The role of actin tyrosine phosphorylation in spore dormancy, and germination of Dictyostelium discoideum.

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UMI
THE ROLE OF ACTIN TYROSINE PHOSPHORYLATION IN SPORE DORMANCY, AND GERMINATION OF DICTYOSTELIUM DISCOIDEUM

by

Christopher H. Clements

A Thesis
Submitted to the Faculty of Graduate Studies and Research
Through the Department of Biological Sciences
In Partial Fulfillment of the Requirements for the Degree of Master of Science
At the University of Windsor

Windsor, Ontario, Canada
1998
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ABSTRACT

The regulation of cellular actin is critical in numerous dynamic processes in cells including cytokinesis, cytoplasmic streaming, and vesicle trafficking. In the slime mold *Dictyostelium discoideum*, a 43 kDa tyrosine phosphorylated protein, in dormant spores, has been shown to be actin. In this investigation similar patterns also have been found in *Dictyostelium purpureum* and *Dictyostelium mucoroides*.

The use of heat activation temperatures also were used to determine if these exposures caused actin dephosphorylation directly or indirectly by stimulating germination. This result revealed that dephosphorylation is not direct and non-viable spores do not undergo a dephosphorylation.

Treatments following spore activation that block germination, such as dinitrophenol, azide, anaerobic conditions, osmotic pressure, or phosphatase inhibitors, maintain tyrosine phosphorylation of actin which is detectable by Western blot analysis. The results indicate that a correlation exists between actin tyrosine phosphorylation and spore dormancy. The results further suggest that dormancy may be linked to restriction of mitochondrial function.

A second aspect of this study employs the use of the protein synthesis inhibitor, cycloheximide, to investigate the stage specific patterns of actin tyrosine phosphorylation in dormant, swollen, and germinating spores. These cycloheximide studies show that decreases in actin tyrosine phosphorylation are correlated to specific stages of spore germination.
Another aspect of this work employed trifluoperazine (TFP), an inhibitor of calmodulin function. When TFP was added following spore swelling, the spores collapsed and resembled dormant spores in morphology. The actin tyrosine phosphorylation pattern revealed no increase in phosphorylation following the TFP mediated collapse. The results indicate that unlike deactivation of spores following activation or addition of endogenous autoinhibitor which both yield dormant spore morphologies, actin tyrosine phosphorylation is not involved in the spore swelling collapse following addition of TFP.

Stages of the sexual life cycle of D. mucoroides were sampled and analyzed for the presence of actin tyrosine phosphorylation. The results indicate that only the mature macrocyst possess any detectable actin tyrosine phosphorylation.

The final aspect of this study investigated the actin tyrosine phosphorylation patterns of several mutants including RasG, which have been implicated in cytoskeleton mobilization.
ACKNOWLEDGEMENTS

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<td>germination adenyl cyclase</td>
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<tr>
<td>APS</td>
<td>ammonium persulfate</td>
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<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
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<tr>
<td>BCIP</td>
<td>5-bromo-3-chloro-indoylphosphate</td>
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<tr>
<td>BME</td>
<td>beta-mercaptoethanol</td>
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<td>BPV</td>
<td>potassium bisperoxo(1,10-phenanthroline)oxovanadate</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>double distilled water</td>
</tr>
<tr>
<td>DIF</td>
<td>differentiation-inducing factor</td>
</tr>
<tr>
<td>DNP</td>
<td>dinitrophenol</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>LP</td>
<td>lactose peptone</td>
</tr>
<tr>
<td>NBT</td>
<td>nitro blue tetrazolium</td>
</tr>
<tr>
<td>PAO</td>
<td>phenylarsine oxide</td>
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<tr>
<td>PKA</td>
<td>cAMP dependent protein kinase</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethylsufonyl fluoride</td>
</tr>
<tr>
<td>PP2B</td>
<td>protein phosphatase 2B</td>
</tr>
<tr>
<td>PVDF</td>
<td>polyvinylidene fluoride</td>
</tr>
<tr>
<td>SM/2</td>
<td>standard medium/2</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
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TEMED  N,N,N',N'-tetramethylethylenediamine
TFP    trifluoperazine
Tween 20 polyoxyethylenesorbitan monolaurate
INTRODUCTION

The cellular slime molds are a small group of eukaryotes. They are considered ideal organisms for studying morphological and biochemical aspects of development. Their ability to form a multicellular stage from a unicellular state and their ability to enter one of three developmental pathways are the intriguing factors making it a model organism. The most common pathway studied is the asexual life cycle (Fig. 1) of Dictyostelium discoideum which terminates with the formation of a fruiting body. Many other Dictyostelids follow this pathway including Dictyostelium mucoroides, and Dictyostelium purpureum. The major focus of the asexual life cycle in this study is on the germination of spores and the maintenance of spore dormancy in the sorocarp.

I. Maintenance of Spore Dormancy in the Asexual Life Cycle of Dictyostelium discoideum

Following aggregation and sporulation, a fruiting body is formed consisting of dead basal disk cells, dead stalk cells, and the sorus (Raper, 1984). The sorus houses the differentiated prespore cells that have matured into encapsulated spores. It is within the sorus that the mechanisms for the maintenance of spore dormancy are found. Within the sorus the spore mass is surrounded by a viscous fluid matrix containing proteins, yellow pigments, and the autoinhibitor which prevents spores from germinating. The autoinhibitor has been suggested to be an adenine derivative called discadenine (Abe et al., 1976). However, at this time the mode of action of discadenine is still unknown.
Figure 1. Asexual life cycle of *Dictyostelium discoideum*. The following stages are diagrammed: (a) spore dispersal; (b) spore germination; (c) vegetative growth; (d) nutrient starvation to pre-aggregation, time 0-5 hours; (e) beginning of aggregation, time 6 hours; (f) middle of aggregation, time 8 hours; (g) late aggregation, time 9 hours; (h) tipped aggregation, time 11 hours; (i) standing slug, time 13 hours; (j) initiation of pseudoplasmodium (slug) migration, time 16 hours; (k) end of short slug migration period, time 18 hours; (l) re-establishment of vertical polarity in preparation for culmination, time 19 hours; (m) initiation of culmination, time 20 hours; (n) early culmination, time 21 hours; (o) middle culmination, time 22 hours; (p) culmination complete, time 24 hours; and (q) fruiting body and spore maturation, time 1-10 days. Borrowed from Cotter *et al.* (1992).
It has also been shown that osmotic pressure prevents spore germination. A variety of solutions have been employed to osmotically prevent spore germination including 0.2 M sucrose. This treatment deactivates heat activated spores and returns them to the dormant stage within six hours (Cotter, 1977). Recently, a membrane bound adenylyl cyclase (ACG) which is specific to spores (Pitt et al., 1992) has been found to produce cAMP in response to osmotic pressure (van Es et al., 1996). The cAMP produced by the osmotic pressure via ACG inhibits spores by activating a cAMP dependent protein kinase (PKA) which in turn phosphorylates a variety of unknown proteins leading to dormancy (Fig. 2; van Es et al., 1996). The osmotic pressure that initiates maximal cAMP production has been shown to be generated by solutions of 100 to 200 mM sucrose and 100 mM NaCl. These concentrations are similar to the previous levels reported by Cotter (1977) that inhibit spore germination (van Es et al., 1996).

Additionally, high levels of ammonium phosphate are also detected in the fruiting body which may contribute to the maintenance of dormancy through ACG (Cotter et al., 1998). Spores have been shown to be inhibited from germinating in the presence of 69 mM ammonium ion and still retain their ability to germinate once washed free of the ammonium ion (Cotter et al., 1998). Through the use of the ACG null mutant (acg¹), which is insensitive to high osmotic stress (van Es et al., 1996), it also has been found that 50 mM ammonium phosphate will inhibit the premature maturation mutant SG1 which can spontaneously germinate and not the ACG null mutant from germinating. Both SG1 and acg- germinate in the 50 mM potassium phosphate control buffer. This indicates that ammonium phosphate blocks spore germination via ACG through an
Figure 2. Signal transduction pathway for osmoregulation of spore germination.

The presence of an osmotic pressure activates an adenylyl cyclase specific for the germination stage (ACG). This osmotic pressure activates ACG producing cyclic adenosine monophosphate (cAMP) that binds to the regulatory subunit (R) of a cAMP dependent protein kinase (PKA). The binding of cAMP to the regulatory subunit of PKA causes the catalytic subunit (C) to disassociate and become active preventing spore germination. Borrowed from van Es et al. (1996).
osmotic pressure

Figure 2.
alternative method other than the osmosensing mechanism (Cotter et al., 1998). These factors working in concert with one another act dynamically to ensure the maintenance of spore dormancy in the sori of fruiting bodies.

II. **Spore Germination in the Asexual Life Cycle of Dictyostelium discoideum**

The initial stage of the asexual life cycle of *D. discoideum* is the germination of nascent amoebae from their spore capsules. Germination of dormant spores consists of four stages; activation of dormant spores, post activation lag, swelling of dormant spores, and emergence of nascent myxamoebae (Fig. 3; Cotter, 1981; Cotter and Raper, 1968a; Cotter et al., 1992). The activation stage of germination is now defined as the period of time spores are exposed to an activating condition. Post activation lag is the phase between termination of the activation stimulus and spore swelling (Cotter et al., 1992). The activation of dormant spores is regulated by a number of environmental factors including pH, temperature, spore density, oxygen tension, osmotic pressure, autoinhibitors, and autoactivators (Cotter, 1975, 1981; Cotter et al., 1979). Interestingly, following spore activation and the initiation of spore germination, deactivation of the process can occur returning the spores to dormancy. However, deactivation can only take place during the lag phase prior to spore swelling (Cotter, 1977).

Wild-type strains NC4 and V12, along with their premature maturation daughter mutants SG1 and SG2 (spontaneous germinating mutants), exhibit the same morphological changes throughout germination (Cotter and Dahlberg, 1977; Dahlberg,
Figure 3. The stages of spore germination. Phase contrast microscopy of four stages in spore germination of *D. discoideum*: (A) dormant encapsulated spore; (B) early spore swelling characterized by phase darkening and a small protrusion; (C) late spore swelling characterized by a phase dark image and an oval shape; (D) emerged amoeba.

Magnification 1000X. Borrowed from Cotter and Raper (1968a).
and Cotter, 1978). Wild-type strains of *D. discoideum* spores can acquire spontaneous germination characteristics once aged 10-14 days. This ability has been suggested to be due to late spore maturation genes (Cotter and Glaves, 1989). Similarly, the addition of autoactivator, a low molecular weight, phosphorylated adenine derivative, will activate aged spores and overcome the inhibition of the autoinhibitor, discadenine, thus producing synchronous germination (Cotter and Glaves, 1989; Glaves and Cotter, 1989). Young spores (2-3 days old) of *D. discoideum* have been shown to be exogenously activated by heat shock (Cotter and Raper, 1966; 1968a), DMSO (Cotter et al., 1976), urea (Cotter and O’Connell, 1976), peptone, and a racemic mixture of the hydrophobic amino acids tryptophan, phenylalanine, and methionine (Cotter and Raper, 1966). The employment of heat shock, 30 minutes at 45°C, and DMSO will also result in synchronous germination (Cotter et al., 1976; Cotter and Raper, 1966; 1968). The activating agent, DMSO, is suggested to disrupt mitochondrial function which is accompanied by a rapid uptake of oxygen following the removal of the protein denaturant (Cotter et al., 1976). Interestingly, mitochondrial inhibitors such as azide and cyanide, that inhibit the function of cytochrome c oxidase during electron transfer, cause spore deactivation (Cotter et al., 1976).

The premature maturation mutants SG1 and SG2 have the ability to spontaneously germinate upon formation of the mature fruiting body and to release autoactivator thus stimulating spore germination in the rest of the population (Dahlberg, and Cotter, 1978). To date the gene or genes responsible for the spontaneous
germination phenotype are unknown. The events that take place to allow mature spores to germinate spontaneously are also unknown.

It has also been shown by Dahlberg and Cotter (1978) that protein synthesis is required for spore swelling in autoactivated spores of the premature maturation mutant SG1. Moreover, protein synthesis is also needed for emergence of heat activated wild-type spores. It has been suggested that the autoactivator may be a product of a protein produced during early spore swelling which is inhibited during the incubation of the spores with the protein synthesis inhibitor, cycloheximide (Cotter and Glaves, 1989; Dahlberg and Cotter, 1978).

III. Vegetative Growth, Aggregation, and the Developmental Phase of the Asexual Life Cycle of Dictyostelium discoideum

Following spore germination, the emerged amoebae become vegetative cells and begin the growth stage of the life cycle where bacteria as a food source allow numerous cell divisions to occur (Fig. 1). About 6-8 hours after the exhaustion of food sources a few cells within the population acquire the ability to secrete pulses of cyclic adenosine monophosphate (cAMP) and to recruit other cells toward them which in turn respond by secreting their own cAMP (Bonner, 1967). These groups of approximately $10^5$ cells form an aggregate around the cAMP centre that eventually forms the mound stage within the life cycle (Loomis, 1982). At this time cells have already begun to differentiate and enter the multicellular stage of development. From this mound of cells two particular cell types emerge; prestalk and prespore. Cells with prestalk characteristics are found in the tip of the mound while prespore cells constitute the entire lower portion of the
mound (Jermyn et al., 1989). These cells will terminally differentiate into the cells that form the mature fruiting body; stalk and spore cells, respectively. The tip of the mound, consisting of prestalk cells, elongates upwards forming a slug or pseudoplasmodium that topples over and migrates horizontally in search of a food source (Raper, 1940). In the absence of food the slug will round upon itself entering the next stage of development, culmination. Interestingly, this stage of development can also be initiated by light and low humidity levels (Raper, 1984). During culmination the anterior cells of the slug differentiate from prestalk to stalk cells and form the structure that supports the sori. The sori will be composed of the posterior area of the slug where prespore cells will differentiate into spore cells. Throughout this differentiation process the stalk cells elevate the prespore region to where the mature spores will form.

There are three morphogens that play a key role in the differentiation event (Cotter et al., 1992). The first, cAMP, which also plays a vital part in aggregation (Bonner, 1967), is critical in the differentiation of prespore cells into spore cells (Riley and Barclay, 1986). Produced in large quantities by prestalk cells (Pan et al., 1974), cAMP inhibits prestalk cells from differentiating (Berks and Kay, 1988). Contrary to this, the second morphogen, differentiation-inducing factor (DIF), inhibits prespore formation while stimulating the transformation of prestalk to differentiated stalk cells (Kay and Jermyn, 1983). The final morphogen that affects differentiation is ammonia. Ammonia acts to inhibit prestalk cells while stimulating prespore cells (Cohen, 1953). It has also been suggested that ammonia specifically increases the intracellular cAMP levels by inhibiting cAMP secretion (Riley and Barclay, 1990). In turn, this causes an
increase in intracellular pH and an inhibition in DIF activity (Van Lookeren Campagne et al., 1989).

Cotter et al. (1992) suggested a source and sink model to explain the interactions of the above morphogens. They have theorized that both cAMP and ammonia are produced by prestalk cells to stimulate prespore differentiation following transport, while DIF is manufactured in prespore cells to initiate prestalk differentiation following its secretion. As well, each morphogen inhibits differentiation at their manufactured site until they are transported away. These interactions lead to the formation of a mature fruiting body (Fig. 1) and may also play a role in maintaining spores in the sori.

IV. Sexual Life Cycles of the Dictyostelids

In addition to the asexual life cycle many species of the Dictyostelids can also enter a sexual phase of the life cycle producing macrocysts (Fig. 4). Macrocysts are dormant structures that initially are formed by the fusion of two amoebae. There are two types of macrocysts that can be formed; homothallic, which are formed by the fusion of cells from identical strains (D. mucoroides, strain Dm7) and heterothallic, which are formed by the fusion of cells from two different mating types within a species (D. discoideum NC4 and V12, SG1 and SG2; see Raper, 1984).

The formation of macrocysts is influenced by a number of factors including the absence of light, high humidity, low phosphate, very small amounts (ppm) of magnesium, nitrate, sulphate, chloride, and calcium (Erdos, et al., 1976; Raper, 1984). Early in the sexual life cycle small amoeboid cells called gametes can be distinguished
Figure 4. Sexual life cycle of *Dictyostelium discoideum*. After growth the amoebae either differentiate as macrocysts or undergo multicellular development to form a fruiting body. The formation of a fruiting body (asexual life cycle) is seen in Fig. 1 (a-q). The events leading to the formation of macrocysts (sexual life cycle) are outlined. Aggregation, cells aggregate together and two vegetative cells of the opposite mating type fuse to form a zygote (1); developing macrocysts, the zygote engulfs surrounding amoebae which become endocytes and a primary wall is formed (2); maturing aggregates, most amoebae are engulfed and a secondary wall is formed (3); aged macrocyst, endocytes are degraded and the cytoplasm shrinks away from the outer wall (4). Aged macrocysts are dormant and can be stimulated to germinate and release nascent amoebae. Borrowed from Cavallo (1996).
within the culture (Lydan and O’Day, 1988; O’Day et al., 1987). Gamete fusion takes place approximately 10 hours into development. Cells are characterized by two nuclei and an increase in the volume of the cytoplasm (Lydan and O’Day, 1988; O’Day et al., 1987). At approximately 18 hours nuclear fusion takes place resulting in zygote or giant cell formation (McConachie, and O’Day, 1987). Once the giant cells are formed they begin to attract amoebae by cAMP mediated chemoattraction. This stage constitutes the precyst (Urushihara, 1992) until an amoeba is engulfed and internalized in a vacuole, becoming an endocyte (Raper, 1984). While amoebae are being engulfed a primary wall forms surrounding a group of endocytes (O’Day, 1979). When the amoebae are completely ingested a secondary and tertiary cellulose wall is constructed forming a mature macrocyst. Aged macrocysts also constitute a stage of the sexual life cycle. The aged macrocyst is characterized by the degradation of the endocytes and the resulting dark and granular cytoplasm (Raper, 1984). The aged macrocyst now enters a period of dormancy that is controlled by environmental factors that will determine the time at which the macrocyst will germinate and release amoebae. Environmental conditions also contribute to whether the amoebae produced enter the asexual life cycle or return to the sexual life cycle to form macrocysts again (Urushihara, 1992). The homothallic macrocysts produced by D. mucoroides, strain Dm7, were used in this work.
V. Actin Cytoskeleton

Microfilaments (called F-actin or actin filaments) are composed of the protein actin. Each actin molecule (globular or G-Actin) is composed of two subunits, each consisting of two subdomains. These subunits become organized into strands that form a double helix in the presence of adenosine triphosphate (ATP), thus forming an actin filament. Each actin subunit also exhibits polarity and is therefore connected in the same direction, forming actin filaments with polarity. As a result, actin filaments are composed of a plus and a minus end. In vitro addition of actin monomers occurs primarily at the plus end while depolymerization of actin takes place from the minus end (Karp, 1996).

Actin filaments have been implicated as playing a functional role in cytoskeletal reorganization including cell motility, membrane trafficking and cell morphology (Rauchenberger et al., 1997). The associations between actin and the force generating myosin proteins is critical to the cytoskeleton. There are two classes of myosin; the conventional or myosin II class and the unconventional or myosin I class. Without exception, all the studied myosins move toward the plus ends of the actin filaments generating force. Myosin II is generally associated with cytokinesis, while myosin I functions have been implicated in membranous organelles, locomotion, and vesicle movement along actin filaments (Karp, 1996). It is not the objective of this thesis to extensively review the interactions of this relationship, but to focus primarily on the regulation and role of actin. Thus, with few exceptions, it can be assumed that myosin proteins are involved in the actin cytoskeleton.
Actin-binding proteins play a vital part in the characteristics of actin filaments. These include those affecting their assembly, their physical properties, and their interactions with cellular organelles and one another. There are several categories of actin-binding proteins such as monomer-sequestering proteins, capping proteins, cross-linking proteins, filament-severing proteins and membrane-binding proteins (Fig. 5). Monomer-sequestering proteins such as profilin act to bind monomeric actin and are critical in the cytoplasm of non-muscle cells due to the high levels of G-actin (50-200 μM). Conditions in the cytoplasm generally promote polymerization of G-actin. Therefore, a change in the levels of monomer-sequestering proteins can significantly affect the balance between filamentous and globular actin in the cell (Karp, 1996). Capping proteins which include cap-Z and β-actinin are involved in determining the length and numbers of actin filaments. These proteins can bind to the plus end of an actin filament terminating its elongation. This allows depolymerization to occur at the minus end if required. Such capping can also stimulate more actin filaments to form leading to numerous smaller helices. Cross-linking proteins possess two actin binding sites allowing for three dimensional organization via the formation of parallel actin bundles and cross-linked filaments (Fig. 5) that form at right angles. Filamin and ABP280 are involved in the formation of right angled filaments while globular shaped villin and fimbrin maintain actin filaments in tight bundles (Karp, 1996). Filament-severing proteins act to slice existing actin filaments into separate sections. This rapidly reduces their lengths and can lead to a drastic decrease in the viscosity of the cytoplasm. Proteins such as severin and fragmin may also contribute to the depolymerization of
Figure 5. Actin binding proteins in *D. discoideum* and their function. Actin binding proteins influence the equilibrium between G-actin and filamentous actin as well as the structural organization of the network of actin filaments. They either bind to monomeric actin and inhibit polymerization, or cap, sever, anchor, crosslink or move actin filaments by binding to the ends or along filaments. Borrowed from Noegel *et al.* (1996).
Figure 5.
actin filaments by increasing the number of free ends available. This result allows monomeric G-actin to assemble. The final category of actin-binding proteins play a role in membrane movements including cell locomotion, cytokinesis, and phagocytosis. These membrane-binding proteins link actin filaments to plasma membranes and consist of several families of proteins.

Recently, the regulation ability of a number of these actin-binding proteins has been shown to be dependent on their phosphorylation and dephosphorylation (Bretscher, 1989; De Corte et al., 1997; Huang et al., 1997). Similarly, actin itself has been shown to be regulated by phosphorylation. In fibroblasts, actin can be phosphorylated on serine residues following exposure to epidermal growth factors (van Delft et al., 1995). In the true slime mold, *Physarum polycephalum*, phosphorylation has been shown to occur on threonine residues 202 and 203 in complex with the capping protein fragmin (De Corte et al., 1996). An actin-fragmin kinase in *Physarum* that specifically phosphorylates the actin-fragmin heterodimer on actin threonine residues has also been uncovered (Eichinger et al., 1996). During the formation of macrocysts from plasmodia, under desiccating conditions, *Physarum* phosphorylates approximately 50% of its actin at threonine residue 203. Following the phosphorylation of the actin no polymerizing activity exists. It is suggested by the authors that the phosphorylation of actin is involved in the transformation from plasmodium to macrocyst (Furuhashi et al., 1998). It is obvious that phosphorylation plays a key regulatory role on actin through either phosphorylation of the actin-binding proteins or actin itself.
VI. Actin Cytoskeleton of *D. discoideum*

Actin genes are highly conserved in eukaryotes making the unicellular slime mold, *D. discoideum*, a model system for studies of this protein. In *D. discoideum*, actin filaments have been shown to exist in both the cytoplasm and the nucleus (Fukui, 1978; Kishi et al., 1994; Sameshima et al., 1994). In dormant spores of the wild-type NC4, F-actin bundles have been observed in the cytoplasm (Sameshima et al., 1994) as well as in the nucleus under non-stress conditions (Kishi et al., 1994).

In *D. discoideum* vegetative cells, actin has been shown to be tyrosine phosphorylated in the presence of the mitochondrial uncoupler, 2,4-dinitrophenol (dnp), azide, heat stress, and Cd$^{2+}$. This phosphorylation is quickly reversed following the removal of these stresses (Jungbluth et al., 1994, 1995). Interestingly, recent discoveries of tyrosine phosphorylation have been made in many organisms including prokaryotes such as cyanobacteria and bacteria of several Archaea subdomains (McCartney et al., 1997; Smith et al., 1997). Actin phosphorylation in *D. discoideum* has been shown to be at the tyrosine-53 residue following inhibition of oxidative phosphorylation in vegetative cells (Jungbluth et al., 1995). Additionally, 10 to 15% of the total actin has been found to be phosphorylated at tyrosine residues in vegetative cells starved and returned to growth medium. These cells became round, lost pseudopodial extensions, and had reduced attachment to the substratum showing non-phosphorylated actin is critical to these functions. A similar study also found that phosphotyrosine phosphatase PTP1 is partially involved in the dephosphorylation of actin (Howard et al., 1993). Recently, dormant spores of *D. discoideum* have been shown to display high levels of actin
tyrosine phosphorylation while germinating spores exhibited progressively lower tyrosine phosphorylation patterns. Emerged amoebae displayed little or no actin tyrosine phosphorylation. The 43 kDa protein, detected by Western blot analysis and presumed to be actin, has been confirmed by two groups using anti-actin antibodies and two dimensional electrophoresis (Gauthier et al., 1997; Kishi et al., 1998).
VII. Objectives of This Investigation

From recent work, it is clear that further characterization of the role of actin tyrosine phosphorylation in *D. discoideum* is required. This particular study will focus primarily on increasing the understanding of the dynamics associated with actin regulation in regards to *D. discoideum* spore germination. The specific areas or subjects follow:

A. **Determine if the dephosphorylation of actin during the germination of *D. discoideum* spores is dependent on the morphological stage of germination.**

Alternative activation methods that shorten the germination process will be employed. Samples will be collected each half hour during the germination program thus allowing the stage specific phosphorylation to be visualized via Western blotting.

B. **Determine whether continuous heat activation temperatures induce dephosphorylation of actin in *D. discoideum*.** Heat activation methods cause dormant spores to germinate if placed at room temperature (Cotter and Raper, 1968). Spores will be incubated at heat activation temperatures for 5 hours and sampled. The incubation may determine if heat activation is responsible for actin tyrosine dephosphorylation or if it stimulates other mechanisms that will lead to the eventual dephosphorylation of actin.
C. **Determine if deactivation of spore germination following activation is accompanied by actin tyrosine phosphorylation.** Using a variety of deactivation methods, including phosphatase, mitochondrial, and protein synthesis inhibitors as well as osmotic pressure it will be determined if a correlation exists between spore dormancy and actin tyrosine phosphorylation. Furthermore, a possible relationship will be evaluated between mitochondrial activity and spore germination.

D. **Determine if inhibition of spore germination by ammonia is accompanied by actin tyrosine phosphorylation.** Spores, from the premature maturation mutant SG1, germinated in solutions of ammonium phosphate, and potassium phosphate, at different pH levels, will be analyzed for phosphorylated actin levels. It will be determined if ammonium phosphate at pH 7.2, which blocks spore germination (Cotter *et al.*, 1998), results in spores with more phosphorylated actin compared to the other treatments and to dormant spores. Phosphorylated actin via ammonia inhibition may also further substantiate the correlation between dormancy and actin tyrosine phosphorylation.

E. **Determine when the majority of actin tyrosine phosphorylation is observed relative to the stages of spore germination.** Using the protein synthesis inhibitor cycloheximide, spore germination will be inhibited prior to spore swelling and emergence. Removal of the inhibition will initiate a rapid advance to the next germination stage. This technique will hopefully allow for the determination of the stage specific dephosphorylation of actin.
F. Determine if the rapid collapse of swollen spores induced by the addition of trifluoperazine correlates with a phosphorylation event. Trifluoperazine (TFP), an inhibitor of calmodulin function, causes swollen spores to rapidly collapse upon its addition (Lydan and Cotter, 1994). Sampling during this event and analysis via Western blotting will determine if detectable phosphorylation occurs as a result of the collapse.

G. Determine if actin tyrosine phosphorylation occurs in alternative species of the *Dictyostelids*, the sexual life cycle, and in mutants of *D. discoideum* that have been implicated in cytoskeletal reorganization. The cellular slime molds *D. purpureum*, and *D. mucoroides* will be examined for actin tyrosine phosphorylation in dormant spores as well as dephosphorylation during germination. Several stages of the sexual life cycle of *D. mucoroides*, strain Dm7, will also be studied for actin tyrosine phosphorylation. Finally, a variety of mutants including Ddras G-, which have been implicated in cytoskeletal mobilization, will be analyzed for actin phosphorylation.
MATERIALS AND METHODS

I. Culture Conditions

Numerous strains of the cellular slime molds were used in this study. All cultures were maintained in the laboratory on either glucose salts medium (containing: 1.0 g NH₄Cl, 6.0 g Na₂HPO₄, 3.0 g KH₂PO₄, 0.13 g MgSO₄ and 20.0 g Bacto Agar (Difco, Detroit, MI) in 1L of double distilled water (ddH₂O); 10 mL of 0.4 g/mL of sterile glucose (Fisher Scientific, Nepean, ON, Canada) was added after the medium was autoclaved) or standard medium/2 (SM/2) (containing: 5.0 g glucose, 5.0 g Bactopeptone (Difco, Detroit, MI), 0.5 g yeast extract, 1.1 g KH₂PO₄, 0.5 K₂HPO₄, 0.5 MgSO₄, and 15.0 g Bacto Agar (Difco, Detroit, MI) in 1 L of ddH₂O adjusted to pH 6.5 using KOH) in association with Escherichia coli B/r or Klebsiella aerogenes. Each agar plate contained 15-20 mL of the medium (Cotter & Raper, 1968a).

To obtain sufficient samples for experiments, spores from the stock cultures were aseptically transferred to a conical tube containing spore germination buffer along with either Escherichia coli B/r or Klebsiella aerogenes. The 0.01 M phosphate germination buffer consisted of 1.36 g KH₂PO₄ in 1 L of ddH₂O. The germination buffer was adjusted to pH 6.5 prior to sterilization using KOH. The spore suspension was mixed by vortexing and 1.5 mL of the solution was added to glucose salts or SM/2 agar plates. The plates were gently shaken to facilitate an even spread of the spore solution and then incubated at room temperature. After 24 hours following the initial inoculation, the plates were again shaken to further promote an even distribution of the spores and
bacteria. Fruiting body formation occurred roughly 3 to 4 days after the initial inoculation.

II. **Spore Germination Conditions**

Spores were harvested by collecting the sori from the agar surfaces with a glass microscope slide. Following collection the spores were rinsed from the microscope slide into a 50 mL beaker containing 5 mL of ddH$_2$O. The spore suspension was then transferred to a 15 mL conical centrifuge tube and centrifuged in a table top IEC Clinical Centrifuge at 4500 x g for 3 minutes to produce a pellet. The supernatant, which contained crude matrix material, was saved while the pellet was resuspended by vortexing in spore germination buffer and centrifuged again at 4500 x g for 3 minutes. Washing of the spores was repeated twice as above.

A. Autoactivation

Pelleted spores were suspended in germination buffer (pH 6.5) at a concentration of $3 \times 10^7$ spores/mL in 1 X 10 cm glass test tubes along with 6 mm magnetic stir bars. Initial and final spore concentrations were determined using a hemacytometer. To allow sufficient oxygen exchange spore suspensions were kept at or below 4 mL and placed on a magnetic stirrer for the duration of the germination experiment. Wild-type strain NC4 were aged 14 days prior to harvesting while the mutant strains SG1 and SG2 were aged 3-4 days.
B. Heat activation

Strains NC4, V12, SG1, and SG2 all germinate under heat shock conditions regardless of spore age or density (Cotter & Glaves, 1989; Dahlberg & Cotter, 1978). Pelleted spores were suspended in germination buffer (pH 6.5) at a concentration of 1-5 x 10^7 spores/mL in 1 X 10 cm glass test tubes and placed in a Braun Thermomix II circulating water bath and heated for 30 minutes at 45°C. Following activation, 6 mm magnetic stir bars were added to the glass test tubes and the spores were placed at room temperature on a magnetic stirrer and allowed to germinate (Cotter, 1975; Cotter, 1981). The removal of the spores from the heat activating conditions was regarded as time 0 at which a sample was collected, pelleted, and then rapidly frozen at -20°C in an alcohol bath until needed.

C. DMSO activation

Pelleted SG1 spores were suspended in germination buffer (pH 6.5) containing 20 % DMSO (Cotter et al., 1976; Ennis and Sussman, 1975) at a concentration of 3 x 10^7 spores/mL in 1 X 10 cm glass test tubes for 1 hour. Following incubation in the 20 % DMSO solution, spores were washed twice as above in germination buffer and resuspended to a final spore concentration of 3 x 10^7 spores/mL. Magnetic stir bars (6 mm) were added to the glass test tubes and the spores were placed at room temperature on a magnetic stirrer to germinate (Cotter et al., 1976). The removal of the spores from the DMSO activating conditions was regarded as time 0 at which a sample was collected, pelleted, and then rapidly frozen at -20°C in an alcohol bath until needed.
D. Peptone activation

Pelleted SG1 spores were suspended in a 1 % peptone solution (containing: 10 g Bactopeptone (Difeo, Detroit, MI) in 1 L of germination buffer at pH 6.5) at a concentration of $4 \times 10^4$ spores/mL in a 1 L flask (Cotter and Raper, 1968a). The suspension was placed at room temperature on The Belly Dancer (Stovall Life Sciences, Inc., Greensboro, NC) at a setting of 2 to allow for sufficient oxygen exchange.

For all germination methods the suspensions were scored every hour using a Zeiss phase contrast microscope. Spores were recorded as either dormant (phase-bright, and capsule-shaped), swollen (phase-dark, and oval or irregular-shaped), or emerged (myxamoebae free of the spore cases). A minimum of 200 objects was counted. At the times indicated samples were centrifuged at 4500 x g in a table top IEC Clinical Centrifuge for 3 minutes and the supernatants discarded (Cotter & Raper, 1968a). The samples were then rapidly frozen at -20°C in an alcohol bath until needed.

III. Extended Heat Exposure Studies

Pelleted spores of wild-type NC4 and the premature maturation mutant SG1 were suspended separately in germination buffer (pH 6.5) at a concentration of $1-5 \times 10^7$ spores/mL in 1 X 10 cm glass test tubes and placed in a Braun Thermomix II circulating water bath and heated for 5 hours at 45°C. Again, samples were scored every hour to determine the germination stage of the spore suspensions. At the times indicated samples were centrifuged at 4500 x g in a table top IEC Clinical Centrifuge for 3
minutes and the supernatants discarded. The samples were then rapidly frozen at -20°C in an alcohol bath until needed.

IV. Spore Deactivation Agents

Various treatments were employed to deactivate wild-type NC4 spores, thus preventing their germination after activation by heating at 45°C for 30 minutes. Samples were continuously exposed to either 200 μM potassium bisperoxo(1,10-phenanthroline) oxovanadate (BPV) (Calbiochem, LaJolla, CA), 7 μM phenylarsine oxide (PAO) (Sigma-Aldrich Chemical Co., Mississauga, ON, Canada), 100 mM sucrose (Anachemia Science, Montreal, Que., Canada), 2 mM azide (Matheson, Coleman, & Bell Manufacturing Chemists, Norwood, OH), 1 mM 2,4-dinitrophenol (DNP) (Sigma Chemical Co., St Louis, MO), or a mineral oil overlay for 5 hours following activation. Samples were scored every hour microscopically as dormant, swollen, or emerged. At the times indicated samples were centrifuged at 4500 x g in a table top IEC Clinical Centrifuge for 3 minutes and the supernatants discarded. The samples were then rapidly frozen at -20°C in an alcohol bath until needed. Spore viability was tested by washing the spores, as above, and allowing them to germinate on 1 % peptone agar plates.

V. Ammonium Inhibition

Spores from the premature maturation mutant SG1 were collected as above and exposed to either 50 mM ammonium phosphate at pH 6.2, 50 mM ammonium phosphate at pH 7.2, 50 mM potassium phosphate at pH 6.2, or 50 mM potassium phosphate at pH
7.2 for 5 hours. The pH levels were altered by the addition of KOH. Samples were scored every hour microscopically as dormant, swollen, or emerged. At the times indicated samples were centrifuged at 4500 x g in a table top IEC Clinical Centrifuge for 3 minutes and the supernatants discarded. The samples were then rapidly frozen at -20°C in an alcohol bath until needed.

VI. Cycloheximide Studies

For heat activation (30 minutes at 45°C), cycloheximide added prior to heating allows spores to quickly swell but not to release nascent myxamoebae. Cycloheximide is removed by washing, as above, prior to resuspension in germination buffer (see Dahlberg and Cotter, 1978). Spores of the premature maturation mutant SG2, exposed to 200 µg/mL of cycloheximide do not swell. Crude autoactivator preparations from 10⁷ spores/mL, at a 1:2 dilution, will overcome the cycloheximide induced dormancy of SG2 spores (Dahlberg and Cotter, 1978). In this experiment, spores were collected as above and exposed to 200 µg/mL of cycloheximide (Sigma Chemical Co., St Louis, MO) in germination buffer for autoactivation (SG2) and heat activation (NC4) experiments to determine when the majority of actin dephosphorylation occurred in germinating spores. Samples were scored every hour microscopically as dormant, swollen, or emerged. At the times indicated samples were centrifuged at 4500 x g in a table top IEC Clinical Centrifuge for 3 minutes and the supernatants discarded. The samples were then rapidly frozen at -20°C in an alcohol bath until needed.
VII. Trifluoperazine Studies

Pelleted spores of the premature maturation mutant SG2 were suspended in germination buffer (pH 6.5) at a concentration of $3 \times 10^7$ spores/mL in 1 X 10 cm glass test tubes along with 6 mm magnetic stir bars and placed at room temperature on a magnetic stirrer to germinate. Trifluoperazine (TFP) (Sigma Chemical Co., St Louis, MO) was added to swollen spores to initiate a collapse of the cells. Treatment with 5 μM TFP elicited the desired effect (Lydan & Cotter, 1994). The TFP was either washed out with germination buffer, as above, at 1 hour after addition or remained in the spore suspension throughout the experiment. Samples were scored every hour microscopically as dormant, swollen, or emerged. At the times indicated samples were centrifuged at 4500 x g in a table top IEC Clinical Centrifuge for 3 minutes and the supernatants discarded. The samples were then rapidly frozen at -20°C in an alcohol bath until needed.

VIII. Formation of Homothallic Macrocysts using *D. mucoroides*, Strain Dm7

Spores were collected as above and 10 mL of the spore suspension was placed on each 0.1 % lactose peptone (LP) agar plate (containing: 1 g lactose, 1 g peptone, and 15.0 g Bacto Agar (Difco, Detroit, MI) in 1 L of ddH₂O), mixed, and placed in the dark. After approximately 1 day pre-aggregate cells were washed from plates, using 0.01 M phosphate germination buffer, into 15 mL conical centrifuge tubes and centrifuged in a table top IEC Clinical Centrifuge at 4500 x g for 3 minutes to produce pellets. The supernatants were discarded and the samples rapidly frozen until needed at -20°C in an
alcohol bath. At 2 days, early macrocysts were collected and late macrocysts at 4 days. Finally, 5 days after the initial inoculation mature macrocysts formed. Each stage was collected and stored as above.

IX. Cell Breakage

Samples for electrophoretic protein separation were at a spore number of $6 \times 10^7$. The frozen spore aliquots were resuspended in 60 µL lysis buffer (containing: 1% Triton X-100, 1 µg/mL leupeptin, and 1 mM phenylmethylsulfonyl fluoride (PMSF)). To mechanically disrupt the spores, 0.2 mL of small glass beads (0.25-0.32 mm in size) were added to the spore suspension. The resuspended spores were then vortexed for 5 minutes at 30 second intervals to reduce excess heating and protein degradation (North & Cotter, 1984). Between vortexing, the samples were also kept on ice. The suspension was then removed from the glass beads using a micropipette and transferred to a microcentrifuge tube. A similar technique was used for the breakage of macrocysts and their early stages of formation. To mechanically disrupt the macrocysts, 0.2 mL of mixed glass beads (0.25-0.32 mm and 1 mm in size) were added to the suspensions containing 60 µL of lysis buffer. The resuspended cells were then vortexed for 2 minutes at 15 second intervals as described above (North & Cotter, 1991). Between vortexing, these samples were also kept on ice. The efficiency of breakage for both techniques was observed microscopically to be 85-90%.
X. **Protein Quantification**

Soluble protein was quantified according to the method of Bradford (1976) using the BioRad Protein Assay (BioRad Laboratories, Mississauga, ON, Canada). Protein standards were made using bovine serum albumin (BSA) (Sigma Chemical Co., St Louis, MO). Sample dilutions were prepared at 1:40 in 10 mM KH$_2$PO$_4$ prior to the reaction with BioRad protein dye concentrate solution (BioRad Laboratories, Mississauga, ON, Canada). The final volume of 200 µL, prepared in a sterile microtitre plate, was gently mixed using a 20 µL micropipette and allowed to develop for 10 minutes. The samples were measured spectrophotometrically at 595 nm using a Microplate Reader (Mandel Scientific Co., Guelph, ON, Canada).

XI. **One Dimensional Electrophoresis**

Samples with 20 µg of protein were prepared by the addition of 5X sample buffer (containing: 250 mM Tris-HCl (pH 6.8), 50 % glycerol, 10 % sodium dodecyl sulfate (SDS), 25 % beta-mercaptoethanol (BME) and 0.25 % bromophenol blue) and the appropriate volume of protein as determined using the BioRad Protein Assay. The prepared samples and the prestained colour molecular weight markers (Sigma Chemical Co., St Louis, MO) were boiled for 3 minutes prior to loading onto the 10 % SDS polyacrylamide gels.
In all cases, 20 μg of protein was separated using the BioRad Mini Protein II Electrophoresis System by the method of Laemmli (1970). The 10% separating gel solution (containing: 3.35 mL 30% bis-acrylamide (BioRad Laboratories, Mississauga, ON, Canada), 2.5 mL 1.5 M Tris-HCl (pH 8.8), 100 μL 10% SDS, 4.0 mL ddH₂O) was thoroughly mixed prior to the addition of the polymerizing agents (10 μL N,N,N',N'-tetramethylethylenediamine (TEMED) and 50 μL 10% ammonium persulfate (APS)). The solution was poured into gel frames and overlaid with ddH₂O. After polymerization, at 4°C for 45 minutes, a 4% stacking gel was formed above the separating gel (containing: 1.5 mL 30% bis-acrylamide (BioRad), 2.5 mL 0.5 M Tris-HCl (pH 6.8), 100 μL 10% SDS, 5.9 mL ddH₂O) and thoroughly mixed prior to the addition of the polymerizing agents (10 μL TEMED and 50 μL 10% APS). A 10 well comb template was quickly inserted into the stacking gel and allowed to polymerize for 45 minutes at 4°C. Following polymerization, the wells were flooded with ddH₂O and the combs removed. Samples of 20 μL, at a concentration of 1 μg/μL, were loaded in each lane, accompanied by 5 μL of molecular weight markers in an adjacent lane in order to determine the relative molecular weights of the proteins. The electrophoretic chamber was immersed in running buffer (containing: 9.0 g Tris base, 43.2 g glycine, 3.0 g SDS in 600 mL of ddH₂O at a pH of 8.3) and connected to a BioRad PowerPac 200 power supply (BioRad Laboratories, Mississauga, ON, Canada) at 4°C. The voltage was initially set at 60 Volts (V) until the bromophenol blue marker dye front had reached the separating gel. At this point the voltage was increased to 90-100 V and remained constant until the bromophenol blue marker dye front reached the bottom of the gel.
XII. Western Blotting

Protein profiles were transferred from 10 % SDS polyacrylamide gels to 0.45 micron polyvinylidene fluoride (PVDF) microporous membranes (Immobilon-P, Millipore Corp., Bedford, MA) in a BioRad mini-trans-blot cell (BioRad Laboratories, Mississauga, ON, Canada) using ice cold blotting buffer (containing: 25 mM Tris base, 193 mM glycine, and 20 % methanol in a 1 L volume with ddH₂O at a pH of 8.3). The PVDF membranes were activated prior to transferring by immersion in 100 % methanol for 15-20 seconds. Transfer was completed by applying a constant voltage of 25 V for 16-18 hours employing a frozen cooling unit.

The blots were blocked with 1 g dry skim milk powder in 250 mL of the blocking buffer (containing: 10 mM Tris HCl, 140 mM NaCl, and 0.1 % polyoxyethylenesorbitan monolaurate (Tween 20) at a pH of 7.4) for 1 hour at room temperature. The membranes were then probed for phosphotyrosine containing proteins using the recombinant anti-phosphotyrosine antibody RC20-E120AP (Transduction Laboratories, Lexington, KY) preconjugated with alkaline phosphatase at a 1:2500 unit dilution in blocking buffer. The membranes were incubated for 30 minutes at room temperature and for 30 minutes at 37°C. Following incubation with the antibody the blots were washed twice with ddH₂O for 2 minutes. Visualization of the phosphotyrosine containing proteins was with the chromogenic substrates for alkaline phosphatase (1.65 mg/mL 5-bromo-3-chloro-indoylphosphate (BCIP) and 12.7 mg/mL nitro blue tetrazolium (NBT) (GibcoBRL, Burlington, ON, Canada) in alkaline phosphatase buffer containing 0.1 M Tris-HCl, 0.1 M NaCl, and 50 mM MgCl₂ in 1 L of ddH₂O at pH 9.5).
RESULTS

I. Actin Tyrosine Phosphorylation Patterns During Germination of *D. discoideum* Spores by Alternative Activation Methods

Previously, actin tyrosine phosphorylation has been shown to be at high levels in dormant spores of *D. discoideum* while the phosphorylation levels progressively decrease throughout the germination process. Upon emergence of amoebae little or no actin tyrosine phosphorylation is present (Gauthier *et al.*, 1997; Kishi *et al.*, 1998). This pattern was observed in all previous activation methods employed. Therefore, it is of interest to investigate alternative activation methods to determine if these same patterns exist.

A. DMSO Activation

The premature maturation mutant SG1 was incubated with the protein denaturant, DMSO at a 20% concentration for 1 hour (see materials and methods). The activation of SG1 spores by DMSO has been thoroughly examined and has been shown to facilitate rapid germination (Cotter *et al.*, 1976). A comparison between the heat activated actin tyrosine phosphorylation pattern (Fig. 6) and the actin tyrosine phosphorylation pattern from DMSO activation reveals that both are similar except that the time required for dephosphorylation is decreased in the alternative activation method.

B. Peptone Activation

Rapid spore germination also occurs in the presence of a 1% peptone solution (see materials and methods). Previously, peptone has been shown to initiate spore germination at an earlier time and to result in more rapid kinetics than both heat shock and autoactivation
Figure 6. Actin tyrosine phosphorylation pattern during heat activation of young wild-type strain NC4 spores. Young spores (3–4 days old) of the wild-type strain NC4, were heat activated and allowed to germinate. Germination kinetics are shown in panel A, where (■) represents the percent of swollen spores in the population and (▲) represents the emerged nascent amoebae. At time 0 (lane 0), following 30 minutes of heat exposure, and each subsequent hour during the germination, aliquots were collected and ground. A 10 % SDS-PAGE gel was used to separate 20 μg/μL of protein in each lane. Panel B illustrates Western blot analysis using anti-phosphotyrosine antibodies which revealed 43 kDa proteins containing phosphotyrosyl residues.
Figure 6.
Figure 7. Actin tyrosine phosphorylation pattern during DMSO activation of the premature maturation mutant SG1. Spores from the premature maturation mutant SG1 were DMSO activated and allowed to germinate. Germination kinetics are shown in panel A, where (■) represents the percent of swollen spores in the population and (▲) represents the emerged nascent amoebae. At time 0 (lane 0), following 1 hour of DMSO exposure, and each subsequent half hour during the germination, aliquots were collected and ground. A 10 % SDS-PAGE gel was used to separate 20 μg/μL of protein in each lane. Panel B illustrates Western blot analysis using anti-phosphotyrosine antibodies which revealed 43 kDa proteins containing phosphotyrosyl residues.
Figure 7.
Figure 8. Actin tyrosine phosphorylation pattern during peptone activation of the premature maturation mutant SG1. Spores from the premature maturation mutant SG1 were peptone activated and allowed to germinate. Germination kinetics are shown in panel A, where (■) represents the percent of swollen spores in the population and (▲) represents the percentage of emerged nascent amoebae. At time 0 (lane 0) and each subsequent half hour during the germination, aliquots were collected and ground. A 10% SDS-PAGE gel was used to separate 20 μg/μL of protein in each lane. Panel B illustrates Western blot analysis using anti-phosphotyrosine antibodies which revealed 43 kDa proteins containing phosphotyrosyl residues.
Figure 8.
(Cotter and George, 1975; Cotter and Raper, 1968). As seen in Fig 8 the actin tyrosine phosphorylation pattern is typical to that in Fig 6 but as shown with DMSO activation the dephosphorylation is more rapid.

In both alternative activation methods the actin tyrosine phosphorylation patterns were similar and corresponded to the patterns obtained by previous methods excluding the time periods. The rapid germination by DMSO and peptone activation show that a further correlation exists between the morphological stage in germination and the actin tyrosine phosphorylation levels.

II. The Effects of Continuous Heat Activation Temperatures on Actin Tyrosine Phosphorylation Levels in D. discoideum

Heat activation of spores at 45°C for 30 minutes prior to incubation at room temperature will activate dormant spores, independent of spore density or age, and lead to synchronous germination (Cotter & Raper, 1968). As shown by Gauthier et al. (1997), heat activation of dormant spores will lead to a decrease in the actin tyrosine phosphorylation levels during germination (see Fig 6). To expand on this data, dormant NC4 and SG1 spores were heat activated for a period of 5 hours following initial activation (45°C for 30 minutes) to determine if a decrease in actin tyrosine phosphorylation could be detected. No decrease in actin tyrosine phosphorylation was observed during the heat exposure for either NC4 (Fig. 9) or SG1 (Fig. 10) spores. Spore viability was undetectable following heat exposure for 5 hours (Cotter and George, 1975) suggesting that non-viable spores do not rapidly dephosphorylate their actin tyrosine residues.
Figure 9. Actin tyrosine phosphorylation pattern of young wild-type strain NC4 spores exposed to continuous heat activation temperatures. Young spores (3-4 days old) of the wild-type strain NC4, were heat activated and continuously exposed to 45°C for 5 hours. Germination kinetics are shown in panel A, where (●) represents the percent of dormant spores in the population, (■) represents the percent of swollen spores, and (△) represents the percentage of emerged nascent amoebae. At time 0 (lane 0), following 30 minutes of heat exposure, and each subsequent hour during the incubation, aliquots were collected and ground. A 10% SDS-PAGE gel was used to separate 20 μg/μL of protein in each lane. Panel B illustrates Western blot analysis using anti-phosphotyrosine antibodies which revealed 43 kDa proteins containing phosphotyrosyl residues.
Figure 9.
Figure 10. Actin tyrosine phosphorylation pattern for spores of the premature maturation mutant SG1 exposed to continuous heat activation temperatures. Spores of the mutant strain SG1, were heat activated and continuously exposed to 45°C for 5 hours. Germination kinetics are shown in panel A, where (●) represents the percent of dormant spores in the population, (■) represents the percent of swollen spores, and (▲) represents the percentage of emerged nascent amoebae. At time 0 (lane 0), following 30 minutes of heat exposure, and each subsequent hour during the incubation, aliquots were collected and ground. A 10% SDS-PAGE gel was used to separate 20 μg/μL of protein in each lane. Panel B illustrates Western blot analysis using anti-phosphotyrosine antibodies which revealed 43 kDa proteins containing phosphotyrosyl residues.
Figure 10.
III. Inhibition of *D. discoideum* Spore Germination and the Effects on Actin Tyrosine Phosphorylation

Using phosphatase inhibitors (PAO and BPV), mitochondrial inhibitors (azide, DNP, and anaerobic conditions), and osmotic pressure (100 mM sucrose), activated spores were inhibited from germinating. Through Western blot analysis the actin tyrosine phosphorylation levels were analyzed at 5 hours for each inhibitor (Figs. 11 and 12). The results clearly show high actin tyrosine phosphorylation levels for all the inhibitor treatments and indicate that actin tyrosine phosphorylation may be correlated with spore dormancy. Spores remained viable after all of the incubations.

IV. Actin Tyrosine Phosphorylation Pattern of Spores Exposed to Ammonium and Potassium Phosphate

Spores from the premature maturation mutant SG1 were exposed to either 50 mM ammonium phosphate at pH 6.2, 50 mM ammonium phosphate at pH 7.2, 50 mM potassium phosphate at pH 6.2, or 50 mM potassium phosphate at pH 7.2 for 5 hours. Fig. 13a shows that the spores in 50 mM ammonium phosphate at pH 7.2 are inhibited from germinating while germination occurred in all the other treatments. The actin tyrosine phosphorylation patterns (Fig. 13b) reveal that the inhibition by 50 mM ammonium phosphate at pH 7.2 correlates with actin tyrosine phosphorylation.
Figure 11. Actin tyrosine phosphorylation patterns at 5 hours for young wild-type strain NC4 spores exposed to anaerobic conditions (mineral oil overlay), 2 mM azide, and 1 mM DNP following heat activation. Young spores (3-4 days old) of the wild-type strain NC4, were heat activated and exposed to either mineral oil overlay, 2 mM azide, or 1 mM DNP for 5 hours. Germination kinetics are shown in panel A, where (●) represents the percent of dormant spores in the population, (■) represents the percent of swollen spores, and (▲) represents the percentage of emerged nascent amoebae. At 5 hours of the incubation, aliquots were collected and ground. A 10 % SDS-PAGE gel was used to separate 20 μg/μL of protein in each lane. Panel B illustrates Western blot analysis using anti-phosphotyrosine antibodies which revealed 43 kDa proteins containing phosphotyrosyl residues.
Figure 11.
Figure 12. Actin tyrosine phosphorylation patterns at 5 hours for young wild-type 
strain NC4 spores exposed to 100 mM sucrose, 200 μM BPV, and 7 μM PAO following 
heat activation. Young spores (3-4 days old) of the wild-type strain NC4, were heat 
activated and exposed to either 300 mM sucrose, 200 μM BPV, or 7 μM PAO for 5 hours. 
Germination kinetics are shown in panel A, where (●) represents the percent of dormant 
spores in the population, (■) represents the percent of swollen spores, and (△) represents 
the percentage of emerged nascent amoebae. At 5 hours of the incubation, aliquots were 
collected and ground. A 10 % SDS-PAGE gel was used to separate 20 μg/μL of protein in 
each lane. Panel B illustrates Western blot analysis using anti-phosphotyrosine antibodies 
which revealed 43 kDa proteins containing phosphotyrosyl residues.
Figure 12.
Figure 13. Actin tyrosine phosphorylation patterns at 5 hours for autoactivated SG1 spores in the presence of ammonium and potassium phosphate. Spores of the mutant strain SG1 were autoactivated and incubated in the presence of either 50 mM ammonium phosphate at pH 6.2, 50 mM ammonium phosphate at pH 7.2, 50 mM potassium phosphate at pH 6.2, or 50 mM potassium phosphate at pH 7.2 for 5 hours. Germination kinetics are shown in panel A, where (○) represents the spores in 50 mM potassium phosphate at pH 7.2 that germinated in the population, (□) represents the spores in 50 mM ammonium phosphate at pH 7.2 that germinated, (▲) represents the spores in 50 mM potassium phosphate at pH 6.2 that germinated, and (◆) represents spores in 50 mM ammonium phosphate at pH 6.2 that germinated. Germinated spores are the combined number of spores that are swollen and emerged in the population. After 5 hours of the incubation, aliquots were collected and ground. A 10 % SDS-PAGE gel was used to separate 20 μg/μL of protein in each lane. Panel B illustrates Western blot analysis using anti-phosphotyrosine antibodies which revealed 43 kDa proteins containing phosphotyrosyl residues.
Figure 13.
V. Characterization of Actin Tyrosine Phosphorylation Patterns in *D. discoideum* Employing the Protein Synthesis Inhibitor Cycloheximide

Cycloheximide is a protein synthesis inhibitor that interferes with translation and prevents autoactivation in premature maturation mutants (Dahlberg & Cotter, 1978) as well as emergence of heat activated wild-type spores (Cotter & Raper, 1970; Giri & Ennis, 1978; Yagura & Iwabuchi, 1976).

The cycloheximide studies conducted under both autoactivation (Fig 14) and heat activation conditions (Fig 15) and the subsequent removal or overriding of the cycloheximide have allowed the actin tyrosine phosphorylation patterns to be further characterized to specific stages. The cycloheximide experiments using crude autoactivator to override the effects of the protein synthesis inhibitor allow for rapid swelling to occur and for the maintenance of that stage. The results in Fig 14 show that a majority of dephosphorylation occurs between the dormant and swollen stages in germination. However, some actin phosphorylation is still present. The use of heat activated spores in cycloheximide (Fig 15) causes swelling and the removal of the inhibitor permits accelerated emergence. This technique indicates that some additional actin tyrosine dephosphorylation is occurring in the emergence process.
Figure 14. Actin tyrosine phosphorylation pattern of autoactivated SG2 spores in the presence of 200 \( \mu \text{g/mL} \) of cycloheximide overridden with autoactivator. Spores of the mutant strain SG2 were autoactivated and incubated in the presence of 200 \( \mu \text{g/mL} \) of cycloheximide where they remained dormant. At 3 hours autoactivator was used to initiate rapid swelling. Germination kinetics are shown in panel A, where (■) represents the percent of swollen spores in the population that have been stimulated by the addition of autoactivator after 3 hours in 200 \( \mu \text{g/mL} \) cycloheximide and (♦) represents the spores that remained in 200 \( \mu \text{g/mL} \) cycloheximide. At time 0 (lane 0) and each subsequent hour during the incubation and after, aliquots were collected and ground. A 10 % SDS-PAGE gel was used to separate 20 \( \mu \text{g/mL} \) of protein in each lane. Panel B illustrates Western blot analysis using anti-phosphotyrosine antibodies which revealed 43 kDa proteins containing phosphotyrosyl residues. Autoactivator has been added to the samples of 4', 5', 6', 7'.
Figure 14.
Figure 15. Actin tyrosine phosphorylation pattern of heat activated NC4 spores in the presence of 200 μg/mL of cycloheximide and its removal. Young spores (3-4 days old) of the wild-type strain NC4, were heat activated in the presence of 200 μg/mL of cycloheximide and rapidly became swollen following activation. At 4 hours the cycloheximide was removed to initiate rapid emergence. Germination kinetics are shown in panel A, where (■) represents the swollen spores in the population following heat activation in 200 μg/mL of cycloheximide, (□) represents the swollen spores following the removal of the inhibitory cycloheximide, (▲) represents the emerged nascent amoebae following heat activation in 200 μg/mL of cycloheximide, and (△) represents the emerged nascent amoebae following the removal of the inhibitory cycloheximide. At time 0 (lane 0), following 30 minutes of heat exposure, and each subsequent hour during the incubation and after, aliquots were collected and ground. A 10 % SDS-PAGE gel was used to separate 20 μg/mL of protein in each lane. Panel B illustrates Western blot analysis using anti-phosphotyrosine antibodies which revealed 43 kDa proteins containing phosphotyrosyl residues. The cycloheximide has been removed by repeated washing in the samples 4', 4.5', and 5'.
Figure 15.
VI. The Effect of Calmodulin Inhibition on Actin Tyrosine Phosphorylation Levels in *D. discoideum*

TFP is an inhibitor of the calcium-dependent regulatory protein calmodulin, which is a positive mediator of spore swelling in *D. discoideum*. Previously, TFP was used to induce a rapid collapse in swollen spores (Lydan and Cotter, 1994). Phase contrast microscopy of germinating spores under these conditions are shown in Fig 16. It is clear that the swollen spores (Fig 16b) that have been exposed to the TFP (Fig 16c) resemble dormant spores (Fig 16a) in morphology. Two experiments were completed to determine if actin tyrosine phosphorylation was involved in the collapse.

In the first study, spores were allowed to swell for up to three hours and the TFP was added inducing spore collapse (Fig 17a). Throughout the following hour spores resembled dormant structures seen prior to spore activation. At 4 hours the TFP was removed and the spores rapidly completed germination (Lydan and Cotter, 1994). As seen in Fig 17b, actin tyrosine phosphorylation did not increase upon addition of TFP and the actin tyrosine phosphorylation patterns were similar to those seen in normal germination patterns (Fig 6).

The second experiment used similar approaches as above but the TFP was added at 3 hours and was continued for a period of 8 hours to determine if extended treatments of TFP exposure would lead to a change in actin tyrosine phosphorylation (Fig 18a). The actin tyrosine phosphorylation pattern, in Fig 18b, shows no increase in phosphorylation throughout the exposure as compared to control phosphorylation patterns (Fig 6). These results suggest that unlike deactivation of heat activated spores by harsh environmental conditions or the presence of endogenous autoinhibitor(s), which both yield dormant spore
Figure 16. Spore morphology during germination in the presence of trifluoperazine.

Spores of the mutant strain SG2 were autoactivated and allowed to swell. At 3 hours 5 μM TFP was added eliciting a collapse of the swollen spores. At 4 hours the TFP was removed and the spores continued germination. Representative germination kinetics are shown in panel A, where (●) represents the percent of dormant or unswollen spores in the population, (■) represents the percent of swollen spores, and (▲) represents the percentage of emerged nascent amoebae. Panel B is a photograph using a phase-contrast Leitz Daiplan photomicroscope illustrating the morphological state of the spores in the following experiments. a) Time 0 hour - dormant spores b) Time 3 hours - swollen spores c) Time 4 hours - morphologically dormant spores. Borrowed from Lydan and Cotter (1994).
A.

![Graph showing percentage over hours.]

B.

a.)

b.)

c.)

Figure 16.
Figure 17. Actin tyrosine phosphorylation pattern of autoactivated SG2 spores after the addition and removal of 5 μM trifluoperazine during swelling. Spores of the mutant strain SG2 were autoactivated and allowed to swell. At 3 hours 5 μM TFP was added eliciting a collapse of the swollen spores. At 4 hours the TFP was removed and the spores continued germinating. Germination kinetics are shown in panel A, where (●) represents the percent of dormant or unswollen spores in the population, (■) represents the percent of swollen spores, and (▲) represents the percentage of emerged nascent amoebae. At time 0 (lane 0) and each subsequent hour during the germination, aliquots were collected and ground. A 10 % SDS-PAGE gel was used to separate 20 μg/μL of protein in each lane. Panel B illustrates Western blot analysis using anti-phosphotyrosine antibodies which revealed 43 kDa proteins containing phosphotyrosyl residues.
Figure 17.
Figure 18. Actin tyrosine phosphorylation pattern of autoactivated SG2 spores after the addition of 5 μM trifluoperazine during swelling. Spores of the mutant strain SG2 were autoactivated and allowed to swell. At 3 hours 5 μM TFP was added eliciting a collapse of the swollen spores. The spores were continuously incubated in the TFP for 8 hours. Germination kinetics are shown in panel A, where (●) represents the percent of dormant or unswollen spores in the population, (■) represents the percent of swollen spores, and (▲) represents the percentage of emerged nascent amoebae. At time 0 (lane 0) and each subsequent hour during the incubation, aliquots were collected and ground. A 10 % SDS-PAGE gel was used to separate 20 μg/μL of protein in each lane. Panel B illustrates Western blot analysis using anti-phosphotyrosine antibodies which revealed 43 kDa proteins containing phosphotyrosyl residues.
Figure 18.
morphologies, the collapse of swollen spores by the addition of TFP does not involve actin tyrosine phosphorylation.

VII. Determination of the Presence of Actin Tyrosine Phosphorylation in Alternative Species of the Dictyostelids

Two alternative species of the Dictyostelids were tested to determine if the actin tyrosine phosphorylation patterns found in D. discoideum were confined only to that species. D. purpureum was germinated by heat activation and elicited an actin tyrosine phosphorylation pattern (Fig 19) similar to heat activated wild-type NC4 spores (Fig 6). Additionally, dormant spores of D. mucoroides also exhibited high levels of actin tyrosine phosphorylation (Fig 19). Clearly, within the cellular slime molds this phenomenon is not exclusive to D. discoideum.

VIII. Determination of the Presence of Actin Tyrosine Phosphorylation in Various Mutants of D. discoideum

Numerous mutants of D. discoideum have been implicated in the mobilization of the cytoskeleton. Several of these mutants were obtained to determine the amounts of actin tyrosine phosphorylation present. The mutants RasG−, RegA−, CN−, SpiA−, YelA−, and the wild-type NC4 at time 0 hour were analyzed for their actin tyrosine phosphorylation levels (Fig 20b). The actin tyrosine phosphorylation was further analyzed using densitometry, shown graphically in Fig 20a as O.D. x mm² units. The results reveal that similar phosphorylation levels to the wild-type were found in all mutants excluding the YelA− strain. RegA− showed the highest phosphorylation activity (7.67 units) while the wild-type NC4
Figure 19. Actin tyrosine phosphorylation patterns of heat activated *D. purpureum* spores and dormant spores of *D. mucoroides*. Spores of *D. purpureum* were heat activated and allowed to germinate. Germination kinetics are shown in panel A, where (●) represents the percent of dormant spores in the population, (■) represents the percent of swollen spores, and (▲) represents the percentage of emerged nascent amoebae. At time 0 (lane 0) and each subsequent hour during the germination, aliquots were collected and ground. Dormant spores of *D. mucoroides* were also collected and ground. A 10% SDS-PAGE gel was used to separate 20 μg/μL of protein in each lane. Panel B illustrates Western blot analysis using anti-phosphotyrosine antibodies which revealed 43 kDa proteins containing phosphotyrosyl residues.
Figure 19.
Figure 20. Actin tyrosine phosphorylation patterns in dormant spores of RasG-, RegA-, PKA-C, CN-, SpiA-, and YetA- mutants. Spores of the various mutant strains were collected as dormant spores. Relative actin tyrosine phosphorylation activity using densitometry is shown in panel A. The dormant spore aliquots were collected and ground. A 10 % SDS-PAGE gel was used to separate 20 µg/µL of protein in each lane. Panel B illustrates Western blot analysis using anti-phosphotyrosine antibodies which revealed 43 kDa proteins containing phosphotyrosyl residues.
Figure 20.
displayed slightly lower levels (7.61 units). This result is expected as RegA- mutants produce super-dormant spores (Meima et al., 1997). RasG- (6.31 units), CN- (5.89 units), and SpiA- (6.51 units) spores displayed similar amounts of actin tyrosine phosphorylation which was slightly lower than the wild-type NC4. YelA- (2.93 units) showed the lowest levels of actin tyrosine phosphorylation activity, as expected, since YelA- mutants arrest at the tight mound stage and form defective spores that quickly lose their viability (Osherov et al., 1997). Therefore, the results reveal that the mutants all have some level of actin tyrosine phosphorylation and these levels differ depending on the mutation.

IX. Actin Tyrosine Phosphorylation in the Sexual Life Cycle of D. mucoroides, Strain Dm7

Four stages of the sexual life cycle of D. mucoroides were collected at the times indicated (see materials and methods). Each stage was sampled to determine the relative levels of actin tyrosine phosphorylation. The pre-aggregate, early macrocyst, and late macrocyst stages elicited no detectable actin tyrosine phosphorylation while the mature macrocyst showed high levels of phosphorylation (Fig 21).
Figure 21. Actin tyrosine phosphorylation patterns of the sexual life cycle of \textit{D. mucoroides}, strain Dm7. Four stages of the sexual life cycle of \textit{D. mucoroides} were collected. The stages included pre-aggregate, early and late macrocyst, as well as the mature macrocyst. Samples at the indicated stages were stored and ground. A 10 \% SDS-PAGE gel was used to separate 20 \( \mu g/\mu L \) of protein in each lane. The Western blot using anti-phosphotyrosine antibodies is illustrated which revealed 43 kDa proteins containing phosphotyrosyl residues.
Figure 21.
DISCUSSION

The experiments performed in this study have elucidated novel information regarding actin tyrosine phosphorylation in *D. discoideum*. Recently, it has been shown that 50% of the total actin in spores of *D. discoideum* is tyrosine phosphorylated (Kishi *et al.*, 1998). This indicates that most of the actin network is affected in dormant spores.

Using the alternative activation methods, 1% peptone and 20% DMSO, spore germination is accelerated with the vast majority of spores releasing amoebae within 3.5 hours. Both of these activation methods result in a reduced germination period compared to the methods used by Gauthier *et al.* (1997). Samples taken throughout these germination experiments were analyzed via Western blotting and showed that the increased rate of germination was paralleled by similar actin tyrosine dephosphorylation (Fig. 7, and 8). Upon comparison with heat activated actin tyrosine phosphorylation patterns (Fig. 6) it is clear that a more rapid dephosphorylation has occurred. These methods were used to establish that the dephosphorylation of actin is stage specific, independent of time and thus dependent on the mode of activation. In this discussion, stage specific phosphorylation refers to the notion that each spore stage (dormancy, swelling, and emergence) has particular levels of actin tyrosine phosphorylation. In these experiments, in comparison with previous dephosphorylation patterns, it appears stage specific patterns do exist.

To further substantiate the occurrence of stage specific phosphorylation patterns and also to determine when the majority of dephosphorylation takes place, cycloheximide, a protein synthesis inhibitor, was employed. Spore swelling requires protein synthesis to occur and autoactivated spores will not undergo this process in the presence 200 μg/mL of
cycloheximide (Dahlberg and Cotter, 1978). Similarly, spores heat activated in 200 
µg/mL of cycloheximide will swell but not release myxamoebae (Dahlberg and Cotter, 
1978). These properties were utilized to allow rapid changes between morphological 
germination stages so that the actin tyrosine phosphorylation patterns between these stages 
could be contrasted at their maximum. SG2 spores were inhibited from autoactivating by 
incubation in cycloheximide. The addition of the autoactivator preparation at 3 hours 
permitted the dormant spores to rapidly swell and remain in the swollen state. Analysis of 
the actin tyrosine phosphorylation pattern revealed that a majority of dephosphorylation 
occurred during the transition from dormant spores to swollen spores (Fig. 14, Panel B). 
Spores were also heat activated in cycloheximide to attain swollen spores that, once washed 
free from the inhibition after 4 hours, quickly emerged. Western blot analysis revealed that 
the swollen spores had low levels of detectable actin tyrosine phosphorylation and the 
emerged amoebae liberated from the swollen spore displayed only a slight decrease in actin 
tyrosine dephosphorylation (Fig. 15, Panel B). Hence, it can be inferred that the majority 
of actin tyrosine dephosphorylation takes place prior to spore swelling.

These results support the notion that the dephosphorylated actin is required for 
spore swelling and emergence. The mechanical processes of swelling and emergence may 
require readily accessible actin pools that can be rapidly polymerized. Recently, Kishi et 
_{al.} (1998) showed that the use of nutrient media with germination promoters, possibly 
secreted polysaccharides originating from the bacterial cell wall of _Klebsiella aerogenes_ 
(Ihara _et al._, 1990), induced rapid dephosphorylation of actin prior to spore swelling. Actin 
tyrosine phosphorylation in _D. discoideum_ has also been shown not to inhibit 
polymerization nor stimulate depolymerization _in vitro_ (Kishi _et al._, 1998). This is in
contrast to the findings in the true slime mold, *Physarum*, where phosphorylation on a threonine residue of actin prevents its polymerizing capabilities (Furushashi et al., 1998). It has also been established that actin phosphorylation in *Physarum* occurs in complex with the actin-binding protein fragmin *in vivo* (De Corte et al., 1996). These data suggest phosphorylation of actin in *D. discoideum* may inhibit polymerization *in vivo* in association with an actin-binding protein other than fragmin which has yet to be uncovered in *D. discoideum*. Regardless, actin tyrosine dephosphorylation has been established as a prerequisite for spore swelling by both cycloheximide studies and alternative activation methods as well as by other recent studies (Kishi et al., 1998). Similarly, it is obvious that the phosphorylation levels of actin are stage specific.

The heat activation method introduced by Cotter and Raper (1966; 1968a) has been utilized in numerous studies (Lydan & Cotter, 1994; Cotter and Glaves, 1989; Cotter et al., 1976). In particular Gauthier et al. (1997) used this particular method on several strains of *D. discoideum* in determining the actin tyrosine phosphorylation pattern of each strain. By heat activating (45°C for 30 minutes) wild-type NC4 and SG1 spores and then continuing the exposure at the activation temperature (45°C), an attempt was made to determine if the heat activation temperatures were directly responsible for the dephosphorylation of actin or indirectly through the initiation of a dephosphorylation cascade. Additionally, non-viable spores were also examined to ascertain whether or not they retained tyrosine phosphorylated actin. Extended heat activation temperatures produced NC4 and SG1 spores that did not germinate after 5 hours (Fig. 9, and 10; Panel A) and spores that were not viable. Actin tyrosine phosphorylation analysis for each hour of the incubation for both strains did not show any dephosphorylation of actin (Fig. 9 and
10; Panel B). Therefore, it can be said that the heat activation temperatures (30 minutes at 45°C) do not directly initiate actin dephosphorylation. It has also been established that non-viable spores, killed by this method, do not undergo actin tyrosine dephosphorylation. Spore germination has been shown to be highly sensitive to temperature. A 3°C shift below the activation temperature produces no germination while a 1.5°C decrease in temperature produces only 50% germination (Cotter, 1973). Clearly, spore germination as well as actin tyrosine phosphorylation are induced by specific activating temperatures.

The understanding of actin tyrosine phosphorylation in *D. discoideum* has been expanded by the use of germination inhibitors to deactivate wild-type NC4 spores following heat activation. The first set of inhibitors all act to restrict mitochondrial function (Fig. 11, Panel A). The mineral oil overlay treatment simulates an anaerobic environment by reducing the amount of oxygen available for ATP production. Azide acts to inhibit the function of cytochrome c oxidase in the electron transport chain. Cytochrome c oxidase transfers electrons from cytochrome c to oxygen and is a critical event in electron transfer that is blocked by azide (Stryer, 1988). The final treatment employed that restricts mitochondrial function was dinitrophenol. This agent dissipates the proton gradient in the mitochondria thus reducing ATP production (Gerisch, 1962). All of these treatments restrict mitochondrial function leading to reduced ATP production that deactivates spores causing them to remain dormant.
Osmotic Pressure was also used to deactivate spores following activation. In spores of *D. discoideum* a membrane bound adenylyl cyclase (ACG) senses increases in osmotic pressure and responds by producing cAMP. The cAMP binds to the regulatory subunit of PKA releasing the catalytic subunit initiating a phosphorylation cascade leading to spore dormancy (Fig. 2 and Fig. 12 Panel A; van Es *et al.*, 1996).

Finally, two phosphatase inhibitors, PAO and BPV, were also used to deactivate spores. Phosphatase inhibitors function to reduce the dephosphorylation capabilities of the cell and therefore alter the balance between phosphorylated and dephosphorylated proteins. Since actin is phosphorylated in dormant spores the use of a phosphatase inhibitor after activation should slow down or stop the dephosphorylation affect that occurs in germinating spores. Spores treated with phosphatase inhibitors after activation return to the dormant state.

Samples at 5 hours for all inhibitors displayed dormant spores that were viable. Similarly, all 5 hour samples analyzed via Western blotting revealed high actin tyrosine phosphorylation levels (Fig11, Panel B and 12 Panel B). These results indicate that a correlation exists between spore dormancy and actin tyrosine phosphorylation that is independent of the mode of deactivation. As well, these results establish that dephosphorylation of actin may be required for spore swelling. Along with the dephosphorylation of actin, previous studies by Lydan and Cotter (1994; 1995) have shown that activated calmodulin, a calcium-dependent regulatory protein, is also required for spore swelling in *D. discoideum*. Calmodulin has been suggested to be stimulated by autoactivator through a phospholipase C pathway that increases internal Ca\(^{2+}\) levels (Lydan and Cotter, 1994; 1995). Calmodulin or the associated binding proteins may in
fact interact with the actin cytoskeleton allowing spore swelling. Similarly, the previous study employing cycloheximide prevented autoactivation. However, this study used the premature maturation mutant SG2, activated by autoactivation, rather than heat activated wild-type NC4 spores. Regardless, this finding further strengthens the correlation for the requirement of actin phosphorylation for spore dormancy and actin dephosphorylation for spore swelling.

Actin tyrosine phosphorylation studies in amoebae of *D. discoideum* support the idea that phosphorylation of actin plays a role in the maintenance of the immobile cell structure of spores. Jungbluth *et al.* (1994; 1995) exposed vegetative amoebae to similar inhibitors as used in this investigation such as dnp, azide, and anaerobic conditions. Amoebae under these conditions readily roundup and become immovable. Vesicles and mitochondria also become static and actin tyrosine phosphorylation levels increase. This suggests a relationship between the actin phosphorylation and the resting state induced by these treatments. These findings in amoebae are similar to the results in spores which are deactivated by these agents following activation. Thus, a correlation exists between the resting state and actin phosphorylation in both cases. Finally, it also appears that the inhibition of mitochondrial function may serve as a signal that initiates actin tyrosine phosphorylation.

Ammonia, which inhibits spore germination, may contribute to the maintenance of dormancy through ACG (Cotter *et al.*, 1998). Using the techniques from Cotter *et al.* (1998), spores from the premature maturation mutant SG1 were incubated in either 50
mM ammonium phosphate at pH 6.2, 50 mM ammonium phosphate at pH 7.2, 50 mM potassium phosphate at pH 6.2, or 50 mM potassium phosphate at pH 7.2 for 5 hours. The various pH levels alter the amount of ammonia or potassium ion present in the corresponding solutions. According to Cotter et al. (1998), a pH decrease from from 7.2 to 6.2 decreases the concentration of ammonia by 90%. These experiments suggested that ammonia was responsible for the inhibition of germination (Cotter et al., 1998). As expected, samples germinated in all cases except for the spores incubated in 50 mM ammonium phosphate at pH 7.2. As well, a slight lag in germination occurred in spores in 50 mM ammonium phosphate (Fig. 13, Panel A). These germination patterns are comparable to those found in Cotter et al. (1998). Actin tyrosine phosphorylation levels of each sample at 5 hours was determined using Western blot analysis (Fig. 13, Panel B). Spores in 50 mM ammonium phosphate at pH 7.2 showed high levels of actin phosphorylation similar to dormant spores while the other samples displayed little or no actin tyrosine phosphorylation. Phosphorylation of actin by ammonia inhibition reveals a downstream effect related to the presence of ammonia. Ammonia has been suggested to prevent spore germination via ACG through an alternative method other than the osmosensing mechanism (Cotter et al., 1998). These results show that both osmotic pressure and ammonia prevent spore germination leading to actin tyrosine phosphorylation.

Another aspect of this investigation, employed TFP in two experiments to determine if actin tyrosine phosphorylation was involved in the rapid collapse induced by TFP addition to swollen spores. TFP is an inhibitor of the calcium-dependent regulatory protein calmodulin, which is a positive mediator of spore swelling in D. discoideum.
(Lydan and Cotter, 1994; 1995). When TFP is added to swollen spores a rapid collapse occurs causing them to resemble dormant spores in morphology. The collapse that occurs in the swollen spores is reversible and upon the removal of the TFP, by repeated washings, the spores will again swell and continue through germination producing emerged nascent amoebae (Lydan and Cotter, 1994; 1995). Figure 16 displays the morphological stages found in these experiments under phase-contrast microscopy.

In the first experiment spores were allowed to swell up to three hours and TFP was added inducing spore collapse (Fig. 17, Panel A). Throughout the following hour spores resembled dormant structures seen prior to spore activation (Fig. 16). At 4 hours the TFP was removed and the spores rapidly completed germination. Western blot analysis revealed that actin tyrosine phosphorylation did not increase after the addition of TFP (Fig 17, Panel B) and the actin tyrosine phosphorylation patterns were similar to those seen in normal germination experiments (Fig. 6). This technique was established by Lydan and Cotter (1994) and was employed in this experiment in accordance with the procedures outlined. The results above suggest that actin tyrosine phosphorylation of actin is not involved in the rapid collapse induced by TFP addition.

The second experiment used similar approaches but the TFP was added at 3 hours and was continued for a period of 8 hours to determine if extended treatments of TFP exposure would lead to a change in actin tyrosine phosphorylation (Fig 18, Panel A). As expected the spores collapsed upon the addition of the TFP and remained in an unswollen state throughout the incubation. The actin tyrosine phosphorylation pattern (Fig. 18, Panel B) showed no increase throughout the exposure as compared to control phosphorylation patterns (Fig. 6). These results suggest that unlike deactivation of heat
activated spores by harsh environmental conditions or the presence of an endogenous autoinhibitor(s), which both yield dormant spore morphologies, the collapse of swollen spores by the addition of TFP does not involve actin tyrosine phosphorylation. An examination of the actin tyrosine phosphorylation patterns using these techniques was initiated to determine if a phosphorylation event could be established following the initiation of swelling. Only dephosphorylation events have been shown to occur after spore swelling during the germination program (Gauthier et al., 1997; Kishi et al., 1998). As described earlier, Jungbluth et al. (1994; 1995) showed that amoebae of D. discoideum will phosphorylate their actin under stress conditions and it was suggested above that the dephosphorylation of actin was required for spore swelling. Therefore, it was presumed that the reversal of spore swelling would be accompanied by actin phosphorylation. However, it is reasonable to assume the opposite effect because swollen spores that are collapsed by TFP are not deactivated and will germinate following the removal of the inhibitor. Previously, it has been established that spores that begin to swell irreversibly lose any spore resistance or deactivation properties (Cotter and Raper, 1968b). It is possible that spores that proceed through the post activation lag stage into spore swelling undergo dephosphorylation in a percentage of their actin. Furthermore, they may not have the capability to re-phosphorylate that actin returning them to the dormant stage.
Actin tyrosine phosphorylation in *D. discoideum* has been a fairly well documented phenomenon (Gauthier et al., 1997; Howard et al., 1993; Jungbluth et al., 1994; 1995). However, actin tyrosine phosphorylation in other species of the *Dictyostelids* had not yet been established. Therefore, *D. purpureum* and *D. mucoroides*, two alternative species of the slime molds, were investigated for actin tyrosine phosphorylation. Spores of *D. purpureum* were heat activated and samples were collected every hour and subjected to Western blot analysis. Spores of *D. mucoroides*, which do not germinate under conditions that yield sufficient samples, were collected from fruiting bodies and analyzed for actin tyrosine phosphorylation. Both *D. purpureum* and *D. mucoroides* displayed high levels of actin tyrosine phosphorylation in dormant spores (Fig. 19). Heat activated *D. purpureum* spores also exhibited progressively lower actin tyrosine phosphorylation levels as germination progressed. Emerged amoebae displayed little or no actin tyrosine phosphorylation. These actin tyrosine phosphorylation patterns were similar to those found in heat activated spores of *D. discoideum* (Fig. 6). These results indicate that the actin tyrosine phosphorylation found in *D. discoideum* is not an isolated mechanism and exists in other species of the *dictyostelids*. The actin tyrosine phosphorylation established in *D. discoideum* has only previously been comparable to the true slime mold, *Physarum polycephalum*, where phosphorylation has been shown to occur on threonine residues 202 and 203 in complex with the capping protein fragmin (De Corte et al., 1996).

In similar fashion, the sexual life cycle of the *dictyostelids* has also been analyzed, via Western blotting, to determine if actin tyrosine phosphorylation occurs
during the formation of mature macrocysts. Four stages of the sexual life cycle of *D. mucoroides*, strain Dm7, which forms homothallic macrocysts, were investigated for actin tyrosine phosphorylation. The four stages consist of pre-aggregate cells, early macrocysts, late macrocysts, and mature macrocysts. This investigation revealed that only the mature macrocysts contained any detectable phosphorylated actin (Fig. 21). These findings are similar to those in *Physarum* that establish that, under desiccating conditions, macrocysts form plasmodia and phosphorylate approximately 50% of their actin at threonine residue 203. Furuhashi *et al.* (1998) suggest that the phosphorylation of actin is involved in the transformation from plasmodium to macrocyst. Thus, actin tyrosine phosphorylation may play a vital role in maintaining mature macrocysts in the cellular slime molds as well as the true slime molds.

Formation of a mature macrocyst may have some similarities with the formation of encapsulated spores. Both form dormant structures which are resistant to numerous environmental conditions such as starvation, and elevated temperatures although macrocysts are less resistant than spores (Cotter and Raper, 1966, 1968a,b; Raper, 1984). As well, it has recently been observed that actin tyrosine phosphorylation occurs during the formation of mature spores during late development. Actin tyrosine phosphorylation in spores has been shown just prior to the end of culmination before a mature fruiting body is formed; at approximately 23-24 hours (Kishi *et al.*, 1998). Similarly, actin tyrosine phosphorylation is only found in mature macrocysts. The phosphorylation of actin at these late stages may be a final mechanism that "locks" the spores or macrocysts into their dormant structures. Prior to the phosphorylation of actin during the formation of spores and macrocysts actin is possibly required for cellular activities such as vesicle movement.
Alternatively, during germination the actin is dephosphorylated or "unlocked" prior to spore swelling as discussed previously.

The final aspect of this investigation utilized a variety of *D. discoideum* mutants that have been implicated in either cytoskeletal mobilization, spore germination or differentiation. Spores of the mutants RasG−, RegA−, CN−, SpiA−, YelA−, and the wild-type NC4 at time 0 hour were analyzed for their actin tyrosine phosphorylation levels (Fig. 20). RasG− mutants lack ras activity and show aberrant cytoskeletal morphologies such as lack of cell polarity, and abnormal filopodia (Tuxworth *et al.*, 1997). RegA− mutants are deficient in an intracellular cAMP phosphodiesterase specific for the regulatory subunit of PKA (Shaulsky *et al.*, 1996). The mutant CN− lacks calcineurin or protein phosphatase 2B (PP2B) activity which binds specifically to calcium and calmodulin (Lydan *et al.*, *unpublished work*). The spiA gene is expressed in pre-spore cells during culmination and the spiA− spores that form quickly lose their viability (Richardson and Loomis, 1992). The final mutant investigated, YelA−, lacks the gene products that essentially regulate terminal differentiation and arrest at the tight mound stage (Osherov *et al.*, 1997). Western blot analysis revealed that the wild-type NC4 and all the mutants, excluding YelA−, showed similar high levels of actin tyrosine phosphorylation (Fig. 20). The actin tyrosine phosphorylation levels were further analyzed using densitometry, shown graphically in Fig. 20, Panel A as O.D. x mm² units. As above, this analysis reveals that similar phosphorylation levels to the wild type were found in all mutants excluding the YelA−. RegA− showed the most phosphorylation activity (7.67 units) followed by the wild-type NC4 (7.61 units). This result is expected
as RegA- mutants produce super-dormant spores (Meima et al., 1997). RasG- (6.31 units), CN- (5.89 units), and SpiA- (6.51 units) spores displayed similar amounts of actin tyrosine phosphorylation which were slightly lower than the wild-type NC4. YelA- (2.93 units) showed the lowest levels of actin tyrosine phosphorylation activity, as expected, since YelA- mutants arrest at the tight mound stage and form defective spores that quickly lose their viability (Osherov et al., 1997). As discussed earlier, Kishi et al. (1998) observed that actin tyrosine phosphorylation occurs during the final hours of development, thus YelA- mutants do not reach this developmental stage where actin tyrosine phosphorylation is detected. Otherwise, these results reveal that the mutants all have some level of actin tyrosine phosphorylation and these levels differ depending on the specific mutation.

Similar work using another D. discoideum mutant, SplA, which is a gene null mutant that encodes for a dual-specificity kinase (Nuckolls et al., 1996), has also been examined for actin tyrosine phosphorylation levels. The spores that form from this mutant are only dormant for a short time before germination takes place. No tyrosine phosphorylation of actin was detected in the SplA mutants suggesting that this dual-specificity kinase may be a possible candidate that phosphorylates actin during the late stages of development or another protein upstream of this event (Kishi et al., 1998).

Recent work has presented new ideas concerning the regulation of spore dormancy and germination. The main characters appear to be PKA and RegA, an intracellular cAMP phosphodiesterase specific for the regulatory subunit of PKA (Shaulsky et al., 1996). RegA activity plays a key regulatory role upon PKA which is
required to maintain spore dormancy (van Es et al., 1996). RegA activity has been shown to be affected via a transmembrane, two-component, histidine kinase transduction cascade DHKB and is activated by phosphorylation (Thomason et al., 1998; Zinda and Singleton, 1998). In dormant spores, this cascade has been suggested to be initiated by the autoinhibitor, discadenine, which through a series of phosphoryl relays phosphorylates a response regulator which inactivates RegA (Zinda and Singleton, 1998). The inactive RegA allows internal cAMP levels to rise which causes a disassociation of the regulatory subunit of PKA from the catalytic subunit thus stimulating PKA activity. These findings along with other work (van Es et al., 1996) has increased the information concerning signalling related to spore dormancy and germination. We propose a general model that collects a number of ideas dealing with signalling and consequently actin tyrosine phosphorylation (Fig. 22, Panel A). The model encompasses the ideas presented above dealing with RegA deactivation via DHKB as well as inhibition of spore germination by osmotic pressure and ammonia through ACG. As described earlier ACG is activated by either osmotic pressure or ammonia and produces cAMP which can bind to the regulatory subunit of PKA (van Es et al., 1996) depending on the activity of RegA. The response regulator that dephosphorylates RegA to inactivate it is likely a type of phosphatase. Additionally, SplA, whose mutant spores do not possess actin tyrosine phosphorylation, may act downstream of PKA playing a role in actin phosphorylation (Kishi et al., 1998).
Figure 22. A model for the regulation of *D. discoideum* spore dormancy.
**DORMANT SPORE**

Osmotic Pressure

ACG

\[ \text{cAMP} \]

(inactivated)

RegA

\[ \text{P} \]

Response regulator

?-phosphatase

\[ \text{splA (?)} \]

\[ \text{ACTIN} \]

Dormancy

**Figure 22.**
Actin tyrosine phosphorylation appears to be a vital component of spore dormancy and its maintenance. The understanding of actin regulation has led to a more complete understanding of the signalling process associated with it as well.
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