Actin Tyrosine Phosphorylation in Microcysts of Polysphondylium pallidum

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
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University of Toronto

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Abstract

High osmolarity causes amoebae of the cellular slime mould *Polysphondylium pallidum* to individually encyst, forming microcysts. During microcyst differentiation, actin is tyrosine phosphorylated. Tyrosine phosphorylation of actin is independent of encystment conditions and occurs during the final stages of microcyst formation. During microcyst germination, actin undergoes dephosphorylation prior to amoebal emergence. Renewed phosphorylation of actin in germinating microcysts can be triggered by increasing the osmolarity of the medium which inhibits emergence. Immunofluorescence reveals that actin is dispersed throughout the cytoplasm in dormant microcysts. Following the onset of germination, actin is observed around vesicles where it co-localizes with phosphotyrosine. Prior to emergence, actin localizes to patches near the cell surface. Increasing osmolarity disrupts this localization and causes actin to redistribute throughout the cytoplasm, a situation similar to that observed in dormant microcysts. Together, these results indicate an association between actin tyrosine phosphorylation, organization of the actin cytoskeleton, and microcyst dormancy.
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Chapter 1
Actin Tyrosine Phosphorylation in Microcysts of Polysphondylium pallidum

1 Introduction

1.1 General

Amoebae of the class Dictyostelia are studied as models for cell signalling, differentiation, and chemotaxis (reviewed in Urushihara, 2009). As social amoebozoans, they are characterized by the ability to alternate between solitary unspecialized and multicellular differentiated stages. During the developmental cycle upon starvation, thousands of amoebae aggregate and undergo development to form a fruiting body (reviewed in Urushihara, 2008). This multicellular structure consists of a stalk and a sorus containing dormant spores. When conditions are favourable, the spores germinate to release individual amoebae and the life cycle begins anew. In addition to spore formation, some species may enter one of two alternative dormant stages. The first is the macrocyst, a sexual structure that develops as a result of cell fusion, aggregation, and cannibalism (reviewed in O’Day et al., 1995). The second is the microcyst, which forms when a cellulose-rich cell wall is secreted around an individual amoeba in response to high osmolarity (Blaskovics and Raper, 1957; Toama and Raper, 1967a).

The purpose of this work was to investigate tyrosine phosphorylation of actin during microcyst differentiation and germination in the slime mould Polysphondylium pallidum. This species is capable of fruiting body, macrocyst, and microcyst formation as shown in Figure 1.1 (Francis et al., 1977). A recent phylogeny of social amoebae places Polysphondylium pallidum in a group more basal to the model organism Dictyostelium discoideum (Schaap et al., 2006). Dictyostelids from the most derived group, including Dictyostelium discoideum, appear to have lost the ability to form microcysts (Schaap et al., 2006).

Although Dictyostelium discoideum is the most widely studied dictyostelid, the recent genome sequencing of Polysphondylium pallidum and other social amoebae provides an opportunity to investigate the genetic differences between these species and to explore their
Figure 1.1: Alternative life cycles in Polysphondylium pallidum. Amoebae may enter into fruiting body, microcyst, or macrocyst developmental pathways. Figure after Francis et al., 1977.
evolutionary basis. This is significant because species differences exist in characteristics such as fruiting body and spore morphology, chemoattractants that induce aggregation during development, and the ability to form macrocysts or microcysts (Schaap et al., 2006). Preliminary sequence data for *Polysphondylium pallidum* and other species, produced by the Jena Centre for Bioinformatics, are available through the Social Amoebas Comparative Genome Browser (http://sacgb.fli-leibniz.de/cgi/index.pl).

1.2 Microcysts of *Polysphondylium pallidum*

Microcyst formation is triggered when cells do not undergo aggregation and fruiting body development, primarily due to increased osmolarity coupled with the presence of certain ions (Toama and Raper, 1967a). Divalent cations (Toama and Raper, 1967a), ammonia (Lonski, 1976; Choi and O’Day, 1982), and methionine starvation (Lonski and Pesut, 1977) enhance microcyst production. The intracellular levels of cyclic adenosine 3′, 5′-monophosphate (cAMP) undergo a dramatic increase prior to encystation, most likely mediated by the osmosensing adenyl cyclase, ACG (Ritchie et al., 2008). The subsequent activation of cAMP-dependent protein kinase (PKA) is necessary and sufficient for microcyst formation (Ritchie et al., 2008). ACG also appears to maintain microcyst dormancy in the presence of high osmolyte concentrations (Ritchie et al., 2008).

During encystment, amoebae become enclosed by a cell wall composed of cellulose, proteins, lipids, and a glycogen-like glucose polymer (Toama and Raper, 1967b). The cell wall consists of two layers, an inner dense layer and an outer loose layer (Hohl et al., 1970). Cell wall formation appears to occur via release of cellulose to the extracellular surface by calcofluor-positive vesicles (Choi and O’Day, 1984). Other biochemical events also take place during microcyst formation. The overall content of RNA and protein decreases, while levels of polysaccharides increase (Githens and Karnovsky, 1973). The phosphodiesters glycerophosphocholine and glycerophosphoethanolamine and the disaccharide trehalose, all nearly absent in amoebae, accumulate to high amounts in microcysts (Klein et al., 1992). Diphosphoinositol phosphate levels also increase, particularly 5,6-bis-PP-InsP₄, which rises 20-fold during encystment (Laussman et al., 2000). In addition, microcysts contain a high level of free amino acids (Gwynne and O’Day, 1978; Ennis, 1981).

Microcyst germination is induced by low osmotic conditions (reviewed in O’Day, 1977). It is an ATP-dependent aerobic process (Klein et al., 1992). Unlike spores, microcysts do not require additional activation to germinate (Cotter and Raper, 1968). Germination involves swelling of the microcyst followed by emergence of an amoeba through gaps in the cyst wall (Hohl et al., 1970). During the swelling stage, polyvesicular bodies enlarge, the endoplasmic reticulum cisternae temporarily dilate, and the cyst wall loosens (Hohl et al., 1970). This change in the endoplasmic reticulum probably corresponds to a peak in protein synthesis, which is required for emergence but not swelling (Hohl et al., 1970; Cotter and Raper, 1968; O’Day, 1974). Protein synthesis begins immediately after microcysts are incubated under germination conditions (O’Day et al., 1976; Ennis et al., 1978; Gwynne and O’Day, 1978). Synthesis of polypeptides may account for the observation that free amino acid concentrations tend to decrease during germination (Gwynne and O’Day, 1978; Ennis, 1981). The proteins made during germination include those synthesized throughout the process as well as those whose production is limited to specific stages, indicating that synthesis is under developmental control (Ennis et al., 1978; Dowbenko and Ennis, 1982). RNA synthesis also occurs following the onset of germination (O’Day et al., 1976; Ennis et al., 1978; Gwynne and O’Day, 1978). However, germination proceeds even in the presence of RNA synthesis inhibitors, suggesting pre-existing mRNA’s are used for the necessary protein synthesis (O’Day, 1974; O’Day et al., 1976).

In addition, changes in hydrolytic enzyme activity occur over the course of germination. The activities of several glycosidases and acid proteases accumulate in the extracellular medium, therefore the excretion of these enzymes may aid in cell wall digestion (O’Day, 1974; O’Day, 1976). Also of note is a three to four-fold increase in intracellular alkaline phosphatase activity (O’Day, 1974; O’Day et al., 1976). The increase in activity of alkaline phosphatase and the
Dormant Microcyst (0h)

- Polyvesicular bodies swell
- Endoplasmic reticulum dilates
- Protein synthesis begins (but not required)
- Cell wall loosens

Swollen Microcyst (3h)

- Polyvesicular bodies and endoplasmic reticulum shrink
- Protein synthesis required
- RNA synthesis begins (but not required)
- Cell wall digested

Emerged Amoeba (6h)

Figure 1.2: Events of microcyst germination in *Polysphondylium pallidum*. Enzyme system I depends on post-translational activation while enzyme system II requires protein synthesis. Scale bars indicate 10 \( \mu \text{m} \). Figure after O’Day, 1977.
glycosidases β-glucosidase, trehalase, and carboxymethyl cellulase depends on protein synthesis (O’Day, 1974; O’Day et al., 1976; Tisa and Cotter, 1979; O’Day and Paterno, 1979). The requirement of protein synthesis for some enzymes suggests that two enzyme systems operate during germination, as shown in Figure 1.2 (O’Day, 1976; O’Day et al., 1976; O’Day and Paterno, 1979). System I enzymes function during the swelling phase to allow initial cell wall loosening and depend on post-translational activation. System II enzymes rely on protein synthesis and are responsible for subsequent cell wall removal.

Finally, the levels of glycerophosphocholine, glycerophosphoethanolamine, and trehalose decrease (Klein et al., 1992). Consumption of the phosphodiesters is likely due to synthesis of membrane phospholipids, while trehalose acts as a carbon and energy source (Klein et al., 1992). The diphosphoinositol phosphates that accumulated to high levels during microcyst differentiation are degraded when cells germinate at low density (Laussman et al., 2000).

### 1.3 Actin

Actin is a cytoskeletal 375-amino acid protein of approximately 42 kDa (reviewed in Kabsch and Vandekerckhove, 1992). It is an abundant eukaryotic protein, especially in muscle cells where it accounts for 10% of cellular protein by weight (Lodish et al., 2000). With amoeba and animal actins sharing 80% identity, this protein is also one of the most conserved (Lodish et al., 2000).

Actin is the major component of microfilaments. Monomers of globular G-actin assemble into filamentous F-actin. F-actin is polar, and elongation proceeds more efficiently at the plus end. Polymerization at the plus end coupled with dissociation from the minus end results in actin treadmilling (reviewed in Bugyi and Carlier, 2010). This drives cell movement, where elongation of filaments forces the cell membrane forward and generates a protrusion at the leading edge (reviewed in Ananthakrishnan and Ehrlicher, 2007). The protruding leading edge adheres to the substratum while the trailing edge detaches from the substratum and retracts into the cell body, which is pulled forward. This process continues as the cell crawls across a surface. Besides cell motility, actin contributes to cell adhesion, cytokinesis, endocytosis, vesicle and organelle transport, and governs cell shape (reviewed in Pollard and Cooper, 2009).
1.4 Actin Phosphorylation

The ability of actin to polymerize depends on ATP binding, ionic strength, and actin-binding proteins. It is also regulated by covalent modifications such as phosphorylation. The earliest experiments examined the effects of phosphorylation on actin \textit{in vitro}. Purified actin phosphorylated by incubation with liver plasma membranes loses its ability to polymerize (Grazi and Magri, 1979). Similarly, phosphorylation of actin in \textit{Amoeba proteus} homogenate blocks its polymerization (Sonobe \textit{et al}., 1986). Phosphorylation of purified actin by the catalytic subunit of protein kinase A reduces its rate of polymerization (Walsh \textit{et al}., 1981; Ohta \textit{et al}., 1987). Conversely, phosphorylation of purified actin by protein kinase C increases its ability to polymerize (Ohta \textit{et al}., 1987).

More recently, actin phosphorylation has been demonstrated \textit{in vivo}. Serine phosphorylation of actin in mouse fibroblasts following stimulation by epidermal growth factor is associated with actin polymerization at the cell cortex and formation of membrane ruffles (van Delft \textit{et al}., 1995). In porcine renal epithelial cells, serine phosphorylation of actin induced by phosphatase inhibition coincides with actin depolymerization, disorganization of the actin cytoskeleton, and cell shape changes (Gu \textit{et al}., 2003). Actin phosphorylation by the serine/threonine kinase PAK1 following stimulation with opioids causes reorganization of the actin cytoskeleton in opossum kidney cells (Papakonstanti and Stournaras, 2002). In \textit{Physarum polycephalum} sclerotium, dry stress induces threonine phosphorylation of actin making it non-polymerizable (Furuhashi \textit{et al}., 1998). Src kinase-dependent tyrosine phosphorylation of actin in serum-stimulated human colon cancer cells appears to regulate actin remodelling at the cell periphery (Avizienyte \textit{et al}., 2007). In chicken B cells, actin tyrosine phosphorylation following cross-linking of the B cell Ag receptor may induce polymerization while dephosphorylation by SHP-1 is required for depolymerization (Baba \textit{et al}., 2003). In the contact-sensitive plant \textit{Mimosa pudica}, dephosphorylation of tyrosine results in fragmentation of actin filaments, a process that takes place during and is required for plant bending (Kanzawa \textit{et al}., 2006; Kameyama \textit{et al}., 2000). Actin tyrosine phosphorylation induced by the bacterium \textit{Anaplasma phagocytophilum} in tick cells reduces F-actin levels and increases G-actin levels, leads to accumulation of G-actin in the nucleus, and alters gene expression (Sultana \textit{et al}., 2010). Actin tyrosine phosphorylation has also been well-studied in the cellular slime mould \textit{Dictyostelium discoideum}. 
1.5 Tyrosine Phosphorylation of Actin in *Dictyostelium discoideum*

In *Dictyostelium discoideum*, a social amoeba related to *Polysphondylium*, specific conditions induce tyrosine phosphorylation of actin. Actin phosphorylation is observed in starving amoebae transferred to nutrient medium, where it undergoes a transient increase (Schweiger *et al*., 1992; Howard *et al*., 1993). It also occurs in amoebae that have reached maximum density in culture medium (Liu *et al*., 2006). Phosphorylation is induced in growing amoebae treated with the tyrosine phosphatase inhibitor phenylarsine oxide (PAO) (Schweiger *et al*., 1992). It is also induced in amoebae subjected to stress conditions, including oxygen depletion, inhibition of ATP synthesis by 2,4-dinitrophenol (DNP), heat shock, heavy metal ions, and osmotic shock (Jungbluth *et al*., 1994; Jungbluth *et al*., 1995; Zischka *et al*., 1999). Dormant spores also contain tyrosine phosphorylated actin (Gauthier *et al*., 1997; Kishi *et al*., 1998).

Actin phosphorylation occurs on a single tyrosine residue at position 53 of the protein’s sequence (Jungbluth *et al*., 1995). Phosphorylated and unphosphorylated actin co-polymerize *in vitro* and co-localize in amoebae (Kishi *et al*., 1998; Liu *et al*., 2006). However, studies with purified actin show that phosphorylation inhibits nucleation and elongation at the pointed end, reduces elongation at the barbed end, slows ATP hydrolysis, and destabilizes filaments (Liu *et al*., 2006). These properties may result from stabilization of actin’s D-loop by hydrogen bonding with the phosphate group, which is the major conformational difference between phosphorylated and unphosphorylated actin (Baek *et al*., 2008). Assays with purified actin in which Tyr-53 has been replaced by Glu or Ala reveal similar effects on actin properties to those induced by tyrosine phosphorylation (Liu *et al*., 2010). In aggregating *Dictyostelium discoideum* amoebae expressing actin constructs where Tyr-53 has been replaced by Ala, a fragmented cytoskeleton is observed along with disrupted cAMP signalling leading to inhibited streaming and defective development (Shu *et al*., 2010).

Actin tyrosine phosphorylation in amoebae is associated with morphological changes and correlates with the reorganization of the actin cytoskeleton in its final stages (Kishi *et al*., 1998). It occurs after cells lose pseudopods, form blebs, and become round and immobile (Schweiger *et al*., 1992; Howard *et al*., 1993; Jungbluth *et al*., 1994). In PAO-treated cells, the intensity of fluorescent phalloidin labelling of actin filaments decreases (Schweiger *et al*., 1992). In DNP-
treated cells, actin filaments form irregular clusters in the cytoplasm (Jungbluth et al., 1994). These clusters co-localize with the motor protein myosin II and the actin capping protein cap32/34 (Jungbluth et al., 1994). In amoebae subjected to osmotic shock, reorganization of the cell cortex and associated morphological changes are followed by actin phosphorylation (Zischka et al., 1999). Tyrosine phosphorylation may deactivate the actin cytoskeleton and help maintain the cell in a resting state (Zischka et al., 1999).

Half of the actin molecules are phosphorylated in dormant spores (Gauthier et al., 1997; Kishi et al., 1998). Phosphorylation first becomes detectable in mature spores following the culmination stage of development (Kishi et al., 1998). The autoinhibitor, an endogenous molecule that prevents spore germination, appears to promote actin phosphorylation during dormancy (Gauthier et al., 1997). Triggers for inducing dephosphorylation in dormant spores include heat shock, certain metabolizable hexoses (in young spores), various other saccharides (in aging spores), or removal of germination inhibitory factors and a decrease in osmotic pressure (in aged spores) (Kishi et al., 2000). Dephosphorylation occurs rapidly when spores are activated to germinate (Gauthier et al., 1997; Kishi et al., 1998). Dephosphorylation appears to be involved in spore swelling and subsequent amoebal emergence during germination, but is not by itself sufficient for the process (Kishi et al., 2000). Both actin tyrosine phosphorylation and viability decline at similar rates as dormant spores age, suggesting an interrelationship (Kishi et al., 1998).

In dormant spores, actin exists as rods extending across the nucleus and cytoplasm (Sameshima et al., 1994). Actin rods are composed of closely packed actin tubules which are bundles of three actin filaments (Sameshima et al., 2000). Rods first appear during mid-culmination, go on form in more than 90% of mature spores, and disappear during germination (Kishi et al., 1994; Sameshima et al., 2001). Rod formation appears important for normal spore shape and viability (Sameshima et al., 2000). The rods first appear as modules of hexagonally arranged actin tubules which go on to associate, leading to rod elongation (Sameshima et al., 2001). The actin tubules comprising the rods then become closely packed, an event that coincides with an increase in actin tyrosine phosphorylation (Sameshima et al., 2001). When spores are activated to germinate and phosphorylation levels decrease, the actin tubules become unpacked and rods disassemble (Sameshima et al., 2001). Therefore actin tyrosine phosphorylation may drive rod maturation (Sameshima et al., 2001). The transcription factor
SrfA appears important for the process, since null mutants have decreased levels of phosphorylation, defective rod elongation, and compromised spore maturation (Escalante et al., 2004). The actin-binding proteins that cross-link filaments and tubules to form rods are unknown, but $\alpha$-actinin, ABP-120, and EF-1$\alpha$ are not required for the process (Sameshima et al., 2000). Myosin is also not detected on the rods (Sameshima et al., 2001). Cofilin is a component of the cytoplasmic rods but not the nuclear rods (Sameshima et al., 2000). In addition, S-adenosyl-L-homocysteine hydrolase is sequestered into both cytoplasmic and nuclear rods, an event that appears to correlate with actin tyrosine phosphorylation (Kishi et al., 2001).

Currently little is known about how actin tyrosine phosphorylation is regulated. The kinase responsible is unknown, although SplA is a candidate since actin tyrosine phosphorylation is not detected in spores of null mutants (Kishi et al., 1998). Conversely, the tyrosine kinases Zak1, Zak2, and PkyA are not components of the phosphorylation pathway (Liu et al., 2006). The phosphotyrosine phosphatase PTP1 contributes to actin dephosphorylation while PTP2 and PTP3 do not (Howard et al., 1993; Howard et al., 1994; Gamper et al., 1999).

### 1.6 Objective

Because tyrosine phosphorylation of actin is associated with spore dormancy, microcyst formation as an alternative dormant state may provide additional insight into the role of actin tyrosine phosphorylation. Microcysts were studied in the basal species *Polysphondylium pallidum* since the encystment pathway has been lost in *Dictyostelium discoideum* (Schaap et al., 2006). In addition, microcysts are ancestral to spores (Kawabe et al., 2009). Consequently the role of actin phosphorylation in spores may be derived from that in microcysts. Finally, differentiation and germination appear to be less complex processes in microcysts than in spores, since they mainly depend on the osmolarity of the medium (Toama and Raper, 1967a; Cotter and Raper, 1968). Therefore microcysts provide an alternative model system in which to study tyrosine phosphorylation of actin.

### 2 Materials and Methods

#### 2.1 Culture methods

Wild-type *Polysphondylium pallidum* was obtained through the dictyBase stock center (http://dictybase.org/StockCenter/StockCenter.html, strain ID DBS0236811). Stock cultures
were grown with *Escherichia coli* strain B/r on SM (Sussman, 1987) agar plates (0.5 g/l yeast extract, 5 g/l proteose peptone #3, 5 g/l glucose, 18 g/l agar, 2.31 g/l KH2PO4, 1.3 g/l Na2HPO4, pH 6.5). When fruiting bodies had formed, the plates were refrigerated for long-term storage. Amoebae were grown in axenic liquid cultures of modified Sussman’s medium, or MSM (15 g/l proteose peptone, 1.45 g/l KH2PO4, 0.72 g/l Na2HPO4, 0.4 g/l lecithin, 1 g/l glucose) to induce encystment (Sussman, 1963; O’Day, 1974). In brief, *Polysphondylium pallidum* spores were resuspended in 5 ml of MSM containing 1 g/l streptomycin sulfate salt (Sigma Aldrich, Oakville, ON) and 0.5 g/l ampicillin sodium salt (Sigma Aldrich, Oakville, ON). When the spores had germinated, 0.5 ml of the culture was transferred to 50 ml of fresh MSM without antibiotic. Amoebae cultured in this manner reached the stationary phase by 2-3 days and encystment was complete (at least 95% cysts) within 6-8 days. For microcyst formation in non-nutrient buffers, amoebae from MSM cultures aged 2 days were pelleted and resuspended at a concentration of 1 x 10^7 cells/ml in KCl buffer (0.08 M KCl in 0.01 M potassium phosphate buffer, pH 6.5) or sucrose buffer (0.144 M sucrose in 0.01 M potassium phosphate buffer, pH 6.5). For germination, microcysts from MSM cultures aged 7-9 days were pelleted and resuspended in 0.01 M potassium phosphate buffer, pH 6.5, at a concentration of 1 x 10^7 cells/ml. Microcyst formation and germination were monitored using phase contract microscopy, where at least 200 cells were counted and scored as microcyst or amoeba based on morphology. In cultures treated with potassium bisperoxo (1,10-phenanthroline) oxovanadate (V) (Santa Cruz Biotechnology Inc., Santa Cruz, CA), the tyrosine phosphatase inhibitor was added from a 0.01 M stock in doubly distilled water to a concentration of 200 µM in culture.

### 2.2 Cell harvest

Cells were pelleted, resuspended in buffer containing 20 mM Tris-HCl pH 6.8, 1 mM EDTA, and a cOmplete™ Mini EDTA-free protease inhibitor cocktail tablet (Roche Diagnostics, Mississauga, ON) at a concentration of about 3 x 10^8 cells/ml, and disrupted by sonication on ice. Lysates were stored at -80°C until used. In experiments where germinating cultures were separated into amoeba and microcyst fractions, pelleted cells were resuspended in Nonidet® P40 buffer (50 mM Tris pH 7.4, 150 mM NaCl, 0.5% v/v Nonidet® P40, and a cOmplete™ Mini EDTA-free protease inhibitor cocktail tablet), vortexed, and pelleted. 0.5% Nonidet® P40 lyses amoebae without disrupting microcysts (Ennis *et al.*, 1978). Supernatant containing amoeba
Lysate was collected, and the microcyst pellet was resuspended in an equal volume of fresh buffer. Microcysts were disrupted by sonication and lysates stored as described above.

### 2.3 Immunoblotting

Sample volumes corresponding to 10 µg of protein were boiled for 5 minutes in an equal volume of electrophoresis sample buffer (0.16 M Tris-HCl pH 6.8, 16% v/v glycerol, 5.3% w/v SDS, 5.0% w/v dithiothreitol, and 0.0027% w/v bromophenol blue), resolved with 15% SDS-PAGE alongside a Spectra™ Multicolor Broad Range Protein Ladder (Fermentas Canada Inc., Burlington, ON), and transferred to a BioTrace™ PVDF membrane (Pall Corporation, Pensacola, FL). Membranes were blocked in 5% non-fat milk in TTBS buffer (24.8 mM Tris pH 7.6, 2.68 mM KCl, 137 mM NaCl, 0.1% v/v Tween-20) for 1 hour at room temperature.

Antibody incubations were carried out for 1 hour in 2% w/v bovine serum albumin in TTBS at room temperature. Anti-phosphotyrosine mouse antibody P-Tyr-100 (Cell Signaling Technology Inc., Danvers, MA) or anti-β-actin mouse antibody C4 (Santa Cruz Biotechnology Inc., Santa Cruz, CA) were both used at a 1:1000 dilution. Prior to incubation with secondary antibody, membranes were washed for 21 minutes with TTBS washing buffer replaced every 7 minutes. Secondary HRP-conjugated goat anti-mouse antibody (Cedarlane Laboratories Ltd., Burlington, ON) was used at a dilution of 1:16,000. Following incubation with secondary antibody, membranes were washed for 30 minutes with TTBS washing buffer replaced every 10 minutes. Visualization was carried out using Amersham ECL Plus™ detection reagents (GE Healthcare, Buckinghamshire, UK) and Storm™ 840 Phosphorimager/Fluorimager scanner (Molecular Dynamics Inc., Sunnyvale, CA). Protein bands were quantified using ImageQuant™ 5.2 software (Molecular Dynamics Inc., Sunnyvale, CA) with Object Average background correction. Statistical analysis was performed using the R software package, version 2.11.1 (The R Foundation for Statistical Computing, http://cran.r-project.org).

### 2.4 Immunoprecipitation

Microcysts from cultures aged 7 to 8 days were pelleted, resuspended in RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.25% w/v sodium deoxycholate, 1% Nonidet® P40, and a cOmplete™ Mini EDTA-free protease inhibitor cocktail tablet) at a concentration of about 1.6 x 10^8 cells/ml, and disrupted by sonication on ice. Each 100 µl sample contained about 0.26 mg of protein. Samples were stored at -80°C until used, where 0 or 6 µl of anti-β-actin
mouse antibody C4 (Santa Cruz Biotechnology Inc., Santa Cruz, CA) were added. The samples were incubated at 4°C for 1 hour with rotation. Following incubation, 20 µl of Protein G PLUS-Agarose (Santa Cruz Biotechnology Inc., Santa Cruz, CA) suspension were added and the samples were immunoprecipitated at 4°C overnight with rotation. The immunoprecipitate was pelleted, washed 4 times with 100 µl RIPA buffer for each wash, and resuspended in 10 µl electrophoresis sample buffer. Samples were stored at -20°C until used for immunoblotting as described above, where each sample was loaded in its entirety.

2.5 Immunofluorescence staining

The immunostaining procedure, modified from Hagedorn et al., 2006, utilized ultracold methanol fixation followed by indirect immunofluorescence staining. Linkam 13 mm x 0.1 mm coverslips (McCrone Microscopes & Accessories, Westmont, IL) were washed in 95% ethanol for 30 minutes, rinsed with distilled water, and coated with a solution of 0.5% w/v gelatin and 0.05% w/v chromium potassium sulfate to enhance adhesion of microcysts. 200 µl of culture were placed on a coverslip 15-30 minutes prior to fixation. Excess liquid was dabbed from the surface and the coverslip was submerged in -80°C methanol. After 50 minutes, coverslips were washed by dipping in PBS buffer (171 mM NaCl, 8.49 mM KH₂PO₄, 3.73 mM K₂HPO₄) 3-5 times and incubated in blocking buffer (0.2% w/v gelatin, 0.1% v/v Triton® X-100, 1% v/v DMSO in PBS). Following blocking, coverslips were placed face-down on 50-µl drops of primary antibody diluted to 1:50 in blocking buffer. Primary antibodies used were anti-phosphotyrosine mouse antibody P-Tyr-100 (Cell Signaling Technology Inc., Danvers, MA), anti-β-actin mouse antibody C4 (Santa Cruz Biotechnology Inc., Santa Cruz, CA), or anti-actin rabbit antibody A2066 (Sigma Aldrich, Oakville, ON). Secondary fluorophore-conjugated antibodies Alexa Fluor® 555 goat anti-rabbit or Alexa Fluor® 488 goat anti-mouse (Invitrogen, Eugene, OR) were used at a dilution of 1:100. Antibody incubations were carried out at room temperature for 1 hour. Coverslips were washed by dipping in PBS 5 times following each antibody incubation. Prior to visualization by microscopy, coverslips were placed face-down on glass slides and the edges sealed with nail polish. A Nikon Eclipse 50i epifluorescence microscope (Nikon Canada Inc., Mississauga, ON) equipped with B-2E/C and G-2E/C filter cubes and a Nikon DS-Ri1 camera, and NIS-Elements BR imaging software (version 3.00) were used to capture images at 1000 times magnification. At least 5 experiments were performed for each time indicated.
3 Results

3.1 Actin is tyrosine phosphorylated in *Polysphondylium pallidum* microcysts

When cultured in MSM, *Polysphondylium pallidum* amoebae proliferated to concentrations on the order of $10^6$-$10^7$ cells/ml within 2-3 days (Figure 1.3,a). At least 95% encystment within 6-8 days was routine (Figure 1.3,b). Levels of tyrosine phosphorylation in vegetative amoebae were compared to those in dormant microcysts by immunoblotting (Figure 1.4,a). An abundant phosphoprotein that migrated between the 42- and 52-kDa markers was detected in microcysts. The intensity of the phosphoprotein band was significantly higher in microcysts than in amoebae (Figure 1.4,b). Based on the molecular weight and intensity of the band, the protein was hypothesized to be actin. In keeping with this, the relative amount of tyrosine phosphorylated actin was expressed as the intensity ratio of the actin bands detected with anti-phosphotyrosine antibody P-Tyr-100 and anti-actin antibody C4. In dormant microcysts, this amount varied from 0.392 to 1.86 between all experiments performed, with a mean of 0.948 and a standard error of 0.0534 (n=34). Two other intense tyrosine phosphoproteins appeared near the 42-kDa marker just below the actin band.

Immunoprecipitation was performed to confirm the identity of the tyrosine phosphorylated protein as actin (Figure 1.5). Samples of dormant microcysts were immunoprecipitated with the anti-actin antibody C4 and immunoblotted. The anti-phosphotyrosine antibody P-Tyr-100 detected a protein migrating between the 42- and 52-kDa markers in the anti-actin immunoprecipitate, but not in the control to which no antibody was added. Bands corresponding to the anti-actin antibody heavy and light chains (migrating just above the 52- and 26-kDa markers, respectively) were also detected in the immunoprecipitate and antibody lanes but not in the control or supernatant lanes.

3.2 Actin tyrosine phosphorylation increases during encystment

Tyrosine phosphorylation of actin was examined during microcyst formation in MSM. Progress of encystment was determined based on the percentage of microcysts present, where phosphorylation levels in samples with 0-10%, 20-30%, 45-55%, 70-80%, and 90-100% microcysts were analyzed (Figure 1.6,a). Actin tyrosine phosphorylation levels increased
Figure 1.3: Micrographs of live *Polysphondylium pallidum* a) amoebae in MSM after 2 days and b) microcysts from the same culture after 8 days. Scale bars indicate 10 μm.
**Figure 1.4:** Tyrosine phosphoproteins in *Polysphondylium pallidum.* a) Immunoblots of vegetative amoebae from MSM culture after 2 days and dormant microcysts from the same culture after 7 days. Separate blots of the same sample were incubated with anti-phosphotyrosine P-Tyr-100 (top) or anti-actin C4 (bottom). P-Tyr-100 detected an intense band between the 42- and 52-kDa markers in microcysts (black arrow). White arrows indicate molecular weights in kDa. b) Quantification of the indicated protein band’s intensity, expressed as a fraction of the intensity of the actin band. Bars indicate means with standard error based on the average of 5 experiments. The intensity of the protein band was significantly higher in microcysts than in amoebae (one-sided paired t-test, p < 0.001).
Figure 1.5: Immunoprecipitation of actin from dormant *Polysphondylium pallidum* microcysts in cultures after 7-8 days. Samples were immunoprecipitated with 0 or 6 µl of the anti-actin antibody C4 and separated into immunoprecipitate (“IP”) and supernatant (“Super”) fractions. A whole cell microcyst sample and 6 µl of the anti-actin antibody were run alongside the immunoprecipitation samples. The immunoblot was probed with the anti-phosphotyrosine antibody P-Tyr-100. Black arrows indicate the positions of the three major bands detected. White arrows indicate molecular weights in kDa.
exponentially as cells in the culture encysted (Figure 1.6,b).

In addition to MSM (Sussman, 1963; O’Day, 1974), iso-osmotic solutions of KCl and sucrose have been shown to yield approximately equal percentages of microcysts (Toama and Raper, 1967a). To examine whether tyrosine phosphorylation of actin also occurs under these encystment conditions, phosphorylation in microcysts formed in MSM was compared to that of microcysts formed in KCl and sucrose-based encystment buffers (Figure 1.7,a). These non-nutritive encystment media consisted of germination buffer (0.01 M potassium phosphate, pH 6.5) in which the osmolarity had been increased by addition of KCl (0.08 M) or sucrose (0.144 M), concentrations optimal for microcyst formation (Toama and Raper, 1967a). Phosphorylation was detected during microcyst formation in each medium, where it increased to the same level in all three media (Figure 1.7,b). Amoebae in potassium phosphate buffer to which KCl or sucrose had not been added did not encyst and their levels of actin tyrosine phosphorylation did not increase (results not shown).

3.3 Actin tyrosine phosphorylation decreases during germination

When microcysts were transferred to germination buffer, levels of actin tyrosine phosphorylation began to steadily decrease (Figure 1.8,a). By 6 hours of germination, over 90% of microcysts had germinated and actin phosphorylation decreased to less than 2% of original levels (Figure 1.8,b). Emergence of amoebae began at 3 hours of germination, by which time tyrosine phosphorylation of actin had declined to below 7%. The two phosphoproteins which migrated just below actin also appeared to undergo dephosphorylation.

3.4 Effect of bpV(phen) on germination and tyrosine dephosphorylation of actin

To determine whether tyrosine dephosphorylation of actin is required for germination, the tyrosine phosphatase inhibitor potassium bisperoxo (1,10-phenanthroline) oxovanadate (V) (bpV[phen]) was added to a concentration of 200 µM at 0 or 3 hours germination (Figure 1.9,a). When added at 0 hours, bpV(phen) completely inhibited germination, with an average emergence of 4.67% at 6 hours compared to 92.0% in the control (Figure 1.9,b). Average actin tyrosine phosphorylation in the inhibited culture decreased to 44.6% of the level present in dormant microcysts, and to 2.18% in the control (Figure 1.9,c). In cultures treated with
Figure 1.6: Actin tyrosine phosphorylation during *Polysphondylium pallidum* microcyst formation. Samples from MSM cultures were collected when 0-10%, 20-30%, 45-55%, 70-80%, and 90-100% of cells had encysted. a) Separate blots of the same sample were incubated with anti-phosphotyrosine P-Tyr-100 or anti-actin C4. b) Band intensity of tyrosine phosphorylated actin was quantified as a fraction of the band intensity of total actin. Bars indicate means with standard error based on the average of 3 experiments.
Figure 1.7: Encystment of *Polysphondylium pallidum* in MSM and 0.01 M potassium phosphate buffer with 0.08 M KCl or with 0.144 M sucrose. Samples were collected when the encystment cultures contained 45-55% and 90-100% microcysts. a) Separate blots of the same sample were incubated with anti-phosphotyrosine P-Tyr-100 or anti-actin C4. b) Band intensity of tyrosine phosphorylated actin was quantified as a fraction of the band intensity of total actin. Bars indicate means with standard error based on the average of 3 experiments. Analysis of the means by two-way ANOVA found a significant difference in the amount of tyrosine phosphorylation between stages of encystment (p < 0.001), but not between different media (p > 0.05), and that the medium had no significant effect on the amount of phosphorylation at a given stage of encystment (p > 0.05). Letters indicate significantly different means based on Tukey’s contrasts at P = 0.05.
Figure 1.8: Actin tyrosine phosphorylation during germination of *Polysphondylium pallidum* microcysts. Starting at 0 hours germination, samples were collected every hour for 6 hours. a) Separate blots of the same sample were incubated with anti-phosphotyrosine P-Tyr-100 or anti-actin C4. b) Band intensity of tyrosine phosphorylated actin was quantified as a fraction of the band intensity of total actin based on 3 experiments. Progress of germination was expressed as a percentage of amoebae to total cells based on 8 experiments. Results indicate means with standard error.
bpV(phen) at 3 hours, average emergence at 6 hours was 45.5% and actin tyrosine phosphorylation declined to 11.0% of dormant levels. The reversibility of bpV(phen) added at 0 hours, based on the ability of washed microcysts to germinate over a period of 24 hours, was 84.0% (average of 3 experiments).

3.5 KCl induces tyrosine phosphorylation of actin in germinating microcysts

To determine the effect of increasing osmolarity during germination, KCl (0.08 M) was introduced into the culture at 3 hours. Tyrosine phosphorylation of actin increased rapidly in response, reaching levels comparable to those in dormant microcysts within 10 minutes after KCl addition (Figure 1.10). This was followed by a gradual decrease in phosphorylation. An hour after KCl addition, phosphorylation levels declined to about a third to a half of peak levels. These levels of phosphorylation remained significantly higher than those in control cultures and were sustained for at least 24 hours (Figure 1.11). Cultures to which KCl had been added exhibited 23.1% emergence by 6 hours, compared to 75.5% in control cultures.

Since cultures in which germination had been inhibited by KCl contained both emerged amoebae and ungerminated microcysts, the contribution of each type of cell to the total levels of actin tyrosine phosphorylation in the culture was investigated (Figure 1.12). Samples were separated into microcyst and amoeba fractions and actin tyrosine phosphorylation levels in each fraction were examined. At 6 hours germination, microcysts contained significantly higher levels of actin tyrosine phosphorylation than amoebae.

KCl addition at 3 hours germination also induced a transient change in the morphology of germinating microcysts (Figure 1.13). Within 5 minutes of KCl addition, the cells shrunk, assumed an irregular shape, and retracted from the cyst wall. Cell roundness was restored within 1 hour, although cell shape remained slightly irregular. Cells in treated germination cultures also decreased adhesion to the substratum, as fewer were retained on coverslips following KCl addition. Similarly to the change in cell shape, this response was less evident after 1 hour.

3.6 Immunolocalization of actin and phosphotyrosine in dormant and germinating microcysts

The localization of actin and phosphotyrosine was visualized by indirect immunofluorescence
Figure 1.9: Actin tyrosine phosphorylation during germination of Polysphondylium pallidum microcysts treated with the tyrosine phosphatase inhibitor bpV(phen). BpV(phen) was added to a concentration of 200 µM at 0 hours or 3 hours germination and samples were collected at 6 hours. Distilled water with no bpV(phen) was added to the control culture. a) Separate blots of the same sample were incubated with anti-phosphotyrosine P-Tyr-100 or anti-actin C4. b) Progress of germination was expressed as a percentage of amoebae to total cells. c) Band intensity of tyrosine phosphorylated actin was quantified as a fraction of the band intensity of total actin. Bars indicate means with standard error based on the average of 3 experiments. Analysis of the means by one-way ANOVA found significant differences in the amounts of germination in a) (p < 0.001) and in the amounts of tyrosine phosphorylation in b) (p < 0.001). Letters indicate significantly different means based on Tukey’s contrasts at P = 0.05.
Figure 1.10: Actin tyrosine phosphorylation following KCl addition during germination of Polysphondylium pallidum microcysts. At 3 hours germination, KCl was added to a concentration of 0.08 M. Samples were collected prior to KCl addition at 3 hours and every 10 minutes following KCl addition. No KCl was added to the control culture. a) Separate blots of the same sample were incubated with anti-phosphotyrosine P-Tyr-100 or anti-actin C4. b) Band intensity of tyrosine phosphorylated actin was quantified as a fraction of the intensity of total actin. Results indicate means with standard error based on the average of 5 experiments.
Figure 1.11: Actin tyrosine phosphorylation following KCl addition during germination of *Polysphondylium pallidum* microcysts. At 3 hours germination, KCl was added to a concentration of 0.08 M. Samples were collected at 0 and 3 hours prior to KCl addition and at 4, 6, and 24 hours following addition. No KCl was added to the control culture. a) Separate blots of the same sample were incubated with anti-phosphotyrosine P-Tyr-100 or anti-actin C4. b) Band intensity of tyrosine phosphorylated actin was quantified as a fraction of the band intensity of total actin. Progress of germination was expressed as a percentage of amoebae to total cells in germination culture. Results indicate means with standard error based on the average of 5 experiments. Analysis of the means by two-way ANOVA found a significant difference in the amount of tyrosine phosphorylation between germination times (p < 0.001), between control and KCl-treated cultures (p < 0.001), and that the treatment had a significant effect on the amount of phosphorylation at a given germination time (p < 0.01). Letters indicate significantly different means based on Tukey’s contrasts at P = 0.05.
staining. It should be noted that methanol fixation caused some shrinkage of the cells within their cyst walls, although in most cases their round morphology was preserved. For the purpose of this report, localization of the fluorophore-conjugated secondary antibodies will be assumed to represent the localization of actin or phosphotyrosine.

In dormant microcysts, fluorescence was observed throughout the cytoplasm when cells were immunostained for actin (Figure 1.14,a), phosphotyrosine (Figure 1.14,e), or both (Figure 1.14,i). By 1 hour of germination, actin localized as fibrillar projections at the cell periphery and around spherical structures inside the microcyst cytoplasm, most likely vesicles (Figure 1.14,b). These vesicles were also located towards the periphery of the cell. Phosphotyrosine was present around the vesicles (Figure 1.14,f), where it co-localized with actin (Figure 1.14,j), but not with the fibrils at the cell periphery. By 3 hours of germination, actin no longer localized to the vesicles, but to dense patches at the cell periphery (Figure 1.14,c). Phosphotyrosine was detected throughout the cytoplasm (Figure 1.14,g) and did not co-localize with actin (Figure 1.14,k). In emerged amoebae at 6 hours, actin was enriched in pseudopodia (Figure 1.14,d) while phosphotyrosine continued to be distributed throughout the cell (Figure 1.14,h). Actin and phosphotyrosine did not co-localize (Figure 1.14,l). The localization of actin in microcysts during germination is summarized in Figure 1.14,m.

The effect of the transient increase in actin phosphorylation caused by KCl addition at 3 hours of germination on actin and phosphotyrosine localization was also examined. Fifteen minutes after KCl addition, actin was detected throughout the microcyst cytoplasm (Figure 1.15,c). In the control actin continued to localize to patches at the cell periphery (Figure 1.15,a). Phosphotyrosine was distributed throughout the cell (Figure 1.15,g) where fluorescence appeared brighter than in the control (Figure 1.15,e). Within 60 minutes following KCl addition, actin localized to vesicles in the cytoplasm (Figure 1.15,d) along with phosphotyrosine (Figure 1.15,h), or to the cell periphery as seen earlier during germination. In control cultures, actin remained at the cell periphery (Figures 1.15,b) and phosphotyrosine throughout the cytoplasm (Figure 1.15,f).

3.7 Effect of aging on actin tyrosine phosphorylation and viability

To determine the levels of actin phosphorylation in aging microcysts, tyrosine phosphorylation was examined in cultures aged 1-9 weeks (Figure 1.16,a). Viability was measured as the ability
Figure 1.12: Actin tyrosine phosphorylation during germination of *Polysphondylium pallidum* microcysts. The osmolarity of the medium was increased at 3 hours germination by addition of KCl to a concentration of 0.08 M. No KCl was added to the control culture. Samples of whole culture and separate amoeba and microcyst fractions were collected at 6 hours. a) Separate blots of the same sample were incubated with anti-phosphotyrosine P-Tyr-100 or anti-actin C4. b) Band intensity of tyrosine phosphorylated actin was quantified as a fraction of the band intensity of total actin. Bars indicate means with standard error based on the average of 5 experiments. In total culture samples, levels of tyrosine phosphorylated actin were significantly higher in the KCl-treated culture than in the control (one-sided paired t-test, p < 0.05). The microcyst fraction of the KCl-treated culture contained higher levels of tyrosine phosphorylated actin than the amoeba fraction (one-sided paired t-test, p < 0.05).
Figure 1.13: Micrographs of live *Polysphondylium pallidum* microcysts at a) 3 hours germination; b) 3 hours and 5 minutes germination, 5 minutes following KCl addition; and c) 4 hours germination, 1 hour following KCl addition. KCl was added to a concentration of 0.08 M. Scale bar indicates 10 μm.
**Figure 1.14:** Micrographs of *Polysphondylium pallidum* cells stained by indirect immunofluorescence. Dormant microcysts (a, e, i), microcysts at 1 hour germination (b, f, j), microcysts at 3 hours germination (c, g, k), and emerged amoebae at 6 hours germination (d, h, l) were fixed using ultracold methanol. The cells were immunostained with anti-actin mouse antibody C4 and anti-mouse Alexa Fluor® 488 antibody (a-d), anti-phosphotyrosine mouse antibody P-Tyr-100 and anti-mouse Alexa Fluor® 488 antibody (e-h), or anti-actin rabbit antibody A2066 with anti-phosphotyrosine mouse antibody P-Tyr-100 and anti-rabbit Alexa Fluor® 555 antibody and anti-mouse Alexa Fluor® 488 antibody (i-l). A summary of actin localization in germinating microcysts is shown in m). Scale bars indicate 10 μm.
of microcysts to germinate when left in germination medium overnight (Figure 1.16,b). Both actin tyrosine phosphorylation and viability decreased in aging microcysts, but the kinetics of each decline were different. Microcysts from cultures older than 5 weeks experienced a sharp drop in viability, while tyrosine phosphorylation of actin decreased gradually as cultures aged.

4 Discussion

Actin was found to be tyrosine phosphorylated in microcysts of *Polysphondylium pallidum*. While little phosphorylation was detected in vegetative amoebae, during encystment phosphorylation levels increased gradually in the early stages and rapidly at the final stages of microcyst differentiation. This is comparable with previous observations in *Dictyostelium* spores, where actin tyrosine phosphorylation was found to be one of the final events of sporulation (Kishi et al., 1998). Therefore tyrosine phosphorylated actin appears to be an intrinsic characteristic of the dormant state.

Actin phosphorylation was independent of encystment conditions, as it occurred in microcysts formed in various high osmotic induction media. The effectiveness of iso-osmotic buffers based on either KCl or sucrose suggests that encystment is mainly due to osmolarity rather than ionic strength, consistent with previous studies on microcyst formation (Toama and Raper, 1967a; Ritchie et al., 2008).

Besides actin, two other tyrosine phosphoprotein bands which appeared during microcyst differentiation and disappeared during germination were detected. The protein migrating just below the actin band is possibly another actin isoform, since a comparable band was sometimes detected by anti-actin. The other tyrosine phosphoprotein is unknown. However, since the kinetics of tyrosine phosphorylation and dephosphorylation of this protein appear similar to those of actin, they may be regulated the same way. Determining the identity of this protein may provide additional insight into the mechanisms involved in germination.

During microcyst germination, actin underwent dephosphorylation prior to emergence of amoebae. Dephosphorylation began immediately upon germination and was complete within 3-4 hours, when emergence of amoebae began. Dephosphorylation was previously found to also occur during spore germination in *Dictyostelium*, where it was required but not sufficient for emergence (Gauthier et al., 1997; Kishi et al., 1998; Kishi et al., 2000). Treatment with tyrosine
Figure 1.15: Micrographs of germinating Polysphondylium pallidum microcysts fixed with ultracold methanol and stained by indirect immunofluorescence. Control cultures are shown at 3 hours and 15 minutes (a, e, i) or 4 hours germination (b, f, j). Cultures where KCl had been added to a concentration of 0.08 M at 3 hours are shown at 3 hours and 15 minutes (c, g, k) or 4 hours germination (d, h, l). Microcysts were immunostained with primary anti-actin mouse antibody C4 and secondary anti-mouse Alexa Fluor® 488 antibody (a-d), primary anti-phosphotyrosine mouse antibody P-Tyr-100 and secondary anti-mouse Alexa Fluor® 488 antibody (e-h), or primary anti-actin rabbit antibody A2066 with primary anti-phosphotyrosine mouse antibody P-Tyr-100 and secondary anti-rabbit Alexa Fluor® 555 antibody and secondary anti-mouse Alexa Fluor® 488 antibody (i-l). Scale bars indicate 10 μm.
Figure 1.16: Actin tyrosine phosphorylation in aging *Polysphondylium pallidum* microcysts. Lysates of microcysts from MSM cultures were collected every week for 9 weeks. a) Separate blots of the same sample were incubated with anti-phosphotyrosine P-Tyr-100 or anti-actin C4. b) Band intensity of tyrosine phosphorylated actin was quantified as a fraction of the band intensity of total actin. Cultures were left to germinate for 24 hours and amoebae that excysted during this time were counted as viable cells. Results indicate means with standard error based on the average of 5-7 experiments.
phosphatase inhibitors maintained high actin tyrosine phosphorylation in spores and they failed to germinate (Kishi et al., 1998). In microcysts, the tyrosine phosphatase inhibitor bpV(phen) completely inhibited germination and partially maintained phosphorylation when added at 0 hours. However, the addition of bpV(phen) at 3 hours also resulted in some inhibition of germination, despite actin tyrosine phosphorylation having declined to a low level by this time. This suggests a complex effect of bpV(phen) on germinating microcysts, where inhibition of germination cannot be attributed solely to inhibition of dephosphorylation. Another approach, such as identifying and knocking out the phosphatase responsible for actin dephosphorylation in germinating microcysts, may provide more conclusive insight on the requirement of dephosphorylation for germination.

An alternative method of assessing the requirement of actin dephosphorylation during germination is raising tyrosine phosphorylation levels during the process. This was achieved by increasing the osmolarity with KCl at 3 hours of germination, after the majority of actin had been dephosphorylated. KCl addition triggered renewed phosphorylation of actin in germinating microcysts. However, phosphorylation levels decreased to less than half their maximum value within an hour of KCl addition, after which they were maintained. Cultures to which KCl had been added excysted poorly compared to control cultures. These results demonstrate that germinating microcysts regulate actin tyrosine phosphorylation levels in response to changes in osmolarity. The increased phosphorylation levels were associated with inhibited germination, therefore actin dephosphorylation may be required for emergence. This is supported by the finding that in emerged amoebae from KCl-treated cultures, the levels of actin tyrosine phosphorylation were low and comparable to those in control germination cultures, while levels in ungerminated microcysts were significantly higher. Therefore microcysts committed to germination underwent complete dephosphorylation of actin following the transient increase, while the microcysts that discontinued germination sustained tyrosine phosphorylation of actin.

In addition to increasing actin tyrosine phosphorylation levels in germinating microcyst cultures, KCl caused shrinking of the cell inside the cyst wall. A similar response was previously observed in Dictyostelium amoebae, where a reduction in cell volume to 50% occurred in response to hyperosmotic shock (Zischka et al., 1999). The change in cell morphology was followed by a 32-fold increase in actin tyrosine phosphorylation (Zischka et al., 1999). Therefore germinating Polysphondylium microcysts reacted similarly to Dictyostelium amoebae, where a
change in cell shape was correlated with tyrosine phosphorylation of actin in response to high osmolarity. However in germinating microcysts this response was not maintained, as phosphorylation levels decreased and a round shape was restored.

The relationship between tyrosine phosphorylation, organization of the actin cytoskeleton, and cell shape was further studied by observing the changes in actin organization during germination. The germinating microcyst undergoes a transition from a round, immobile cell to an irregular-shaped, motile amoeba. Since cell shape is defined by the cytoskeleton, immunofluorescence was used to determine the organization of actin and its relationship to phosphorylation. In dormant microcysts, both actin and phosphotyrosine were detected uniformly throughout the cytoplasm. The lack of localization to specific structures may suggest that actin filaments are disassembled. Early during germination, however, both actin and phosphotyrosine localized to spherical bodies in the cytoplasm, most likely vesicles. The enlargement of vacuoles and polyvesicular bodies after the initiation of microcyst germination has been described previously and corresponds to the swelling stage (Hohl et al., 1970, O’Day, 1974). It is interesting to note that in dormant Dictyostelium spores tyrosine phosphorylated actin has been observed around vesicles by immunoelectron microscopy (Kishi et al., 1998). It remains possible that tyrosine phosphorylated actin surrounds vesicles during microcyst dormancy as well, but cannot be observed prior to vesicle enlargement due to insufficient resolution. Alternatively, the vesicles seen in swollen Polysphondylium microcysts and dormant Dictyostelium spores may not be corresponding structures. Determining the identity of these vesicles may provide insight about their role during germination. A possible approach would be to investigate co-localization with organelle-specific GTPases or phosphoinositides (reviewed in Behnia and Munro, 2005).

The significance of actin associating with vesicles is unclear, but one possibility is that the vesicles function in actin trafficking. During cytokinesis in Drosophila embryos, endosome-derived vesicles associated with F-actin transport it to the cleavage furrow (Albertson et al., 2008). Therefore vesicle-mediated actin trafficking may also take place in germinating microcysts. Vesicles that sequester actin on their surface could transport it to the cell periphery, where emergence through the cyst wall is facilitated by formation of pseudopodia. This would be consistent with the vesicles initially being observed near the cell periphery and with the later localization of actin to patches near the cell surface. Another possibility is that the vesicles are
involved in the secretion of hydrolytic enzymes, as enzyme activities accumulate during germination and may function in cyst wall digestion (reviewed in O’Day, 1977). In this scenario, actin may contribute to vesicle transport or positioning; vesicles and organelles have been previously shown to be associated with actin and transported on actin filaments by myosin motors (reviewed in DePina and Langford, 1999). It is worth noting that the vesicles in dormant Dictyostelium spores are immobile, but during germination vesicle movement is observed following actin dephosphorylation; therefore tyrosine phosphorylation of actin may keep cells as well as their intracellular components stationary during dormancy (Kishi et al., 1998).

In addition to vesicles, actin localized to the cell periphery in germinating microcysts. It was initially fibrillar or punctate and did not co-localize with phosphotyrosine. This may indicate that actin is undergoing polymerization beneath the cell surface, and that tyrosine phosphorylated actin is not being incorporated into filaments. Therefore tyrosine phosphorylation may be negatively regulating actin’s ability to polymerize. This would be consistent with previous studies with purified actin, which showed that the polymerization rate of tyrosine phosphorylated actin was reduced (Liu et al., 2006).

Just prior to emergence, actin appeared as solid patches at the cell periphery. By this point, the majority of actin had undergone dephosphorylation and actin did not co-localize with phosphotyrosine. Finally, in emerged amoebae, actin was enriched in pseudopodia. The strong localization of actin beneath the cell surface during the later stages of germination may be associated with the re-initiation of motility to enable emergence of the excysting amoeba. This would be consistent with the observation that emergence is achieved by extension of pseudopodia through gaps in the cyst wall (Hohl et al., 1970). If tyrosine phosphorylation of actin converts it to a state that does not favour assembly of filaments, dephosphorylation may be necessary in order for actin polymerization, amoebal emergence, and subsequent motility to take place.

In germinating microcysts treated with KCl at 3 hours, actin became uniform inside the cell within 15 minutes. The distribution of actin resembled that in dormant microcysts, suggesting that the cell may have re-established a dormant-like state. This, along with immunoblotting observations, implies that increased osmolarity leads to cytoskeletal reorganization which correlates with tyrosine phosphorylation of actin. One hour after KCl
addition, however, actin returned to being localized around vesicles and at the cell periphery. Therefore the events following KCl addition appear to mirror those of early germination. Whereas actin is initially tyrosine phosphorylated and dispersed throughout the cytoplasm, the onset of dephosphorylation is associated with the appearance of actin around vesicles followed by redistribution to the cell periphery.

Together, the immunoblotting and immunostaining results suggest a relationship between tyrosine phosphorylation of actin, the state and localization of the actin cytoskeleton, and cell morphology. The association of actin tyrosine phosphorylation with cytoskeleton rearrangement has been previously demonstrated in *Dicyostelium* amoebae, where induction of tyrosine phosphorylation and changes in cell morphology are correlated (Schweiger *et al*., 1992; Howard *et al*., 1993; Jungbluth *et al*., 1994; Zischka *et al*., 1999). Therefore tyrosine phosphorylation appears to regulate organization of the actin cytoskeleton in *Polysphondylium* as it does in *Dictyostelium*.

Additionally, the immunoblotting and immunofluorescence results support a strong association between actin tyrosine phosphorylation and microcyst dormancy. Tyrosine phosphorylation of actin has been observed in dormant states of several amoeboid eukaryotes. These include spores of *Dictyostelium discoideum*, *Dictyostelium mucoroides*, and *Dictyostelium purpureum* (Kishi *et al*., 1998), *Acanthamoeba castellanii* cysts (Liu *et al*., 2006), and now *Polysphondylium pallidum* microcysts. Cataloguing actin tyrosine phosphorylation during dormancy in other amoebozoans would provide insight into its evolution and may contribute to understanding the physiological function and adaptive significance of this event. Since amoebozoans that undergo encystment include pathogens such as *Entamoeba histolytica*, determining whether actin tyrosine phosphorylation occurs in this and other species could be of medical significance.

Another amoebozoan in which actin phosphorylation during dormancy has been documented is the plasmodial slime mould *Physarum polycephalum*. Tyrosine phosphorylation of actin is detected in flagellated swarm cells; however, these are active, not dormant, cells and the tyrosine residue phosphorylated is unknown (Shirai *et al*., 2006). However during dormancy, actin undergoes phosphorylation on a threonine, not tyrosine, residue. In the desiccation-resistant sclerotium state, actin is phosphorylated on a threonine at position 203 of the protein’s sequence.
(Furuhashi et al., 1998). Since threonine-203 phosphorylated actin undergoes loss of polymerizing activity, phosphorylation may inactivate actin during dormancy (Furuhashi et al., 1998; Furuhashi, 2002). Actin dephosphorylation occurs prior to germination of the dormant sclerotium following hydration (Furuhashi, 2002). Actin threonine phosphorylation has also been observed in dormant Physarum spores and microcysts, where dephosphorylation accompanies germination in both (Shirai et al., 2006). Since phosphorylation during dormancy appears to correlate with desiccation, its role may involve preserving the actin cytoskeleton during drought (Shirai et al., 2006). However, the effect of threonine phosphorylation on viability of the dormant states has not yet been characterized in Physarum.

On the other hand, in aging Dictyostelium spores actin tyrosine phosphorylation was found to correlate with viability (Kishi et al., 1998). A dramatic decrease in both occurred in spores older than 20 days (Kishi et al., 1998). Although actin phosphorylation and viability also declined in aging Polysphondylium microcysts, these decreases were not synchronous. Polysphondylium microcysts remained viable in cultures younger than 5 weeks, after which a sharp decline similar to that observed in Dictyostelium spores occurred. However, actin tyrosine phosphorylation experienced a steady decrease as microcysts aged. Based on this observation, maintenance of actin tyrosine phosphorylation in Polysphondylium microcysts does not appear to be as crucial for preserving long-term viability as it does in Dictyostelium spores. The differences in the relationship between actin tyrosine phosphorylation and viability in spores and microcysts may be due to different states of actin in each dormant form. In Dictyostelium spores, actin forms nuclear and cytoplasmic rods (Kishi et al., 1994; Sameshima et al., 1994; Sameshima et al., 2000; Sameshima et al., 2001; Sameshima et al., 2002). In Polysphondylium microcysts, no evidence for such structures has been found. Therefore in spores the role of actin tyrosine phosphorylation may be to stabilize the rods and in turn maintain viability (Kishi et al., 1998; Sameshima et al., 2001). In microcysts, phosphorylation may act mainly to preserve actin in an inactive, possibly disassembled, state until germination. If this is so, the phosphorylation state of actin would not be expected to have as drastic an effect on viability in microcysts as it does in spores. Finally, since aged microcysts in which levels of actin tyrosine phosphorylation are low did not initiate germination in the encystment medium, dephosphorylation alone appears insufficient for excystment and a low osmolarity trigger may be required.
5 Summary and Conclusions

This study found that actin was tyrosine phosphorylated in microcysts of the cellular slime mould *Polysphondylium pallidum*. During dormancy, phosphorylation levels were high and actin was observed throughout the cytoplasm. When germination was initiated, desphosphorylation began and actin and phosphotyrosine localized to cytoplasmic vesicles. Actin was also observed at the cell periphery, where it was initially fibrillar or punctate. However, after dephosphorylation was complete just prior to emergence, actin appeared as solid patches near the cell surface. Finally, in emerged amoebae, actin was enriched in pseudopodia. Increasing the osmolarity by KCl addition about halfway into germination caused renewed actin tyrosine phosphorylation, led to its redistribution throughout the cytoplasm, and impaired emergence. Together, these results indicate an association between actin tyrosine phosphorylation, organization of the actin cytoskeleton, and microcyst dormancy. Although the specific function of actin tyrosine phosphorylation in microcysts remains unclear, it may maintain the cytoskeleton in an inactive state. Thus the cell would be kept immobile for the duration of dormancy. Upon dephosphorylation during germination, the actin cytoskeleton would be restored to activity. Cell motility would be recovered and allow emergence. Since the differentiation of microcysts appears less complex than that of spores and evolved earlier (Kawabe *et al.*, 2009), microcysts of *Polysphondylium pallidum* provide a convenient alternative system in which tyrosine phosphorylation of actin can be studied.
References


Appendix

**Calmodulin Antagonists Inhibit *Polysphondylium pallidum* Microcyst Germination, but not Dephosphorylation of Tyrosine Phosphorylated Actin**

The previous chapter characterized tyrosine phosphorylation of actin in microcysts of the cellular slime mould *Polysphondylium pallidum*. Actin tyrosine phosphorylation increases during microcyst formation, remains high during dormancy, and decreases during germination. Since during germination dephosphorylation occurs prior to and may be required for emergence, studying the signalling events behind this process may lead to insight about its regulation. One protein that may be involved in dephosphorylation signalling is calmodulin. Calmodulin is a highly conserved calcium-binding protein that regulates target proteins by interacting with their calmodulin-binding domains; in the cellular slime mould *Dictyostelium discoideum*, at least five dozen calmodulin-binding proteins exist (reviewed in Catalano and O’Day, 2008). Calmodulin activity is required for germination of *Dictyostelium discoideum* spores (Lydan and Cotter, 1994; Lydan et al., 1994a; Lydan et al., 1994b), a dormant state in which actin is also tyrosine phosphorylated (Gauthier et al., 1997; Kishi et al., 1998). Therefore it was hypothesized that calmodulin would also be required for germination of *Polysphondylium pallidum* microcysts, and whether calmodulin signalling could function in regulating the dephosphorylation of actin during the process was investigated.

The effects of the calmodulin antagonists trifluoperazine (TFP), W7, and W5 on microcyst germination were tested. When added at 0 hours germination, each chemical dose-dependently inhibited emergence at 6 hours (Figure A.1). Whether TFP and W7 addition affected the levels of actin tyrosine phosphorylation was examined by immunoblotting, where concentrations that caused an average decline in emergence to below 15% at 6 hours germination were tested (Figure A.2). Although there was a trend for the actin phosphorylation levels to be higher in treated germination cultures, this difference was not statistically significant. Similarly, preliminary results indicated that TFP and W7 also inhibit emergence when added at 3 hours, without any effect on tyrosine phosphorylation of actin (not shown).

The dose-dependent inhibition of germination by the calmodulin antagonists TFP, W7, and W5 suggests that calmodulin activity is required for germination of *Polysphondylium pallidum* microcysts. This inhibition is unlikely to be due to non-specific effects of the
Figure A.1: Germination of *Polysphondylium pallidum* microcysts treated with the calmodulin antagonists a) trifluoperazine, b) W7, and c) W5. Indicated concentrations of each compound were added from stock solutions in distilled water to the germination culture at 0 hours. Progress of germination was expressed as a percentage of amoebae to total cells at 6 hours. Results indicate means with standard error based on the average of 3 experiments.
Figure A.2: Actin tyrosine phosphorylation during germination of *Polysphondylium pallidum* microcysts treated with the calmodulin antagonists trifluoperazine (15 µM) or W7 (50 µM). The compounds were added at 0 hours germination and samples were collected at 6 hours. Distilled water was added to the control culture. a) Separate blots of the same sample were incubated with anti-phosphotyrosine P-Tyr-100 or anti-actin C4. b) Band intensity of tyrosine phosphorylated actin was quantified as a fraction of the band intensity of total actin. Progress of germination was expressed as a percentage of amoebae to total cells in germination culture. Results indicate means with standard error based on the average of 3-5 experiments. Analysis of the means by two-way ANOVA found a significant difference in the amount of tyrosine phosphorylation between germination times (p < 0.01), but not between different treatments (p > 0.05), and that the treatment had no significant effect on the amount of phosphorylation at a given time (p > 0.05). Letters indicate significantly different means based on Tukey’s contrasts at P = 0.05.
compounds since each one acts in different ways. Also, W5, a structurally related but less potent calmodulin antagonist than W7, required higher doses to inhibit germination. Microcysts from treated cultures germinated when left overnight, indicating that the calmodulin antagonists considerably slow germination. However, dephosphorylation within 3 hours of germination occurred even in their presence. In addition, the calmodulin antagonists inhibited germination even when added at 3 hours, after dephosphorylation had already occurred. Therefore calmodulin activity is required throughout germination, but does not appear to regulate actin dephosphorylation during the process.

Since a significant effect of calmodulin antagonists on actin dephosphorylation during germination was not observed, the role of calmodulin was not pursued further. However, the inhibition of emergence suggests a requirement for calmodulin in some other aspect of germination. Therefore calmodulin signalling during microcyst germination may be worth investigating in future studies.

References


