum* cell motility. A. Random cell motility. B. Chemotaxis. To determine the distance the cells migrated, cells were spotted onto agar with or without an added agent and spot diameters were measured at 0 h and 4 h (M+S.D.). A one-way ANOVA with Tukey’s Test was used to analyze significant differences in spot diameters after 4 h. Data points that do not share a letter are significantly different, *p*≤0.05. This experiment was independently replicated 5 times. Figure 8 from Poloz and O’Day, 2012.

Ca^{2+}, inhibited colchicine-enhanced cell motility by 61.5±7.6%, 91.7±8.7% and 94.0±5.4%, respectively (Fig. 4.8A; Malagodi et al., 1974). We then used null mutant strains to further
Figure 4.8. The effect of agents and mutant strains on colchicine (C)-enhanced random cell motility in *D. discoideum*. A. Cell were spotted onto agar with 50 mM colchicine with or without an added inhibitor or other agent and spot diameters were measured at 0 h and 4 h (M+S.D.). B. Mutant strain cells were spotted onto agar with 50 mM colchicine and spot diameters were measured at 0 h and 4 h (M+S.D.). Data points that do not share a letter are significantly different. *p*≤0.05. This experiment was independently replicated 5 times. Figure 9 from Poloz and O’Day, 2012.

characterize this signaling pathway. DH1 was the parental strain for all mutant strains. Colchicine (50 mM) enhanced random cell motility of DH1 by 161.61±6.75% compared to
untreated control (Fig. 4.8B). Colchicine enhanced cell motility to a smaller extent in DH1 cells than AX3 cells. Random cell motility was not enhanced by 50 mM colchicine in PLC (plc) null, G protein β (Gβ; gpbA) null or G protein α2 (Ga2; gpaB) null strains (Fig. 4.8B). These results confirm that colchicine requires PLC to enhance random cell motility and also suggest the involvement of G proteins, specifically G protein G2. These results suggest that colchicine enhances random cell motility through a Ca\(^{2+}\)-mediated signaling pathway, involving CaM, PLC and G protein G2.

**4.4.8 Colchicine does not affect the expression or cytoskeleton-association of actin or myosin**

Treatment of cells with cytochalasin D, an inhibitor of actin polymerization, inhibited colchicine-enhanced cell motility by 85.6±0.3%, demonstrating the importance of actin (Fig. 4.8A). In order to further elucidate how it enhances cell motility we analyzed colchicine’s effect on actin and myosin, 2 components of the intracellular cytoskeletal network of cells. The levels of total cell actin or myosin or cytoskeleton-associated actin or myosin did not change when cells were treated with colchicine (Fig. 4.9A,B,E). The levels of total cell actin or myosin or cytoskeleton-associated actin also did not change when cells were treated with nocodazole but cytoskeleton-associated myosin was reduced to 69.92±9.06% of control (Fig. 4.9C,D,F). Thus colchicine appears to enhance cell motility by altering the regulatory components of the cytoskeleton rather than actin or myosin directly.

**4.4.9 Colchicine’s developmental effect is rescued through alterations of Ca\(^{2+}\) or antagonism of CaM**

The treatment of cells with BAPTA-AM (0.15 mM), a chelator of intracellular Ca\(^{2+}\), or EDTA (0.5 mM), a chelator of extracellular Ca\(^{2+}\), rescued fruiting body formation of cells developed on 10 mM colchicine-soaked filters (Fig. 4.10A). Calcofluor staining revealed the formation of morphologically normal spore, stalk and basal disc cells (Fig. 4.10B-D). The treatment of cells with W-7 (50 μM), a CaM-antagonist, also rescued fruiting body formation with morphologically normal spore, stalk and basal disc cells (Fig. 4.10A-D). Spores were able to germinate when mixed with *E.coli* on SM agar plates after pretreatment with 0.5% NP-40 (data not shown). Interestingly, the treatment of cells with U-73122 (10 μM), an inhibitor of PLC, did not rescue development and slugs still formed terminally differentiated stalk cells at the posterior
Figure 4.9. The effect of colchicine on the expression and cytoskeletal association of actin and myosin in *D. discoideum*. A-D. Western blot of cells treated with colchicine (Col) or nocodazole (Noc) for 4 h. A. Total cell actin (42 kDa). B. Cytoskeleton-associated actin. C. Total cell myosin heavy chain (240 kDa). D. Cytoskeleton-associated myosin heavy chain. E-F. Quantification of western blots in (A-D) (M+S.D.). Black bars represent total cell protein and grey bars represent cytoskeleton-associated protein. A one-way ANOVA with Tukey’s Test was used to analyze significant changes in protein levels. a is different from control (only significant results are shown). p≤0.05. This experiment was independently replicated 3 times. Figure 10 from Poloz and O’Day, 2012.
Developmental effects of agents on (A-D) 10 mM and (E-F) 50 mM colchicine treated cells. Images show developmental structures after 24 h. Morphology of structures formed after treatment with (A) 10 mM colchicine or (E) 50 mM colchicine and an added agent. B-D. Morphology of calcofluor stained spore, stalk and basal disc cells of structures in (A), respectively. No fruiting bodies formed after treatment with 10 mM colchicine and U-73122 therefore no stalk or spore cells were present. F. Morphology of calcofluor stained cells at the posterior end of structures in (E). Arrows point to terminally differentiated stalk cells. This experiment was independently replicated 3 times. Each time at least 5 fruiting bodies or finger-like structures were stained and analyzed. Figure 11 from Poloz and O'Day, 2012.

Colchicine also arrested morphogenesis at the slug stage (10 mM colchicine) or the tipped mound-like stage (50 mM colchicine) and induced stalk cell differentiation at the posterior end of these structures in PLC null mutant strain (Fig. 4.11A-F). These results suggest that colchicine’s developmental effects result from PLC independent elevation of intracellular
Ca\(^{2+}\) levels, and activation of CaM. The attempts to rescue normal fruiting body formation of cells treated with 50 mM colchicine were unsuccessful (Fig. 4.10E,F). The treatment of cells with BAPTA-AM, EDTA or W-7 inhibited terminal stalk cell differentiation at the posterior end of the tipped mound-like structures but morphogenesis was not rescued (Fig. 4.10E,F). These results indicate that 50 mM colchicine likely pushes cell fate determination towards stalk cell differentiation beyond a state that can be rescued.

4.5 Discussion

Research gathered over the last 50 years has shown that colchicine induces major developmental anomalies in many organisms including Hydra, Tetrahymena, Xenopus, Ambystoma, Mycrophyla, Gallus, Drosophila and Dictyostelium (Diwan 1966, 1967; Corff and Burnett, 1969; Nelsen, 1970; O’Day and Durston, 1978; Koch and Spitzer, 1982, 1983; Alberch and Gale, 1983; Drozdovskaya and Rapoport, 1988). Using *D. discoideum* as a model system, here we have shown that colchicine’s effects are not associated with its microtubule disrupting ability. Instead, we have revealed that colchicine arrests morphogenesis, represses spore cell differentiation and induces stalk cell differentiation by affecting Ca\(^{2+}\) signal transduction leading to changes in gene expression and cell motility. This work has also shown that the developmental effects of colchicine can be rescued through chelation of Ca\(^{2+}\) or antagonism of its primary effector, CaM.

It has been suggested, but not directly demonstrated, that colchicine’s developmental effects are the direct result of its interaction with tubulin, which leads to the depolymerization of intracellular microtubular networks and subsequent mitotic arrest (Diwan, 1966, 1967; Corff and Burnett, 1969; Nelsen, 1970; Durston and O’Day, 1978; Alberch and Gale, 1983; Koch and Spitzer, 1982, 1983). Here we showed that in *D. discoideum* colchicine’s developmental effects are independent of microtubule-depolymerization, as other microtubule-depolymerizing agents failed to cause any developmental effects at microtubule-depolymerizing concentrations. Nocodazole was chosen for this study as it was shown to be a ligand for the colchicine-binding site on tubulin while vinblastine was shown to bind to a different site (Zefirova et al., 2007). EthylN-phenylcarbamate (EPC) and thiabendazole are two other microtubule-depolymerizing agents that have been shown to not disrupt *D. discoideum* morphogenesis or induce stalk cell
differentiation (Kitanishi et al., 1984). These results thus suggest the existence of a yet unidentified-colchicine target in cells.

O'Day and Durston (1978), were the first to show that colchicine disrupts morphogenesis, represses spore cell differentiation and induces stalk cells differentiation and multiple axis formation in *D. discoideum*. We confirmed that colchicine arrests morphogenesis, represses spore cell differentiation and induces stalk cell differentiation. We specifically showed that
colchicine alters gene expression and changes the pattern of cells within the slug. It induced \textit{ecmB} gene expression but repressed \textit{ecmA} and prespore and spore-specific gene expression. Analysis of the changes in spatial gene expression patterns revealed that colchicine induces accumulation of \textit{ecmB}-β-gal-expressing cells at the posterior end of slugs and tipped mound-like structures. We also showed that \textit{ecmA}-β-gal-expressing cells shift towards the posterior, \textit{ecmO}-β-gal-expressing cells expand from their original location and \textit{cotB}-β-gal-expressing prespore cells shift into the prestalk zone. The half-life of β-gal is reported to be more than 8 h in \textit{D. discoideum} (Detterbeck et al., 1994). Thus cells with changing gene expression patterns may still show β-gal activity even if the regulatory region driving β-gal synthesis is no longer stimulated.

Transdifferentiation, the conversion of one cell type into another, occurs in \textit{D. discoideum} naturally on a small scale and when there is an imbalance in the proportion of different cell types (Francis and O’Day, 1971; Abe et al., 1994; Detterbeck et al., 1994; Brown and Firtel, 1999). It has been suggested to proceed in the following manner: prespore \(\leftrightarrow\) ALC \(\leftrightarrow\) prestalk O \(\leftrightarrow\) prestalk A \(\rightarrow\) prestalk AB (Raper, 1940; Bonner, 1952; Sakai, 1973; Sternfeld and David, 1982; Sternfeld, 1992; Abe et al., 1994). The transdifferentiation pattern of prestalk B cells is not known. At 16 h of 10 mM colchicine treatment, the staining pattern of \textit{ecmA}-β-gal, \textit{ecmO}-β-gal and \textit{cotB}-β-gal expression was the same as in control, untreated slugs (data not shown). By 24 h, the pattern changed drastically, suggesting that colchicine induces transdifferentiation of cells at this time. Colchicine likely induces the transdifferentiation of prespore cells into \textit{ecmB}-expressing prestalk cells in the prespore zone. Very few \textit{ecmB}-β-gal-expressing cells were found where prestalk AB cells are normally located and no stalk tube formation was observed. This suggests that colchicine likely induces the differentiation of prestalk B cells or ALCs but not prestalk AB cells. The accumulation of terminally differentiated stalk cells at the posterior end of 10 mM colchicine-treated slugs suggests the premature differentiation of these \textit{ecmB}-expressing cells, that differentiate into the basal disc and upper and lower cup cells of fruiting bodies.

We showed that colchicine and DIF-1 likely act through different signal transduction pathways. Williams et al. (1987), showed that DIF-1 induces \textit{ecmA} and \textit{ecmB} gene expression while colchicine induced \textit{ecmB} but repressed \textit{ecmA} gene expression, again suggesting two different signal transduction pathways are involved. Colchicine also failed to induce nuclear translocation of DimB, a DIF-1 responsive transcription factor that was shown to be essential for normal
responses to DIF-1 (Huang et al., 2005; Zhukovskaya et al., 2005). Prestalk cell types that are not regulated by DIF-1 are known to exist since a mutant strain that cannot produce DIF-1, stlb null, has been shown to only have a 30% reduction in the number of prestalk cells, mainly missing most of the outer basal disc and some lower cup cells but showing normal inner basal disc, stalk and upper cup differentiation (Saito et al., 2008). Several in-situ hybridization studies with probes of numerous developmental genes have also demonstrated that there is heterogeneity in the currently established prestalk cell types and recently a new prestalk cell type, prestalk U, has been identified that differentiates normally in the DIF-1 mutant strain (Maeda et al., 2003; Yamada et al., 2005; Yamada et al., 2010). Thus, colchicine likely induces the differentiation of ecmB-expressing prestalk B cells or ALCs through a signaling pathway that is not utilized by DIF-1. Alternatively, colchicine may utilize a specific branch of the DIF-1 signaling pathway that does not involve the transcription factor DimB. This remains to be further analyzed.

Present work also demonstrated that colchicine enhances random cell motility three- (10 mM colchicine) to five-fold (50 mM colchicine), a microtubule-independent effect, but does not significantly affect the rate of chemotaxis. Colchicine has previously been shown to enhance cell motility in human neutrophils, mouse macrophages and Walker carcinosarcoma cells (Sundharadas and Cheung, 1977; Keller et al., 1984; Keller and Zimmermann, 1986; Niggli, 2003). The signal transduction pathways that regulate random cell motility in D. discoideum are not well understood. It has been shown that PI3K, PLA2, PTEN, Ras and phospholipase D (PLD), but not PLC or G proteins, are important regulators of random cell motility (Zouwail et al., 2005; Lim et al., 2005; Sasaki et al., 2007; Bosgraaf and Van Haastert, 2009; Arai et al., 2010). For example, Ly294002, an inhibitor of PI3K, was shown to inhibit D. discoideum KAX3 random cell motility by more than 85% while null mutant PLC cells migrated at a rate similar to control (Sasaki et al., 2007; Arai et al., 2010). In contrast, we showed that colchicine enhances random cell motility mainly through a PI3K-independent pathway, involving Ca\(^{2+}\), CaM, PLC and G proteins. Colchicine has also been shown to enhance cell motility of human neutrophils through a largely unknown PI3K-independent signaling pathway (Niggli, 2003). Epidermal growth factor-like peptides also enhance random cell motility in D. discoideum through a PI3K-independent signaling pathway, involving Ca\(^{2+}\) and CaM but not PLC (Huber and O’Day, 2009, 2011). The importance of Ca\(^{2+}\) in the movement of amoeboid cells including D. discoideum and
human leukocytes is well documented (Sawyer et al., 1985; Brundage et al., 1991; Newell et al.,
1995).

Intracellular Ca$^{2+}$ transients have been observed in chemotaxing and randomly moving D.
discoideum cells and Ca$^{2+}$ has been shown to be important for efficient chemotaxis and random
cell motility, through a largely unknown mechanism (Abet et al., 1988; Schlatterer et al., 1994;
Newell et al., 1995; Malchow et al., 1996; Yumura et al., 1996; Nebl and Fisher, 1997; Lombardi
et al., 2008; Lusche et al., 2009). CaM, a primary Ca$^{2+}$ sensor in all eukaryotic cells, has also
been shown to be important in cAMP- and folic acid-mediated chemotaxis (Gauthier and O’Day,
2001). Colchicine likely elevates intracellular calcium levels through the influx from
extracellular space and release from the endoplasmic reticulum. Colchicine has previously been
shown to elevate intracellular Ca$^{2+}$ levels in cells, including rat cardiac myocytes and rat cortical
neurons (Kerfant et al., 2001; Zhai et al., 2011). G protein $\beta\gamma$ and $\alpha_2$ (subunits of G protein G2)
are also involved in colchicine’s effect on cell motility since colchicine did not induce random
motility in G protein $\beta$ and $\alpha_2$ null strains. G proteins have been shown to activate PLC in many
different cell types and G protein G2 activates PLC in D. discoideum (Okaichi et al., 1992;
Bominaar et al., 1994). PLC has also been shown to bind to and be activated by Ca$^{2+}$ in D.
discoideum (Drayer et al., 1995). In other cell types, PLC and G protein $\beta\gamma$ subunits are also
regulated by Ca$^{2+}$-bound CaM (Liu et al., 1997; McCullar et al., 2003). Further research is
needed to elucidate the interplay between Ca$^{2+}$, CaM, PLC and G proteins in colchicine-treated
cells.

Additionally, dynamic actin reorganization was essential for colchicine’s effect on motility but
colchicine did not induce actin or myosin production or increase the amount of these proteins in
the cytoskeleton. This result is unique since Ca$^{2+}$ signaling has previously been shown to control
the expression and cytoskeletal association of actin and myosin (Newell et al., 1995; Catalano
and O’Day, 2008; Huber and O’Day, 2011). Overall, colchicine may be enhancing random cell
motility through a novel Ca$^{2+}$-mediated signaling pathway, involving CaM, PLC, and G proteins.
Since differentiation and morphogenesis are largely based on differential cell motility colchicine
may be disrupting the morphogenetic cell movements at culmination by enhancing random cell
motility of cells and altering the specific cell movement patterns. Additionally, colchicine may
be repressing spore cell differentiation and inducing stalk cell differentiation, at least in part, by
enhancing random cell motility.
Finally, we rescued 10 mM colchicine’s developmental effects by lowering intracellular Ca\(^{2+}\) levels (BAPTA-AM) or extracellular Ca\(^{2+}\) levels (with EDTA) or by antagonizing CaM (with W-7). Interestingly, PLC is involved in colchicine-enhanced cell motility but not development, suggesting the two effects are in part separate. G protein G2 mutants were unable to aggregate and therefore were not examined further. These results suggest that colchicine disrupts morphogenesis, represses spore cell differentiation and induces stalk cell differentiation through the elevation of intracellular Ca\(^{2+}\) levels and activation of CaM.

In conclusion, colchicine disrupts morphogenesis, represses spore cell differentiation and induces differentiation of ecmB-expressing stalk cells through a Ca\(^{2+}\) signal transduction pathway involving changes in gene expression and cell motility. Further elucidation of colchicine’s mode of action will provide new insights into the developmental program of D. discoideum and higher eukaryotes.

4.6 Acknowledgements

This work was supported in part by a grant from the Natural Sciences and Engineering Research Council of Canada (DHOD, A6807). A special thank you to Dr. Jeff Williams providing us with the DimB-GFP strain. Thank you also to the Dictyostelium Stock Center and everyone who deposited the strains.

4.7 References


Differentiation, 20, 10-21.


Chapter 5

Ca\textsuperscript{2+} signaling regulates ecmB expression patterns, cell differentiation and slug regeneration in Dictyostelium

This chapter was submitted for publication as is:


I carried out all experiments and data analysis for this chapter. I made all Figures and wrote the chapter. Danton H. O’Day offered assistance in experimental design and edited the manuscript.

5.1 Abstract

Ca\textsuperscript{2+} regulates cell differentiation and morphogenesis in a diversity of organisms and dysregulation of Ca\textsuperscript{2+} signal transduction pathways leads to many cellular pathologies. In Dictyostelium Ca\textsuperscript{2+} induces ecmB expression and stalk cell differentiation. Here we have analyzed the pattern of ecmB expression in intact and bisected slugs and the effect of agents that affect Ca\textsuperscript{2+} levels or antagonize CaM on this expression pattern. We have shown that Ca\textsuperscript{2+} and CaM regulate ecmB expression and pstAB/pstB cell differentiation in vivo. Agents that increase intracellular Ca\textsuperscript{2+} levels increased ecmB expression and/or pstAB/pstB cell differentiation, while agents that decrease intracellular Ca\textsuperscript{2+} or antagonize CaM decreased it. In isolated slug tips agents that affect Ca\textsuperscript{2+} levels and antagonize CaM had differential effect on ecmB expression and cell differentiation in the anterior versus posterior zones. Agents that increase intracellular Ca\textsuperscript{2+} levels increased the number of ecmB expressing cells in the anterior region of slugs, while agents that decrease intracellular Ca\textsuperscript{2+} levels or antagonize CaM activity increased the number of ecmB expressing cells in the posterior. We have also demonstrated that agents that affect Ca\textsuperscript{2+} levels or antagonize CaM affect cells motility and regeneration of shape in isolated slug tips and backs and regeneration of tips in isolated slug backs. To our knowledge, this is the first study detailing the pattern of ecmB expression in regenerating slugs as well as the role of Ca\textsuperscript{2+} and CaM in the regeneration process and ecmB expression.
5.2 Introduction

Ca$^{2+}$ is a ubiquitous cation and a universal signaling molecule found in the intracellular and extracellular environment of virtually all eukaryotic cells (Petersen et al., 2005). It plays crucial roles in cellular processes including cell growth, cell division, cell death, cell motility, biomembrane fusion, fertilization, differentiation and morphogenesis. For example, Ca$^{2+}$ is involved in the differentiation of neurons, cardiomyocytes, osteoblasts, osteoclasts and keratinocytes (Spitzer et al., 2000; Tu et al., 2004; Fu et al., 2006; Zayzafoon, 2006; Zhou et al., 2011). Ca$^{2+}$ is also involved in dorsal-ventral patterning and morphogenetic cell movements during gastrulation of *Xenopus* and *Danio* embryos and left-right axis establishments in *Xenopus*, *Gallus* and *Mus* embryos (Slusarski and Pelegri, 2007). Dysregulation of fine tuned Ca$^{2+}$ homeostasis in cells has been linked to many pathologies including cancer, cardiovascular disease, bone disease and many neurodegenerative diseases including Alzheimer’s (Parkash and Asotra, 2010; Blair et al., 2011; Cartwright et al., 2011; Giacomello et al., 2011).

*Dictyostelium discoideum* is a well established model for the study of signal transduction pathways associated with cell differentiation and morphogenesis (Kimmel and Firtel, 2004; Williams, 2006). Differentiation of stalk cells is coordinated with a slow sustained increase in intracellular Ca$^{2+}$ levels (Schaap et al., 1996). *EcmB* has been used as a marker for stalk cell differentiation (Williams, et al., 1987; Jermyn and Williams, 1991). Differentiation inducing factor-1 (DIF-1), a stalk cell morphogen, induces *ecmB* expression and stalk cell differentiation *in vitro* through the elevation of intracellular Ca$^{2+}$ levels (Kubohara and Okamoto, 1994; Schaap et al., 1996; Kay et al., 1999; Thompson and Kay, 2000; Kay and Thompson, 2001; Saito et al., 2008; Kubohara et al., 2007). An increase in intracellular Ca$^{2+}$ levels resulting from treatments with pharmacological agents like BHQ also induces *ecmB* expression and stalk cell differentiation *in vitro* (Kubohara and Okamoto, 1994; Schaap et al., 1996; Kubohara et al., 2007).

Ca$^{2+}$ levels are significantly higher in prestalk than prespore cells *in vivo* (Abe and Maeda, 1989; Saran et al., 1994; Cubitt et al., 1995). Baskar et al., 2000, have shown that elevation of intracellular Ca$^{2+}$ levels *in vivo* results in slugs with larger prestalk zones and fruiting bodies with larger basal discs and smaller spore masses. Expression of a constitutively active Ca$^{2+}$ pump that decreased intracellular Ca$^{2+}$ levels affected cell aggregation, sorting and pattern formation
(Cubitt et al., 1998). Ca\(^{2+}\) is also important in cell motility and cell adhesion (Wong et al., 2002; Lusche et al., 2009). Thus, Ca\(^{2+}\) plays a major role in stalk cell differentiation and morphogenesis in \textit{D. discoideum}. The downstream components of Ca\(^{2+}\) signal transduction pathways are largely unknown but a few Ca\(^{2+}\) and CaM binding proteins have been identified and showed to have roles in differentiation and morphogenesis in \textit{D. discoideum} (Catalano and O’Day, 2008; O’Day, 2003; Smith et al., 2010). CaM is a primary Ca\(^{2+}\) sensor in all eukaryotic cells that relays Ca\(^{2+}\) signals to an array of downstream effectors (Catalano and O’Day, 2008). Treatment of cells with calmidazolium, a CaM antagonist, has been shown to inhibit BHQ induced \textit{ecmB} expression \textit{in vitro} (Verkerke-van Wijk, 1998). Thus, CaM is likely a major Ca\(^{2+}\) effector during stalk cell differentiation.

DIF-1 has specifically been shown to affect differentiation of \textit{ecmB} expressing pstB cells \textit{in vivo} (Keller and Thompson, 2008; Saito et al., 2008; Yamada et al., 2011). Though DIF-1 acts through a Ca\(^{2+}\) signaling pathway the role of Ca\(^{2+}\) in \textit{ecmB} expression and pstB cell differentiation \textit{in vivo} has not been examined to date. Our first goal was to analyze the effect of agents that affect Ca\(^{2+}\) levels and antagonize CaM on \textit{ecmB} expression patterns in intact and regenerating slugs. Our second goal was to analyze the effect of these agents on the re-establishment of slug shape and motility in regenerating slugs.

To affect Ca\(^{2+}\) levels and CaM we selected specific agents that increase or decrease intracellular Ca\(^{2+}\) levels or antagonize CaM. Bafilomycin A\textsubscript{1} and BHQ have been shown to increase intracellular Ca\(^{2+}\) levels in mammalian cells by inhibiting vacuolar-type H\(^+\) -ATPase and sarco-endoplasmic reticulum Ca\(^{2+}\)-ATPase, respectively (Moore et al., 1987; Bowman et al., 1988). Bafilomycin A\textsubscript{1} and BHQ also increase intracellular Ca\(^{2+}\) levels in \textit{D. discoideum} cells (Rooney and Gross, 1992; Rooney et al., 1994; Schaap et al., 1996). For example, 100 µM BHQ has been shown to increase cytoplasmic Ca\(^{2+}\) concentration in \textit{D. discoideum} cells by more than 2.5 fold (Schaap et al., 1996). CaCl\textsubscript{2} together with the Ca\(^{2+}\) ionophore A23187 also increase intracellular and likely extracellular Ca\(^{2+}\) levels in \textit{D. discoideum} cells (Reed and Lardy, 1972; Lydan and O’Day, 1988a; Baskar et al., 2000). Baskar et al., 2000, have shown that cytoplasmic Ca\(^{2+}\) levels in cells of untreated slugs are around 100 nM and 160 nM in cells of slugs treated with 100 µM CaCl\textsubscript{2} and 7 µM A23187. Verapamil is a Ca\(^{2+}\) channel blocker that has been shown to inhibit Ca\(^{2+}\) influx and stimulate its efflux in several cell types (Opie, 1987; Warburton et al., 1989). Verapamil has been successfully used in \textit{D. discoideum} before (Schapp et al., 1986; Korohoda et
al., 2002). For example, the treatment of *D. discoideum* cells with verapamil inhibited chemotactic response in Ca$^{2+}$ gradients (Korohoda et al., 2002). EGTA and BAPTA-AM are Ca$^{2+}$ chelating agents that are used to lower extracellular and intracellular Ca$^{2+}$ levels, respectively, in *D. discoideum* and mammalian cells (Dieter et al., 1993; Baskar et al., 2000). EGTA together with A23187 have been shown to decrease intracellular Ca$^{2+}$ levels in *D. discoideum* cells by more than 2 fold (Baskar et al., 2000). W-7 is a specific antagonist of CaM that antagonizes CaM binding to its effectors in *D. discoideum* (Hidaka et al., 1981; Lydan and O’Day, 1988b; Winckler et al., 1991; Myre and O’Day, 2004).

To visualize cells that express *ecmB*, *ecmB-ubi*-ile-a-galactosidase expressing cells were histochemically stained for β-galactosidase activity. This particular reporter was chosen as its half-life is about 30 min and it does not accumulate in cells over time (Bachmair et al., 1986; Detterbeck et al., 1994). Thus, changing gene expression patterns in transdifferentiating cells can be visualized relatively accurately. We treated intact and bisected slugs with the agents described above and monitored changes in *ecmB* expression, slug shape and cell/slug motility. We hypothesized that Ca$^{2+}$ and CaM regulate *ecmB* expression and cell differentiation in intact and regenerating slugs.

### 5.3 Materials and Methods

#### 5.3.1 Chemicals, strains and culture conditions

*Dictyostelium* AX3 cells were acquired from the *Dictyostelium* stock center (www.Dictybase.org). Cells were maintained in HL-5 medium at 21 °C shaking at 180 rpm as previously described (Fey et al., 2007). For development on filters, *ecmB-ubi*-ile-a-galactosidase (DNA plasmid was obtained from www.Dictybase.org; deposited by H. MacWilliams) expressing cells were grown on SM agar in association with *E. coli* B/r at 21 °C in the dark. Unless specified otherwise, all generic chemicals were purchased from BioShop Canada (Burlington, ON, Canada) or Sigma-Aldrich (St. Louis, MO, USA).

#### 5.3.2 Transformation of *D. discoideum*

Cells were transformed as previously described (Suarez et al, 2011). In brief, 5 x 10$^6$ cells were washed with H-50 buffer (20 mM HEPES, 50 mM KCl, 1 mM MgSO$_4$, 5 mM NaHCO$_3$, 10 mM NaCl, 1 mM NaH$_2$PO$_4$, pH 7.0) and electroporated with 5 µg of plasmid DNA in 1 mm cuvettes,
pulsed at 0.85 kV, 25 μF, 1.0 ms, 2 pulses 5 s apart. Cells were allowed to recover in HL-5 for 24h. Successfully transformed cells were selected with 10 μg/mL G418 for about 10 days.

5.3.3  Synchronized development and slug manipulation

Cells (2 x 10⁷) were washed free of *E. coli* and developed on white membrane filters as previously described (see Chapter 4; Fey et al., 2007). Before deposition onto the filters cells were stained with 0.1 % neutral red. At 16 hours, cells developed to the slug stage. Slugs were transferred onto filter pads soaked in ddH₂O +/- 20 μM bafilomycin A1, 100 μM 2,5-Di-tert-butylhydroquinone (BHQ; Enzo Life Sciences, Plymouth Meeting, PA, USA), 100 μM CaCl₂ + 7 μM A23187 (Enzo Life Sciences), 100 μM verapamil (Enzo Life Sciences), 1 mM EGTA + 7 μM A23187, 150 μM BAPTA-AM (Enzo Life Sciences) or 100 μM W-7. Alternatively, slugs were cut at the prestalk-prespore border (estimated by neutral red staining) using a syringe needle before transfer onto filter pads soaked with one of the above agents. For the analysis of slug regeneration from bisected tips, the prespore zones were cut off and removed; vice versa was done for the analysis of slug regeneration from bisected backs.

5.3.4  Histochemical staining for β-galactosidase activity

Slug staining was carried out according to a previously described method (Dingermann et al., 1989). In brief, slugs were fixed and permeabilized with 1 % glutaraldehyde and 0.1 % NP-40, respectively, in Z buffer for 15 min. Slugs were washed with Z buffer and stained with 0.4 mg/mL X-gal, 2.5 mM K₄Fe(CN)₆ and 2.5 mM K₃Fe(CN)₆ in Z buffer. Staining was allowed to proceed for a specific period of time that was kept constant for all structures at a specific point of regeneration. Staining was stopped by washing off the staining solution with Z buffer.

5.3.5  Microscopy and image analysis

Slugs and regenerating structures were viewed and photographed using the Nikon Eclipse 50i microscope with the mounted Nikon Digital Sight DS-Ri1 camera (Nikon, Melville, NY, USA). To quantify the spatial staining patterns the slugs were scanned using the profile tool in NIS Elements 3.2Br software (Nikon). The same program was used to count the number of stained cells, measure the migration distances and measure slug shapes. All measurements were analyzed using Microsoft Office Excel.
5.3.6 Statistical analysis

A one-way ANOVA with Tukey’s Test in Minitab16 (Minitab, State College, PA, USA) was used to analyze significant differences in slug shapes, cell counts and migration distances.

5.4 Results

5.4.1 Spatial and temporal ecmB expression patterns in bisected slugs

Transdifferentiation and sorting events have previously been shown to be involved in the regeneration of isolated slug tips and backs into slugs that form properly proportioned fruiting bodies (Raper, 1940). The pattern of ecmB expression in regenerating slug tips and backs has not been analyzed before.

Neutral red stains autophagic vacuoles of prestalk cells (Bonner, 1951). Slugs were cut at the prestalk-prespore border based on the pattern of neutral red staining (Fig. 5.1A,B). Both regions were allowed to regenerate into slugs and form fruiting bodies over the course of 24 h. In uncut slugs, ecmB expressing cells are located in the core of the tip, at the prestalk-prespore border and dispersed throughout the prespore zone (Fig. 5.2A). Cells in the core of the tip are pstAB cells (Jermyn et al., 1989; Ceccarelli et al., 1991). Cells at the prestalk-prespore border and dispersed throughout the prespore zone are pstB cells (Dormann et al., 1996; Jermyn et al., 1996).

Isolated slug backs stopped forward movement immediately after the cut (Fig. 5.2B; Raper, 1940). Within the first hour, cells gathered towards the cut site and formed a mound. PstB cells accumulated at the cut sites after 30 min, before the rest of the cells reached this location. By 1.5 hours the mounds formed single tips. In the tipped mounds ecmB expressing cells were mainly located laterally, at the side of the cut. Very few ecmB expressing cells were found in tips. By 2 h the mounds extended into fingers. In the fingers, ecmB expressing cells were located in a band just posterior to the tip and laterally, at the side of the cut. By 4 h standing slugs were formed. In these slugs ecmB expressing cells were dispersed throughout the posterior but were absent from the tip. Many ecmB expressing cells were specifically located just posterior to the tip but most were located at the base of standing slugs. By 6 h the apparently normal spatial pattern of ecmB expression was restored in now migrating slugs. Most ecmB expressing cells that were present at the base of standing slugs were left behind at the cut site. By 8 h slugs initiated culmination and fruiting bodies were observed by 10 h.
The isolated tips did not form into mounds like the backs but rather persisted with their forward movement away from the cut site (Fig. 5.2C). They elongated over the course of 8 h resembling small slugs. *EcmB* expression was strongly reduced in the anterior core of these slugs by 30 minutes. Some *ecmB* expressing cells were scattered throughout the posterior of the slugs and some were left behind in the slime trail. This pattern persisted for at least 10 h of regeneration. By 24 h fruiting bodies had formed.

### 5.4.2 Agents that modulate Ca^{2+} levels or antagonize CaM affect regeneration of *ecmB* expression in isolated slug backs

Isolated slug backs were transferred onto filter pads soaked with various agents and allowed to regenerate for up to 24 h. Backs were histochemically stained at various time points as described in the previous section. Since the backs regenerated into slugs by 6 h, the effect of agents that affect Ca^{2+} levels and antagonize CaM was examined at this time point (Fig. 5.3). Staining pattern and the timing of events 0-6 h of regeneration were not affected by any of the agents. Regeneration of *ecmB* expression in isolated slug backs was analyzed by quantifying staining...
Figure 5.2. Pattern of *ecmB* expression in regenerating slug tips and backs. A. A representative uncut slug stained for *ecmB*-α-gal activity. B. Pattern regeneration and *ecmB* (staining for *ecmB*-α-gal activity) expression in bisected slug backs over the course of 24 h. C. Pattern regeneration and *ecmB* (staining for *ecmB*-α-gal activity) expression in bisected slug tips over the course of 24 h. All images show representative structures at the specific time points. At least 15 structures were analyzed at each time point in 3 independent replicates. Scale bars represent 100 µm.
Figure 5.3. Pattern of *ecmB* expression in bisected slug backs after 6 h of regeneration. Bisected slug backs were transferred onto filters soaked in ddH$_2$O +/− 20 μm bafilomycin A, 100 μm BHQ, 100 μm CaCl$_2$ + 10 μm A23187, 100 μm verapamil, 1 mM EGTA + 10 μm A23187, 150 μm BAPTA-AM or 100 μm W-7. Slugs were stained for *ecmB* driven α-gal activity. Representative slugs are shown. Scale bars represent 100 μm.
Figure 5.4. The effect of Ca\(^{2+}\) and CaM signal modulation on ecmB expression in bisected slug backs after 6 h of regeneration. Bisected slug backs were transferred onto filters soaked in ddH\(_2\)O +/- 20 μm bafilomycin A (black bars), 100 μm BHQ (grey bars), 100 μm CaCl\(_2\) + 10 μm A23187 (white bars), 100 μm verapamil (red bars), 1 mM EGTA + 10 μm A23187 (green bars), 150 μm BAPTA-AM (orange bars) or 100 μm W-7 (purple bars). The slugs were stained for ecmB driven α-galactosidase activity. The values are expressed as the percentage of staining measured in slugs transferred onto ddH\(_2\)O alone (M+S.D.). The larger the white intensity value the less staining was observed. All slugs were standardized to the length of 1. At least 15 slugs, from 3 independent replicates were analyzed for each treatment. A one-way ANOVA with Tukey’s Test was used to identify significant changes in staining intensity at each position relative to the untreated control at that position. a, b, c are different than the control (only significant changes are shown). p≤0.05.

<table>
<thead>
<tr>
<th>Position along the slug’s length</th>
<th>White intensity (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>150</td>
</tr>
<tr>
<td>0.05</td>
<td>145.20±27%</td>
</tr>
<tr>
<td>0.1</td>
<td>159.81±9.8%</td>
</tr>
<tr>
<td>0.2</td>
<td>158.70±12%</td>
</tr>
<tr>
<td>0.3</td>
<td>157.60±15%</td>
</tr>
<tr>
<td>0.4</td>
<td>156.50±18%</td>
</tr>
<tr>
<td>0.5</td>
<td>155.40±21%</td>
</tr>
<tr>
<td>0.6</td>
<td>154.30±24%</td>
</tr>
<tr>
<td>0.7</td>
<td>153.20±27%</td>
</tr>
<tr>
<td>0.8</td>
<td>152.10±30%</td>
</tr>
<tr>
<td>0.9</td>
<td>151.00±33%</td>
</tr>
<tr>
<td>1</td>
<td>150.00±36%</td>
</tr>
</tbody>
</table>

Intensity along the length of regenerated slugs (Fig. 5.4). All slugs were standardized to the length of 1 (Fig. 5.1B). The anterior end of the slug is position 0 and the posterior end of the slug is position 1. Treatment with BHQ or CaCl\(_2\) + A23187 increased the staining intensity at position 0.05 by 59.81±9.8% and 45.20±27%, respectively. This position corresponds to the location of pstAB cells in slugs. Treatment with bafilomycin A1, verapamil, EGTA + A23187 or BAPTA-AM did not significantly affect ecmB expression at position 0.05. Treatment with W-7 decreased the staining intensity at this position by 78.34±34%. In general, agents that increase intracellular Ca\(^{2+}\) levels increased staining intensity in the anterior core. Agents that decrease intracellular
Ca\textsuperscript{2+} levels did not have an effect but antagonism of CaM led to a decrease in the staining intensity. Position 0.2 corresponds to the approximate border between prestalk and prespore zones in slugs. PstB cells accumulate here during slug migration and prior to culmination (Dormann et al., 1996; Jermyn et al., 1996). Treatment with bafilomycin A1 and BHQ increased staining intensity at this position by 24.73±11.1% and 39.80±15.5%, respectively. Treatment with CaCl\textsubscript{2} + A23187, verapamil, EGTA + A23187 or BAPTA-AM did not significantly affect the staining intensity at this position. Treatment with W-7 decreased the staining intensity at this position by 49.35±4.2%. Thus, agents that increase intracellular Ca\textsuperscript{2+} levels increased the staining intensity while antagonism of CaM led to a decrease in the staining intensity.

Positions 0.3 to 1 correspond to the prespore zone in slugs. The rest of pstB cells are dispersed here and some accumulate at the very posterior end, sometimes referred to as the rearguard zone. Treatment with bafilomycin A1, BHQ or CaCl\textsubscript{2} + A23187 enhanced staining intensity in the prespore zone by a maximum of 29.15±2.3%, 64.41±8.4% and 36.06±3.4% at position 1, respectively. Treatment with verapamil decreased the staining intensity in the prespore zone by a maximum of 40.31±8.2% at position 0.5. Treatment with EGTA + A23187 or BAPTA-AM decreased the staining intensity to a maximum of 43.28±17.0% and 70.78±34.2% at position 0.7, respectively. Lastly, treatment with W-7 decreased the staining intensity in the prespore zone by a maximum of 65.40±42.0% at position 0.3. In general, agents that increase intracellular Ca\textsuperscript{2+} levels increased staining intensity at the prestalk-prespore border and in the prespore zone while agents that decrease intracellular Ca\textsuperscript{2+} levels or antagonize CaM decreased the staining intensity.

5.4.3 Agents that modulate Ca\textsuperscript{2+} levels or antagonize CaM activity affect regeneration of \textit{ecmB} expression in isolated slug tips

\textit{EcmB} expression in isolated slug tips was analyzed at 8 h of regeneration (Fig. 5.5A). Instead of quantifying the staining intensity along the length of each slug the number of stained cells was counted in the anterior 20% and the posterior 80%. Treatment with bafilomycin A1, BHQ and CaCl\textsubscript{2} + A23187 increased the number of stained cells in the anterior 20% of regenerated slugs (Fig. 5.5B). On average, more than 50% of untreated (control) slugs had no staining cells in the anterior 20%. Treatment with bafilomycin, BHQ or CaCl\textsubscript{2} + A23187 resulted in slugs with an average of more than 3, 5 and 1 stained cell(s) in the anterior, respectively. Treatment with
verapamil, EGTA + A23187, BAPTA-AM or W-7 did not have an effect on the number of stained cells in the anterior. Thus, Ca\(^{2+}\) appears to regulate ecmB expression and possibly pstAB cell differentiation in regenerating slug tips.

The opposite was observed in the posterior 80% of regenerating slugs. Treatment with bafilomycin, BHQ or CaCl\(_2\) + A23187 did not affect the number of stained cells in the posterior (Fig. 5.5C). On the other hand, treatment with verapamil, EGTA + A23187, BAPTA-AM or W-7 resulted in slugs with an average of more than 9, 14, 16 and 17 stained cells in the posterior, respectively. Thus, Ca\(^{2+}\) and CaM may negatively regulate regeneration of ecmB expression and likely differentiation of pstB cells in regenerating slug tips. Overall, ecmB expression as well as pstAB and pstB cell differentiation appear to be differentially regulated by Ca\(^{2+}\) and CaM.

5.4.4 Agents that modulate Ca\(^{2+}\) levels or antagonize CaM affect ecmB expression in uncut slugs

We also analyzed the effect of the above agents on ecmB expression in uncut slugs after 4 h of treatment (Fig. 5.6). The staining intensity was measured the same away as for the isolated slug backs. Treatment with bafilomycin A1 or BHQ increased the staining intensity of slugs at position 0.05 by 52.23±9.2 % and 70.10±5.8%, respectively. Treatment with CaCl\(_2\) + A23187, verapamil, or BAPTA-AM did not significantly affect the staining intensity at this position. Treatment of slug with EGTA + A23187 or W-7 decreased the staining intensity at position 0.05 by 65.61±16.0% and 63.97±11.7%, respectively. Thus, these agents have a similar effect at this position in uncut and cut slugs.

Treatment with bafilomycin A1, BHQ or CaCl\(_2\) + A23187 all increased the staining intensity at position 0.2. Treatment with verapamil, EGTA + A23187, BAPTA-AM or W-7 did not significantly affect the staining intensity at this position. In the prespore zone, treatment of slugs with bafilomycin A1 or BHQ enhanced staining intensity by a maximum of 44.52±3.9% and 55.13±1.36% at position 1, respectively. This position corresponds to a group of prestalk cells that accumulate at the back of the slug. Addition of CaCl\(_2\) + A23187 did not have a significant effect on staining intensity at positions 0.3 to 1. Treatment with verapamil, EGTA + A23187, BAPTA-AM or W-7 decreased the staining intensity in the prespore zone by a maximum of 40.84±24.3%, 60.23±17.9%, 46.00±10.2% and 38.00±6.9% at position 0.4, respectively. Thus, Ca\(^{2+}\) and CaM appear to regulate spatial ecmB expression patterns in uncut slugs.
Figure 5.5. Pattern of ecmB expression in bisected slug tips after 8 h of regeneration. A. Bisected slug tips were transferred onto filters soaked in ddH₂O +/- 20 μm bafilomycin A, 100 μm BHQ, 100 μm CaCl₂ + 10 μm A23187, 100 μm verapamil, 1 mM EGTA + 10 μm A23187, 150 μm BAPTA-AM or 100 μm W-7. Slugs were stained for ecmB-α-gal activity. Representative slugs are shown. Scale bars represent 100 μm. B. The number of stained cells in the anterior 20% of regenerated slugs (M+S.D.). C. The number of stained cells in the posterior 80% of regenerated slugs (M+S.D.). At least 15 slugs, from 3 independent replicates were analyzed for each treatment. One-way ANOVA with Tukey’s Test was used to identify significant changes in the number of stained cells. Data points that do not share a letter are different. p≤0.05.
Figure 5.6. The effect of Ca$^{2+}$ and CaM signal modulation on ecmB expression in intact slugs after 4 h of treatment. Slugs were transferred onto filters soaked in ddH$_2$O +/- 20 μm bafilomycin A (black bars), 100 μm BHQ (grey bars), 100 μm CaCl$_2$ + 10 μm A23187 (white bars), 100 μm verapamil (red bars), 1 mM EGTA + 10 μm A23187 (green bars), 150 μm BAPTA-AM (orange bars) or 100 μm W-7 (purple bars). The slugs were stained for ecmB-α-gal activity. The values are expressed as the percentage of staining measured in slugs transferred onto ddH$_2$O alone (M+S.D.). The larger the white intensity value the less staining was observed. All slugs were standardized to the length of 1. At least 15 slugs, from 3 independent replicates were analyzed for each treatment. A one-way ANOVA with Tukey’s Test was used to identify significant changes in staining intensity at each position relative to the untreated control at that position. a and b are both different than the control (only significant changes are shown). p≤0.05.

5.4.5 Agents that modulate Ca$^{2+}$ levels or antagonize CaM inhibit cell movement in isolated slug backs

As mentioned previously, treatment with different agents did not affect the timing of events or the pattern of ecmB expression during the first 6 h of regeneration of slug backs. After all treatments, tipped mounds formed by 1.5 h and extended into fingers by 2 h. Standing slugs formed by 4 h and migratory slugs were present by 6 h. The agents did affect the rate of cell movement during mound formation. To indirectly quantify the rate of cell movement the ratios of back length to width were measured at 1 h of regeneration (Fig. 5.7A,B). Treatment with all
agents increased the ratio of back length to width by an average of 1.5 times suggesting that agents that affect Ca\(^{2+}\) levels and CaM negatively impact cell motility at this point. Treatment with verapamil, EGTA + A23187 or BAPTA-AM was more inhibitory than treatment with the other agents. This suggests that Ca\(^{2+}\) levels and CaM activity are tightly regulated for proper cell movement at this time. After all treatments, ecmB expressing cells were gathered at the cut site by 30 min while unstained cells lagged behind even at 1 h. Judging by the localization of ecmB expressing cells in this study and neutral red stained cells in previous studies, the unstained cells at the posterior end are prespore cells (Sternfeld and David, 1982). The increase in the ratio of back length to width caused by different agents was likely due to inhibition of prespore cell migration.

5.4.6 **Agents that decrease Ca\(^{2+}\) levels or antagonize CaM activity inhibit prestalk zone regeneration in isolated slug backs**

To further elucidate the role of Ca\(^{2+}\) and CaM in differentiation and morphogenesis we analyzed the effect of agents that modulate Ca\(^{2+}\) levels or antagonize CaM on the regeneration of prestalk zones in isolated slug backs (Fig. 5.8). Lengths of prespore zones were measured based on the lengths of unstained areas at the front of slugs. This provided the estimate for the lengths of prestalk zones. Since isolated slug backs regenerated into slugs by 6 h this analysis was done at this time point. In uncut slugs the prestalk zone occupied 13.82±0.5% of slug length. In isolated slug backs the prestalk zone occupied 12.05±2.5% of slug length by 6 h of regeneration. These values were not statistically different. Treatment with bafilomycin A1, BHQ or CaCl\(_2\) + A23187 did not significantly affect tip regeneration by 6 h. In contrast, treatment with verapamil, EGTA + A23187, BAPTA-AM or W-7 negatively affected tip regeneration. After 6h of regeneration in the presence of one of the above agents, the prestalk zone occupied 11.66±0.4%, 9.39±2.6%, 8.76±1.4% and 8.86±1.2% of slug length, respectively. This suggests that agents that decrease intracellular Ca\(^{2+}\) levels or antagonize CaM affect cell transdifferentiation or sorting and thereby anterior prestalk zone regeneration.

5.4.7 **Agents that modulate Ca\(^{2+}\) levels or antagonize CaM affect the regeneration of shape in isolated slug tips and backs**

The shape of developmental structures affects morphogenetic fields and therefore cell differentiation and morphogenesis. It has been previously shown that cells developed on agar
containing EGTA + A23187 produce shorter slugs (Baskar et al., 2000). The role of Ca\(^{2+}\) and CaM on regeneration of slug shape in bisected slugs has not been previously examined. Ratios of slug length to width were determined for isolated slug tips after 8 h of regeneration and for slug backs after 6 h of regeneration (Fig. 5.9A,B). Without treatment, isolated slug tips and backs regenerated a normal slug shape by this time point. Treatment of isolated slug backs with CaCl\(_2\) + A23187 or verapamil resulted in longer and thinner slugs. The ratios of slug length to width were 26.12±1.4 and 24.01±4.1, respectively, compared to 19.03±1.7 for uncut slugs. Treatment with other agents did not affect the regeneration of slug shape. As mentioned previously, verapamil is known to increase Ca\(^{2+}\) efflux, therefore it is likely that it could raise extracellular...
Ca"^{2+}
levels (Warburton et al., 1989). Thus, both agents likely increase extracellular Ca"^{2+}
levels. This suggests that extracellular Ca"^{2+} plays a role in regulation of slug shape regeneration in
isolated slug backs. Treatment of isolated slug tips with verapamil, EGTA + A23187, BAPATA-
AM or W-7 all inhibited the regeneration of normal slug shape. Slugs that formed under these
treatments were shorter and wider. The ratios of slug length to width were 10.98±3.8, 11.02±4.9,
10.09±2.0 and 9.14±1.4, respectively. Thus, intracellular Ca"^{2+} and CaM regulate slug shape
regeneration in isolated slug tips.
Figure 5.9. The effect of Ca\(^{2+}\) and CaM signal modulation on the re-establishment of slug shape in bisected slug backs (A) and tips (B) after 6 and 8 h of regeneration, respectively. Bisected slug backs and tips were transferred onto filters soaked in ddH\(_2\)O +/- 20 μm bafilomycin A, 100 μm BHQ, 100 μm CaCl\(_2\) + 10 μm A23187, 100 μm verapamil, 1 mM EGTA + 10 μm A23187, 150 μm BAPTA-AM or 100 μm W-7. Slug lengths were measured and divided by slug widths (M+S.D.). At least 15 slugs, from 3 independent replicates were measured for each treatment. A one-way ANOVA with Tukey’s Test was used to identify significant changes in the ability of bisected slugs to re-establish normal slug shape. Data points that do not share a letter are statistically different. \(p \leq 0.05\).

### 5.4.8 Agents that affect Ca\(^{2+}\) levels affect migration of regenerating slug tips

Prestalk and prespore cells have different cell motility patterns. Prespore cells are thought to be responsible for traction with the substratum and forward slug movement (Siegert and Weijer, 1991, 1992; Abe et al., 1994; Rieu et al., 2003). We analyzed the distances that isolated slug tips and backs migrated from the cut sites and determined the effect of agents that modulate Ca\(^{2+}\) levels or antagonize CaM on their migration (Fig. 5.10A,B). Untreated slug backs migrated an average of 1677.39±263.9 μm from cut sites by 6 h of regeneration. Treatment with any of the agents did not affect this migration distance significantly. Thus, agents that affect Ca\(^{2+}\) levels or antagonize CaM do not affect the migration of regenerating slug backs. Untreated slug tips migrated an average of 439.62±38.6 μm from cut sites by 2 h of regeneration. Treatment with bafilomycin A1, BHQ or CaCl\(_2\) + A23187 inhibited their migration as the tips migrated...
Figure 5.10. The effect of Ca\(^{2+}\) and CaM signal modulation on the migration of bisected slug backs (A) and tips (B) after 6 and 2 h of regeneration, respectively. Bisected slug backs and tips were transferred onto filters soaked in ddH\(_2\)O +/- 20 µm bafilomycin A, 100 µm BHQ, 100 µm CaCl\(_2\) + 10 µm A23187, 100 µm verapamil, 1 mM EGTA + 10 µm A23187, 150 µm BAPTA-AM or 100 µm W-7. The length of the path travelled by each bisected slug back and tip was measured (M+S.D.). At least 15 slug paths were measured from 3 independent replicates for each treatment. A one-way ANOVA with Tukey’s Test was used to identify significant changes in the distances slugs migrated. Data points that do not share a letter are statistically different. p≤0.05.

211.40±29.1 µm, 158.00±11.3 µm and 322.84±1.9 µm, respectively. Treatment with EGTA + A23187 also inhibited tip migration as the tips migrated 239.67±27.9 µm from the cut sites. Treatment with verapamil, BAPTA-AM or W-7 did not have a significant effect on the distances the isolated tips migrated. Thus, an increase in intracellular Ca\(^{2+}\) or chelation of extracellular Ca\(^{2+}\) inhibited the migration of regenerating slug tips.

5.5 Discussion

Here we analyzed the pattern of ecmB expression in regenerating slug tips and backs. We showed that agents that affect Ca\(^{2+}\) levels or antagonize CaM affect the regeneration of normal pattern of ecmB expression in slug tips and backs and also the pattern observed in uncut slugs.
We have also determined that agents that affect Ca\(^{2+}\) levels or antagonize CaM activity affect cell motility and the regeneration of shape in bisected slug tips and backs.

Regeneration of bisected *D. discoideum* slugs was first observed by Kenneth Raper, the discoverer of *Dictyostelium*, in 1940 (Raper, 1940). He cut the slugs at different locations and observed that each fragment regulated to reform into a slug and then a properly proportioned fruiting body. Since then it has been demonstrated that when the slug tip is cut off the anterior like cells (ALCs; express *ecmA* gene), that are dispersed through the prespore zone sort to reform the tip of the slug (Francis and O’Day, 1971; Sakai, 1973; Sternfeld and David, 1982; Sternfeld, 1992; Shaulsky and Loomis, 1993; Abe et al., 1994; Detterbeck et al., 1994; Brown and Firtel, 1999). In the reformed tip, ALCs transdifferentiate sequentially into pstO, pstA and then pstAB cells. At the same time prespore cells transdifferentiate to replenish the ALC population (Harwood et al., 1991; Shaulsky and Loomis, 1993; Detterbeck et al., 1994). The reverse is thought to happen when the back is cut off. Prestalk cells transdifferentiate to replenish the prespore population. The pattern of regeneration of *ecmB* expressing cells, had not been previously examined.

To analyze *ecmB* expression we transformed cells with a DNA plasmid carrying *ecmB-ubi-ile-α-gal*. Beta-gal expression was regulated by the whole *ecmB* promoter element. Ubiquitin-gal fusion protein is produced when the *ecmB* promoter is stimulated. De-ubiquitination of gal exposes the amino-terminal isoleucine which leads to quick degradation of the protein, according to the N-end rule (Bachmair et al., 1986; Deichsel et al., 1999). Utilization of this reporter allowed us to indirectly visualize cells that express *ecmB*. We have also attempted to transform cells with a DNA plasmid carrying *ecmB-ubi-ile-GFP*. Transformed cells acquired antibiotic resistance but no fluorescence could be seen. This could have been due to low level of *ecmB* expression in slugs and the labile nature of the reporter. The use of the GFP reporter would have allowed us to follow individual cell movements in regenerating slug tips and backs.

Here we have shown that in bisected slug backs *ecmB* expressing pstB cells aggregate at the cut site like *ecmA* expressing cells. On the other hand, *ecmB* expressing cells did not reform the tip like *ecmA* expressing cells, but rather localized just posterior to it and at the base of formed fingers. It is also interesting that when slugs formed and began migrating, the majority of *ecmB* expressing cells were left behind at the cut site. It is peculiar that a regenerating slug, in need of
new prestalk cells, would discard these ecmB expressing cells. This suggests that ecmB expressing pstB cells may not participate in the transdifferentiation process and may be even inhibitory in some way, so they are discarded. EcmB expressing pstAB cells appeared in the tip and more pstB cells appeared in the prespore zone of regenerated slugs by 6 h, restoring the pattern of ecmB expression seen in uncut slugs.

Treatment with agents that increase intracellular Ca\(^{2+}\) levels increased staining in the anterior and posterior of regenerated slugs. This suggests that Ca\(^{2+}\) induces ecmB expression in slugs. On the other hand, treatment with agents that decrease intracellular Ca\(^{2+}\) levels decreased staining in the prespore zone but did not affect staining in the anterior region. This suggests that lowering intracellular Ca\(^{2+}\) inhibits ecmB expression in the prespore zone only. Treatment with W-7, a CaM antagonist decreased ecmB expression in both anterior and posterior of these slugs. Thus, Ca\(^{2+}\) and CaM likely regulate ecmB expression in the anterior and posterior of regenerating slug backs and therefore affect differentiation of pstAB and pstB cells, respectively. These agents had the same effect on ecmB expression in uncut slugs and therefore same conclusions can be drawn.

It was difficult to count individual cells so it is unclear whether Ca\(^{2+}\) and CaM affected the level of ecmB expression in cells or the number of ecmB expressing cells. This issue was further confounded by the heterogeneity in the number of reporter plasmids each cell possessed. Furthermore, it is possible that these agents affect the sorting pattern of cells along the anterior-posterior axis rather than gene expression patterns directly. Analysis of fruiting body parameters, immunological labeling of ecmB expressing cells and live observation of ecmB-GFP expressing cells could help elucidate this question in the future. In fact, cells developed in the presence of agents that increase intracellular Ca\(^{2+}\) have previously been shown to form fruiting bodies with larger basal discs (Baskar et al., 2000). PstAB cells make the inner basal disc, while pstB and ALC cells make the outer basal disc (Sternfeld and David, 1981; Dormann et al., 1996; Jermyn et al., 1996). Agents that decrease intracellular Ca\(^{2+}\) or antagonize CaM also decreased the percentage of slug length occupied by anterior prestalk cells. Anterior prestalk cells that do not express ecmB gene are pstA and pstO cells. These cells were assumed to be the unstained cells in the anteriors of regenerating slug backs. This suggests that intracellular Ca\(^{2+}\) levels and CaM are important in transdifferentiation or sorting of ALCs and regeneration of the anterior prestalk zone.
It is known that pstB cells in the prespore zone shuttle back and forth from the prestalk/prespore boundary during slug migration and that some pstAB cells travel from the anterior core to the back of the slug and are shed in the slime trail (Siegert and Weijer, 1991, 1992; Sternfeld, 1992; Abe et al., 1994). We suggest the cell types that could be found at the specific positions along the slug’s length but the reader should keep in mind the highly dynamic nature of slugs and that these positions can only serve as approximations of the locations of different cell types. Regardless, the work presented here should serve as a foundation for future, more mechanistic studies aimed at analyzing Ca\textsuperscript{2+} signaling pathways that regulate sorting and differentiation of the different cell types.

Unlike in regenerating slug backs, ecmB expressing cells were counted in regenerating slug tips. Most ecmB expressing pstAB cells were discarded in the slime trail during regeneration and a few ecmB expressing cells appeared in the posterior. It was difficult to discern which ecmB expressing cells were pstAB cells travelling through the forming prespore zone to be discarded in the slime trail versus newly differentiating pstB cells of the prespore zone. Therefore the number of ecmB expressing cells in the posterior of regenerating slug tips may be an overestimate of the number of differentiating pstB cells. By 8 h of regeneration, an average of less than 1 ecmB expressing cell (pstAB) was present in the anterior and about 5 cells (pstB) were dispersed throughout the posterior. No accumulation of ecmB expressing cells was observed posterior to the tip like in uncut slugs and regenerated slug backs.

Treatment with agents that increase intracellular Ca\textsuperscript{2+} levels increased the number of pstAB cells in the anterior of regenerating tips. Treatment with agents that decrease intracellular Ca\textsuperscript{2+} levels or antagonize CaM increased the number of pstB cells in the posterior of regenerating tips. This suggests that high intracellular Ca\textsuperscript{2+} levels positively regulate differentiation of pstAB cells but low intracellular Ca\textsuperscript{2+} positively regulates differentiation of pstB cells. Again, these agents could also affect the sorting of cells leading to effects on ecmB expression patterns. The analysis of the effect of agents that affect Ca\textsuperscript{2+} levels or antagonize CaM on prespore cell differentiation could help elucidate this phenomenon. Following labeled ecmB-GFP expressing cells would also provide valuable information. Prespore cells produce DIF-1 that regulates differentiation of pstB cells (Kay et al., 1993; Saito et al., 2008). Possibly, low intracellular Ca\textsuperscript{2+} and CaM activity promote prespore cell differentiation and/or DIF-1 production and therefore induce pstB cell
differentiation. High intracellular Ca\(^{2+}\) has previously been shown to inhibit prespore specific gene expression in vitro (Verkerke-van Wijk et al., 1998; Kubohara et al., 2007).

The effect of agents that modulate Ca\(^{2+}\) levels or antagonize CaM on cell motility and slug motility has also been analyzed. Agents that increase or decrease intracellular Ca\(^{2+}\) levels or antagonize CaM inhibited the rate of cell movement during mound formation in isolated slug backs. Agents that decrease intracellular Ca\(^{2+}\) levels had a greater inhibitory effect. All agents specifically inhibited the migration of unstained prespore cells but not ecmB expressing cells. Prestalk and prespore cells of slugs are through to migrate chemotactically in response to cAMP emitted by anterior prestalk cells (Dormann et al., 2000; Dormann and Weijer, 2001). When the tip is cut off oscillating cAMP waves are no longer detected in slug backs until a new tip is re-established. This suggests that Ca\(^{2+}\) and CaM are not important for sorting of pstB cells to the anterior end of slug backs but are important for subsequent chemotactic movement of prespore cells to the signal, possibly cAMP, that is emitted by ALC or pstB cells. Tight regulation of intracellular Ca\(^{2+}\) levels and CaM may be required for efficient chemotaxis of prespore cells. In fact, Ca\(^{2+}\) and CaM have previously been shown to play an important role in chemotaxis of amoebae during the aggregation stage of development (Gauthier and O’Day, 2001; Lusche et al., 2009).

Motility of slugs formed from isolated slug tips and backs was also analyzed. Unlike during mound formation, agents that modulate Ca\(^{2+}\) levels or antagonize CaM did not affect migration of slugs formed from isolated slug backs. This suggests that Ca\(^{2+}\) and CaM may not be important for efficient slug migration. On the other hand, agents that increase intracellular Ca\(^{2+}\) levels inhibited migration of slugs formed from isolated slug tips. As mentioned previously, elevation of intracellular Ca\(^{2+}\) may inhibit prespore differentiation and prespore cells are the ones that are responsible for forward slug movement (Siegert and Weijer, 1991, 1992; Abe et al., 1994; Rieu et al., 2003). Chelation of extracellular Ca\(^{2+}\) with EGTA also inhibited migration of these slugs. Extracellular Ca\(^{2+}\) may bind to Ca\(^{2+}\) receptors in the cell membrane and initiate intracellular signal transduction events that are important in cell differentiation or motility. Alternatively, chelation of extracellular Ca\(^{2+}\) may affect Ca\(^{2+}\)-dependent cell adhesion. Cells lacking the Ca\(^{2+}\)-dependent cell adhesion molecule, DdCAD-1, have previously been shown to be unable to undergo proper sorting or spore cell differentiation (Wong et al., 2002).
Lastly, we have analyzed the effect of agents that modulate $\text{Ca}^{2+}$ levels or antagonize CaM on the ability of isolated slug backs and tips to re-establish normal slug shape. The shape of a developmental structure affects positional information of cells in that structure. The shape also affects the direction and the distance that the cells have to travel during sorting. Thus, the shape likely affects cell differentiation and morphogenesis. On the other hand, cell differentiation would also affect the shape of the structure. Isolated slug tips and backs regenerated normal slug shape by 8 and 6 h, respectively. Treatment with $\text{CaCl}_2 + \text{A 23187}$ or verapamil both resulted in formation of long and thin slugs from regenerated slug backs. Both agents likely increase extracellular $\text{Ca}^{2+}$ levels. Mutants defective in DIF-1 production also produce long and thin slugs (Saito et al., 2008). DIF-1 is known to induce $\text{Ca}^{2+}$ influx from the extracellular space (Azhar et al., 1997). Further analysis of the role of extracellular $\text{Ca}^{2+}$ and the possible regulation of its levels by DIF-1 may elucidate a link between extracellular $\text{Ca}^{2+}$ levels and slug shape. Treatment of regenerating slug tips with agents that decrease intracellular $\text{Ca}^{2+}$ levels or antagonize CaM resulted in short and wide slugs. As mentioned previously, agents that affect intracellular $\text{Ca}^{2+}$ levels or CaM may affect cell transdifferentiation. The link between cell transdifferentiation and the establishment of shape remains to be investigated.

It is important to comment on our experimental set up, in regard to placing slugs on substratums containing agents. Baskar et al., 2000, have taken slugs that were raised on the same substratum containing different agents and measured intracellular free and sequestered $\text{Ca}^{2+}$ levels in individual cells of these slugs using chlorotetracycline and fura-2. They showed that $\text{Ca}^{2+}$ levels changed consistently in all cells of the slugs. We have previously immunostained slugs raised on the same substratum containing microtubule disrupting agents and also showed that microtubules depolymerize in all cells of these slugs (Poloz and O’Day, 2012). These studies thus confirm that agents are able to diffuse from the substratum to affect all cells of the slugs.

It is of interest to note that not all agents that were expected to increase or decrease intracellular $\text{Ca}^{2+}$ levels affected slugs to the same extent. This phenomenon could be explained by the fact that cells may use different sources of $\text{Ca}^{2+}$ for different events and that not all agents may be able to change $\text{Ca}^{2+}$ levels to the same extent or in the same way. Intracellularly, $\text{Ca}^{2+}$ can be sequestered by several sources including the endoplasmic reticulum, acidic vacuoles and mitochondria (Newell et al., 1995). It is well established that release and uptake of $\text{Ca}^{2+}$ from
these sources differentially regulates many cellular events, including cell differentiation (Newell et al., 1995).

In conclusion, to our knowledge this is the first study demonstrating the localization of $ecmB$ expressing cells in regenerating slug tips and backs and the effect of agents that affect $Ca^{2+}$ levels and CaM on this expression pattern. Agents that affect $Ca^{2+}$ levels and CaM also affected cell motility and regeneration of shape in isolated slug tips and backs and regeneration of tips in isolated slug backs. This study has provided considerable insight into cell differentiation and morphogenesis in $D. discoideum$ and the role of $Ca^{2+}$ and CaM signaling in these events.

5.6 Acknowledgements

This research was supported in part by a grant from the Natural Sciences and Engineering Council of Canada (DHOD, A6807). Thank you to the Dictyostelium stock centre and Dr. Harry MacWilliams for depositing $ecmB$-ubi-ile-$\alpha$-gal plasmid.

5.7 References


Korohoda, W., Madeja, Z., & Sroka, J. (2002). Diverse chemotactic responses of *Dictyostelium discoideum* amoebae in the developing (temporal) and stationary (spatial) concentration gradients of folic acid, cAMP, Ca\(^{2+}\) and Mg\(^{2+}\). *Cell Motility and The Cytoskeleton*, 53, 1-25.


Chapter 6
Summary

6.1 Summary of goals achieved and hypotheses verified

The overall goal of my thesis was to gain insight into the role of Ca\textsuperscript{2+} and CaM in *Dictyostelium* differentiation and morphogenesis. I had 3 specific goals:

6.1.1 Analyze the developmental roles of NumA1 and its binding partners CBP4a and PsaA

As hypothesized, I identified that CaMBP NumA1 and its binding partners, CBP4a and PsaA, are regulated by extracellular signaling molecules and have roles in cell differentiation (Chapter 2, 3). First, I produced, affinity purified and verified the specificity of anti-NumA1 antibody. I then used this antibody, along with anti-CBP4a and anti-PsaA, to analyzing the effect of extracellular signaling molecules, cAMP, DIF-1 and ammonia on expression of these proteins (Chapter 2). Expression of NumA1 and CBP4a was co-regulated by these extracellular signaling molecules. As I hypothesized, during starvation, DIF-1 maintained vegetative NumA1 and CBP4a expression while cAMP and ammonia repressed it. CBP4a is a pstO specific protein (Maeda et al., 2003). PstO cell differentiation is known to be regulated by DIF-1 (Thompson and Kay, 2000a). Thus, NumA1 and CBP4a likely play a role in pstO cell differentiation in *D. discoideum* (Fig. 6.1A). This role remains to be identified.

On the other hand, PsaA expression was regulated by extracellular signaling molecules in a different way. DIF-1 and ammonia repressed PsaA expression while cAMP induced it (Chapter 3). Due to availability of an inhibitor, BME, and PsaA overexpressing strains I decided to focus on the developmental role of PsaA. Induction of PsaA expression by cAMP but inhibition by DIF-1 suggested a role for PsaA in prespore/spore cell differentiation (Fig. 6.1A). Consistent with this, treatment of cells with BME inhibited spore cell differentiation. Less prespore cells differentiated into spores and the ones that did were rounded in shape. The same effect was observed with PsaA overexpression. Other than NumA1, PsaA also interacts with Cdk5 (Huber and O’Day, 2011). Inhibition of Cdk5 function also inhibits spore cell differentiation (Sharma et al., 2002). Additionally, I showed that PsaA is distributed throughout the nucleoplasm and cytoplasm of prestalk/stalk and prespore/spore cells. PsaA without NLS2 was not able to localize
properly to the nucleoplasm and consequently accumulated in the cytoplasm. PsaAΔNLS2 overexpression did not affect spore cell differentiation. In chimeras, PsaA overexpressing cells differentiated into stalk cells while PsaAΔNLS2 overexpressing cells differentiate into spores. Thus, nuclear and cytoplasmic PsaA appear to have different roles in cell differentiation in *D. discoideum*. Further analysis of nuclear and cytoplasmic forms of PsaA will help elucidate their differential function in cell differentiation.

This suggests that differential interaction of NumA1 with CBP4a and PsaA during development regulates stalk and spore cell differentiation, respectively. NumA1 interacting with CBP4a likely regulates pstO cell differentiation, while NumA1 interacting with PsaA likely regulates spore cell differentiation (Fig. 6.1A). Spore cell differentiation is also likely regulated by the interaction of PsaA with Cdk5 (Fig. 6.1A). Further analysis of the developmental role of NumA1, CBP4a and PsaA will help elucidate the dynamic regulation of cell differentiation by CaMBPs and their binding partners.

### 6.1.2 Analyze colchicine’s mechanism of action in disrupting morphogenesis and inducing stalk cell differentiation

I have revealed that colchicine disrupts morphogenesis, enhances cell motility, inhibits spore cell differentiation and induces stalk cell differentiation through a Ca\(^{2+}\) and CaM-dependent signaling pathway (Chapter 4). Thus my hypothesis, that colchicine induces stalk cell differentiation by affecting Ca\(^{2+}\) singal transduction pathways, was correct. I also showed that colchicine specifically induces *ecmB* expression and differentiation of *ecmB* expressing stalk cells. DIF-1 regulates *ecmB* expression and differentiation of stalk cells *in vitro* (Town et al., 1976; Morris et al., 1987; Williams et al., 1987). DIF-1 is known to work through an increase in intracellular Ca\(^{2+}\) levels (Kubohara and Okamoto, 1994; Schaap et al., 1996; Kay et al., 1999; Thompson and Kay, 2000b; Kay and Thompson, 2001; Saito et al., 2008; Kubohara et al., 2007). Increasing intracellular Ca\(^{2+}\) levels with pharmacological agents also induces *ecmB* expression and stalk cell differentiation *in vitro* (Kubohara and Okamoto, 1994; Schaap et al., 1996). Fittingly, I found that colchicine disrupts morphogenesis, inhibits spore cell differentiation and induces stalk cell differentiation through a Ca\(^{2+}\) and CaM-mediated signal transduction pathway. Interestingly, colchicine’s effect was independent of DIF-1 production. I thus proposed that colchicine specifically induces differentiation of *ecmB* expressing cells through a signaling pathway distinct from the one that is utilized by DIF-1 and independent of DIF-1 production.
As hypothesized, I also found that colchicine’s effects are microtubule independent, as other microtubule depolymerizing agents did not have any developmental effects. Colchicine also enhanced random cell motility through a Ca$^{2+}$ and CaM-mediated signal transduction pathway. Lastly, colchicine’s developmental phenotype was rescued by chelation of Ca$^{2+}$ or antagonism of CaM. Thus, colchicine disrupts morphogenesis, inhibits spore cell differentiation and induces stalk cell differentiation through a DIF-1 independent Ca$^{2+}$ and CaM–mediated signal transduction pathway, leading to changes in gene expression and cell motility. This research provides considerable insight into this 50-year old developmental problem, finally revealing that developmental actions of colchicine are not due to microtubular depolymerization. It also emphasizes the role of Ca$^{2+}$ and CaM in cell differentiation and morphogenesis in *D. discoideum*.

In the Appendix I, I have additionally shown that colchicine differentially regulates expression of NumA1, CBP4a, PsaA, Cdk5 and CyrA (Fig. A.1). Colchicine induced expression of NumA1 but repressed expression of CBP4a, PsaA and Cdk5. As mentioned previously, NumA1 interacting with CBP4a likely regulates pstO cells differentiation. In support of this, Cbp4a is expressed specifically in pstO cells and both proteins are regulated by DIF-1 (Maeda et al., 2003). I have shown that colchicine inhibits differentiation of pstO cells. PsaA and Cdk5 regulate spore cell differentiation and colchicine inhibits spore cell differentiation. Colchicine’s inhibition of CBP4a, PsaA and Cdk5 expression therefore fits with the proposed role of these proteins. Induction of NumA1 expression by colchicine was unexpected. This suggests a novel role for NumA1 in the differentiation of *ecmB* expressing cells, since this cell type is specifically induced by colchicine. Further analysis of NumA1 function will help elucidate how this CaMBP regulates cell differentiation, which cell types are specifically affected and how the NumA1 binding partners differentially mediate its functions. Colchicine also induced expression of another CaMBP, CyrA, a matricellular protein that is thought to regulate cell motility during development (Suarez et al., 2011). This provided further insight into the mechanism of action of colchicine. Colchicine may specifically enhance cell motility through the action of CyrA.

### 6.1.3 Analyze the role of Ca$^{2+}$ and CaM in *ecmB* expression, cell differentiation and morphogenesis *in vivo*, in intact and regenerating slugs

As hypothesized, Ca$^{2+}$ and CaM regulate differentiation and morphogenesis *in vivo* (Chapter 5). I analyzed the effect of agents that modulate Ca$^{2+}$ levels or antagonize CaM on *ecmB* expression,
cell differentiation and morphogenesis in intact and regenerating slugs. As mentioned previously, ecmB expression and stalk cell differentiation is regulated by Ca^{2+} in vitro. Until now, no one has been able to show that the same is true in vivo. I have shown that pharmacological agents that increase intracellular Ca^{2+} levels induced ecmB expression and/or pstB and pstAB cell differentiation. Conversely, agents that decrease intracellular Ca^{2+} levels or antagonize CaM repressed ecmB expression and/or pstB and pstAB cell differentiation. Ca^{2+} and CaM also played differential roles in the regeneration of shape, motility and ecmB expression in bisected slugs. *D. discoideum* could serve as a simple model to study cell transdifferentiation and morphogenesis during regeneration as well as to gain further insight into the role of Ca^{2+} signaling in cell differentiation and morphogenesis. Further analysis of Ca^{2+} and CaM downstream signaling pathways, including identification of CaMBPs, will provide insight into differentiation, morphogenesis and regeneration in *D. discoideum* and higher eukaryotes.

### 6.2 Significance

As mentioned in Chapter 1, Ca^{2+} plays a major role in differentiation and morphogenesis of many organisms including slime molds, fungi, plants and animals (O’Day, 1990; Hepler, 2005; Webb et al., 2005; Kubohara et al., 2007; Slusarski and Palegri, 2007). For example, in *Xenopus* and *Danio* embryos a Ca^{2+} signaling pathway has been implicated in dorsal-ventral patterning and morphogenetic cell movement during gastrulation (reviewed in Slusarski and Pelegri, 2007). A largely unknown Ca^{2+} signaling pathway has also been implicated in the establishment of the left-right axis of *Xenopus, Gallus* and *Mus* embryos (reviewed in Slusarski and Pelegri, 2007). There is additional evidence for a role of Ca^{2+} in neural induction, somite differentiation and hematopoietic and other stem cell differentiation in numerous vertebrates (reviewed in Webb et al., 2005; Slusarski and Pelegri, 2007). Lastly, regeneration of axolotl limbs involves a rise in intracellular Ca^{2+} (reviewed in Stocum and Cameron, 2011). Despite the global importance of Ca^{2+}, little is known about its developmental signaling pathways. Here I used *D. discoideum* as a model to further show that Ca^{2+} plays major roles in differentiation and morphogenesis (Fig. 6.A,B). I identified CaM as a primary Ca^{2+} effector during *D. discoideum* development. I showed that Ca^{2+} works through CaM and different downstream effectors (CaMBPs, including NumA1 and its binding partners) to regulate differentiation and morphogenesis. Perturbation of Ca^{2+} and CaM-dependent signaling pathways with pharmacological agents, including colchicine, leads to drastic developmental effects. Ca^{2+} and CaM also regulate regeneration, transdifferentiation,
motility and the establishment of shape in *D. discoideum*. Further analysis of Ca\(^{2+}\) and CaM-dependent signaling pathways will provide insight into differentiation and morphogenesis in *D. discoideum* and higher eukaryotes.

All my findings are novel. No previous work existed on the regulation of CaMBPs or their binding partners by developmental signaling molecules. PsaA was shown to be important in mouse embryogenesis but no previous work existed on its function in *D. discoideum* development. The mechanism of action of colchicine was not known, though over the past 50 years colchicine was shown to have developmental effects in many organisms. With the new insight into mechanism of action of colchicine, developmental events in different organisms affected by colchicine can be linked to a common Ca\(^{2+}\) and CaM-dependent signaling pathway. Lastly, the role of Ca\(^{2+}\) and CaM in *ecmB* expression, cell differentiation, motility and morphogenesis has not been previously analyzed *in vivo*, in intact or regenerating slugs. In fact, signaling pathways involved in *D. discoideum* regeneration had not been previously analyzed.
6.3 Future studies

6.3.1 Further elucidation of Ca$^{2+}$ and CaM-dependent signaling pathways

As mentioned previously, Ca$^{2+}$ is known to regulate differentiation and morphogenesis in many organisms but little is known about the upstream and downstream components of Ca$^{2+}$ signaling pathways. As I have shown CaM is a primary Ca$^{2+}$ effector during *D. discoideum* development. *D. discoideum* has more than 4 dozen developmentally regulated CaMBPs (Gauthier and O’Day, 2001). Future work should focus on the identification of CaMBPs and characterization of their developmental roles. New CaMBPs can be identified by a previously described CaM-binding overlay technique (CaMBOT), followed by mass spectrometry (O’Day, 2003).

First, the developmental role of NumA1 and its binding partners, CBP4a and PsaA should be further analyzed. NumA1 may have roles in differentiation of pstO, pstB and prespore cells, likely through interaction with different binding partners including CBP4a and PsaA. Insight into how NumA1 may regulate these different cell types can be gained from the analysis of NumA1 null mutant and deletion construct strains. For example, interaction of NumA1 with CBP4a depends on the DEED domain (Myre and O’Day, 2004). Development of cells expressing NumA1ΔDEED will help elucidate the role of the interaction of NumA1 with CBP4a in pstO cell differentiation. Additionally, the developmental phenotype of NumA1 null cells will provide new insight into NumA1 function in differentiation and morphogenesis.

6.3.2 Development of colchicine as a tool to study cell differentiation

Colchicine likely induces differentiation of *ecmB* expressing pstB cells. The signaling pathways that regulate differentiation of this cell type are largely unknown. Here I showed that Ca$^{2+}$ regulates differentiation of this cell type through CaM. Further elucidation of the mechanism of action of colchicine will help identify signaling pathways involved in regulation of this cell type. This can be accomplished in several ways. Production of an anti-colchicine antibody has been previously described but is no longer available (Rouan et al., 1989). Colchicine’s target could be identified through co-immunoprecipitation using an anti-colchicine antibody. In addition, restriction enzyme-mediated integration (REMI) mutagenesis could help identify cell lines resistant to colchicine’s developmental effects. Subsequent analysis of mutated genes should help identify components of colchicine’s signaling pathway. Lastly, treatment of NumA1 and
CyrA null strains with colchicine will reveal the role these CaMBPs play in colchicine’s mechanism of action in cell differentiation and motility, respectively.

6.3.3 The role of Ca\(^{2+}\) and CaM in regeneration

Here I showed that Ca\(^{2+}\), through CaM, regulates cell motility and differentiation during regeneration. I only analyzed regeneration of slugs expressing the ecmB marker. In the future, other markers, including ecmA, ecmO and \(cotB/pspA\) should be analyzed. This will provide a complete picture of cell movement and cell differentiation during regeneration. Instead of galactosidase as a reporter, labile GFP and RFP should be used. This would allow the observation of live cell movement during regeneration and more than one cell type could be followed at the same time. Regeneration experiments should also be performed with Ca\(^{2+}\) signaling mutant strains (ex: NumA1, CyrA etc.). This could provide further information on the developmental roles of these proteins and the role of Ca\(^{2+}\) signaling in regeneration.

6.4 References


Appendix I

The effect of colchicine on expression of developmentally regulated proteins

A.1 Introduction and Results

Colchicine’s effect on protein expression was analyzed to further elucidate the mechanism of action of colchicine and to gain insight into the developmental roles of NumA1, CBP4a, PsaA, Cdk5 and CyrA, proteins of central interest in our laboratory. CBP4a and PsaA are binding partners of NumA1 (Myre and O’Day, 2004; Myre, 2005; Catalano et al., 2011). NumA1 and CBP4a are regulated by DIF-1 and likely play a role in pstO cell differentiation (Chapter 2; Maeda et al., 2003). Colchicine inhibits pstO-specific gene expression (Chapter 4). I hypothesize that colchicine will inhibit NumA1 and CBP4a expression. On the other hand, PsaA regulates spore cell differentiation (Chapter 3). Cdk5 is a binding partner of PsaA that also regulates spore cell differentiation (Sharma et al., 2002; Huber and O’Day, 2011a). Colchicine inhibits spore cell differentiation (Chapter 4). I therefore hypothesize that colchicine will inhibit PsaA and Cdk5 expression. Lastly, CyrA is an extracellular CaMBP that contains epidermal growth factor like (EGFL) repeats that regulate cell motility during development (Suarez et al., 2011). EGFLs have specifically been shown to enhance cell motility through a Ca\(^{2+}\) and CaM-dependent signaling pathway (Huber and O’Day, 2009; 2011b). Colchicine also enhances cell motility through a Ca\(^{2+}\) and CaM-dependent signaling pathway (Chapter 4). Colchicine may enhance cell motility through the action of CyrA. I therefore hypothesize that colchicine induces CyrA expression.

Cells were allowed to develop on filters soaked in colchicine for up to 24 h (Materials and Methods section 3.3.3). Cells were harvested every 4 h and protein expression levels were analyzed using western blotting (Materials and Methods section 3.3.7). Colchicine induced NumA1 expression at 12–24 h of development (Fig. A.1A). The maximum induction was observed at 20 h of development where 10 mM colchicine induced NumA1 expression to 229.17±29.5% of vegetative levels. In comparison, NumA1 expression in untreated cells was 175.00±7.1% of vegetative levels at this time point. This suggests a role for NumA1 in differentiation of ecmB expressing pstB cells, the cell type that is specifically induced by colchicine.
Figure A.1. Effect of 10 mM colchicine (Col) on developmental expression of NumA1 (A), CBP4a (B), PsaA (C), Cdk5 (D) and CyrA (E). Left panels are the western blots and right panels are quantifications of these western blots. One-way ANOVA was used to analyze changes in expression in colchicine treated versus untreated cells at each time point. *p≤0.05
As hypothesized, colchicine inhibited the expression of CBP4a (Fig. A.1B). At 12 h of development CBP4a levels were 270±28.3% of vegetative levels in untreated cells but 135±21.2% of vegetative levels in cells treated with 10 mM colchicine. Also as hypothesized, PsaA and Cdk5 expression was inhibited by colchicine at 8-24 h of development (Fig. A.1C,D). Maximum inhibition of PsaA expression was observed at 12 h, where 10 mM colchicine inhibited PsaA expression to 35.01±4.2% of vegetative levels. In comparison, PsaA expression in untreated cells was 52.01±2.8% of vegetative levels at 12 h of development. Cdk5 expression was maximally inhibited at 20 h of development. At this time point, Cdk5 expression was 130.50±14.1% of vegetative levels in 10 mM colchicine treated cells but 185.32±7.0% of vegetative levels in untreated cells. Lastly, as hypothesized, colchicine induced CyrA expression 4-24 h of development (Fig. A.1E). Maximum induction was observed at 8 h of development. At this time point, CyrA expression in 10 mM colchicine treated cells was 205.25±21.2% of vegetative levels but in untreated cells was 80.20±28.3% of vegetative levels. Thus, colchicine’s effect on cell motility may be the result of increased CyrA expression.

In conclusion, colchicine differentially affects expression of NumA1 and its binding partners, as well as Cdk5 and CyrA, as hypothesized. This has provided further insight into the mechanism of action of colchicine and the developmental role of these proteins.

A.2 References


Copyright Acknowledgements

Chapter 2 was published as part of a manuscript:


Chapter 3 Figures 2, 4 and 5 were published:


Chapter 3 Figures 1, parts of 5 and 6-13 were accepted for publication:


Chapter 4 was accepted for publication as is:


Chapter 5 was submitted for publication as is: