The roles of actin tyrosine phosphorylation and cellulose during dormancy and germination of various Dictyostelium discoideum mutants.

Tracy Lynn. Marr

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THE ROLES OF ACTIN TYROSINE PHOSPHORYLATION AND CELLULOSE DURING DORMANCY AND GERMINATION OF VARIOUS DICTYOSTELIUM DISCOIDEUM MUTANTS

by

Tracy L. Marr

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

2001
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ABSTRACT

Actin tyrosine phosphorylation plays a critical role in maintaining spore dormancy and spore viability. In the slime mold, Dictyostelium discoideum, several strains were explored for spore morphology, actin tyrosine phosphorylation levels, and the presence of cellulose in the spore coat layers of the cell walls in both dormant and germinating spores.

The first experiment done by Western blot analysis revealed that actin tyrosine phosphorylation occurs throughout the 14 days of development, excluding the late culmination stage (22 hours of development) for the D. discoideum strains (wild type NC4, V12, JH10, and mutant strains SG1, SG2, Ax2, Ax3, CN', RegA', and SpiA'). On average the actin tyrosine phosphorylation levels were maximized on the fourth to sixth day and there after slowly decreased as the spores reached the age of 14 days.

Spore morphology was observed and the results show that the strains NC4, V12, SG1, and SG2 are elliptical or oval in shape and large in size; whereas JH10, Ax2, Ax3, CN', RegA', SpiA', and Acg' are oval to round in spore shape and short in spore length; and SplA', and the aca' [PKA-C] overexpressor are round and short, and appear to look more like amoebae, than dormant spores.

The third experiment done by Western blot analysis revealed that actin tyrosine phosphorylation patterns for NC4, SG1, SG2, Ax3, and RegA' show an overall decrease for the 15 °C grown cultures in comparison to the RT grown cultures. The spore morphology also exhibited a decrease, the spores grown at 15 °C are smaller than those at RT.

The last half of this thesis focused on cellulose because it plays an important role in the development of D. discoideum by adding structural strength to slugs, sheaths, stalks, and spore walls. Cellulose studies revealed that cellulose: was present in the middle layer of the spore coat cell wall; was present in dormant, swelling, and emerging spores, but absent in nascent amoebae; demonstrated that there is a certain location within the spore coat cell wall that is a common tearing or splitting point during the natural process (germination), but not for the mechanical process (glass beads); and was present throughout the spore.
ACKNOWLEDGEMENTS

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I thank the entire faculty, staff, graduate students, and lab members for their assistance during my graduate studies. Thanks go to Dave Cervi, Steven Kales, and Dana Mahadeo for providing such a relaxing, enjoyable environment to work in. A special thanks goes to Dave Cervi for his constant help, insight, and guidance throughout my years at the university.

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<tr>
<td>ACG</td>
<td>germination adenylyl cyclase</td>
</tr>
<tr>
<td>APS</td>
<td>ammonium persulfate</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BCIP</td>
<td>5-bromo-3-chloro-indolylphosphate</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<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>GS</td>
<td>glucose salts medium</td>
</tr>
<tr>
<td>KPi</td>
<td>inorganic potassium phosphate buffer</td>
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<tr>
<td>M</td>
<td>molar</td>
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<td>mL</td>
<td>milliliter</td>
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<td>mM</td>
<td>millimolar</td>
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<tr>
<td>NBT</td>
<td>nitro blue tetrazolium</td>
</tr>
<tr>
<td>PKA</td>
<td>cAMP dependent protein kinase</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SM/2</td>
<td>standard medium/2</td>
</tr>
<tr>
<td>TEMED</td>
<td>(N,N,N',N')-tetramethylethylenediamine</td>
</tr>
<tr>
<td>Tween 20</td>
<td>polyoxyethylenesorbitan monolaurate</td>
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<td>(\mu L)</td>
<td>microliter</td>
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INTRODUCTION

More is known of the morphological details of *Dictyostelium discoideum* development than of any other member of the social amoebae. Ever since its discovery by Raper in 1935 from the forest soil in North Carolina, USA, its potential as an experimental organism have been appreciated and it has thus been intensively studied. It is not, by any means, the most common species of cellular slime mold but it is the one most studied in research laboratories, which has recently been designated a model organism by the United States National Institutes for Health (NIH). *D. discoideum* expresses many of the common processes of development. Its appeal as an experimental tool derives from the fact that it is easily cultured, disaggregated, mutated, and chemically dissected making it an ideal organism for studying morphological and biochemical aspects of development. The cells' ability to form a multicellular stage from a unicellular state and their ability to enter one of the three developmental pathways are the intriguing factors that make it a model organism.

I. The Asexual Life Cycle of *Dictyostelium discoideum*.

The most common pathway studied is the asexual life cycle (Fig 1A) of *D. discoideum* which terminates with the formation of a fruiting body. As stated earlier *D. discoideum* is an excellent model organism for developmental studies because of its haploid genotype, and its peculiar life cycle encompassing both unicellular and multicellular stages. Even though studies presented in this thesis focus on the haploid, asexual life cycle, it is important to state that under specific conditions (i.e. darkness and moisture) some species of the *Dictyosteliaceae* may enter a sexual cycle (Fig 1B). Many other *Dictyostelids* follow this pathway including *Dictyostelium mucoroides* and *Dictyostelium purpureum* (Raper, 1984). The major focus of the asexual life cycle in this study is on the maintenance of spore dormancy and the germination of spores in the sorocarp.
Figure 1A. The asexual (haploid) life cycle of *Dictyostelium discoideum*. The following stages are depicted. (A) Spore germination with single amoebae emerging. (B) Nascent amoebae. (C) Aggregating amoebae shown at much lower magnification (time 6 hours). (D) Pseudoplasmodium at beginning of migration (time 16 hours). (E) Beginning of sorocarp formation (culmination) (time 20 hours). (F) Later stage of sorocarp formation with stalk tube almost fully formed (time 24 hours). (G) Mature sorocarp showing cellular stalk and spores embedded in matrix (time 1-10 days).

Figure 1B. The sexual (diploid) life cycle of *Dictyostelium discoideum*. The following stages are depicted. (H) Dikaryotic giant cell that will become a zygote. (I) Macrocyst with diploid nucleus. A series of mitotic events followed by meiotic events leading to the formation of newly emerged haploid amoebae that escape from the cyst. From Alexopolous *et al.*, 1996.
In many regards, the most complex cells are not found inside large plants or animals but rather in some of the smaller eukaryotic organisms, such as the cellular slime mold, *Dictyostelium discoideum*. During the majority of *D. discoideum*’s life cycle, the cells exist as solitary, independent amoebae, crawling over their substratum (forest floor). Each vegetative cell is a complete, self-sufficient organism (Figure 2a). When food supplies become scarce, however, a new type of activity is triggered among the cells and they stream toward each other (Figure 2b) to form an aggregate called a pseudoplasmodium (slug), which migrates slowly over the substratum leaving a trail of “slime” (Figure 2c). The cells at the front end of the slug will become the stalk cells, whereas the cells at the rear end of the slug will become spore cells. The slug then ceases migration, rounds up, and begins to lift off the substratum, and cells that are seen at the upper end become part of the stalk (Figure 2d, 2e & 2f). The fruiting body consists of a slender stalk holding up a mass of spores at its upper end (Figure 2g). Where over time, each spore (Figure 2h) will give rise to an independent amoeba, able to begin the cycle once again (Raper, 1984).

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

The dormant spore of *D. discoideum* has the appearance of being the least dynamic phase of its development. The spores of *D. discoideum* are elliptical in shape (Fig 2h). They vary considerably in size, being approximately 6 to 9 μ long and 2.5 to 3.5 μ in diameter (Bonner, 1967). However, proper control of spore dormancy is probably the most critical step in the slime mold life cycle to ensure regeneration of the organism. Elaborate control mechanisms that regulate spore dormancy are still not fully understood, however their molecular components studied to date have been shown to be imperative.
Figure 2. Asexual life cycle of *Dictyostelium discoideum*. The following stages are outlined: (a) vegetative cell; (b) aggregation; (c) pseudoplasm (slug); (d) initiation of culmination; (e) middle of culmination; (f) completion of culmination; (g) fruiting body and spore maturation; (h) dormant spore. From several sources: Alberts *et al.*, 1994; Karp, 1996; and Raper, 1984.
Figure 2.
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. Surrounding the encapsulated spores resides a viscous fluid matrix containing proteins, yellow pigments, and an endogenous autoinhibitor, discadenine (Abe et al., 1976). The mechanism by which discadenine operates to promote spore dormancy (i.e. prevent spore germination) is only speculative at this point. The structure of discadenine (Fig 3A) was determined to be 3-(3-amino-3-carboxypropyl)-N⁶,²-Ádisopentenyladenine by Abe et al. in 1976. It was also shown that discadenine is synthesized from i⁶Ade (N⁶,²Á-isopentenyladenine) (Fig 3B) by transfer of the 3-amino-3-carboxypropyl moiety of S-adenosyl-methionine, using the enzyme discadenine synthetase (Abe et al., 1976).

Discadenine possesses not only activity for inhibition of spore germination, but also cytokinin activity as expected from its structure. It was discovered that fruiting bodies of D. discoideum contain a large amount of free i⁶Ade, but it is unknown whether i⁶Ade itself has any biological function in D. discoideum or whether it is merely a precursor of discadenine (Ihara et al., 1980). However, it is known that in D. discoideum the activities of a variety of enzymes change during the course of cell development (Loomis, 1975). Regulation of these enzymatic activities is presumably related to specific biological functions of the cells at particular developmental stages, or may even trigger differentiation. In this connection, the enzymes involved in the biosynthesis of discadenine are interesting subjects of study with respect to regulation of enzyme biosynthesis, since discadenine (and possibly i⁶Ade) has a specific physiological function, and so the activities of these enzymes should be coordinated with the overall developmental program.
Figure 3. Chemical structure of (A) discadenine and (B) i\(^6\)Ade. Discadenine is an organic inhibitor found in the spore matrix of *Dictyostelium discoideum* that prevents spore germination. Discadenine is synthesized from i\(^6\)Ade (N\(^6\)-\(\Delta\)-isopentenyladenine) by transfer of the 3-amino-3-carboxypropyl moiety of S-adenosylmethionine and the enzyme discadenine synthetase. From Abe *et al.*, 1976.
Figure 3.
Recent findings suggest two possible mechanisms of maintaining dormancy with in the sorus. There seems to be a high level of phosphorylation on a tyrosine residue of actin molecules (Gauthier et al., 1997; Kishi et al., 1998) found prior to spore swelling. This is believed to occur downstream within a phosphorylation cascade mediated by the low-molecular-weight autoinhibitor, through the activation of a transmembrane histidine kinase (DHKB) (Zinda and Singleton, 1998).

It has also been shown that a high ambient osmolarity can prevent spore germination (Cotter, 1997). Maintenance of high osmotic pressure within the sorus is mediated by low relative humidity within the environment (Cotter et al., 1999). Several solutions have been used to osmotically prevent spore germination including 0.2 M sucrose. This treatment has been shown to deactivate heat activated spores which then returns them to the dormant stage within six hours (Cotter, 1977; van Es et al., 1996).

A membrane bound adenylyl cyclase (ACG) which is specific to spores has been found to produce cAMP in response to osmotic pressure (van Es et al., 1996). The cAMP produced by the osmotic pressure through ACG inhibits spores by activating a cAMP dependent protein kinase (PKA) which in turn phosphorylates a variety of unknown proteins leading to dormancy. Studies conducted by van Es et al. (1996) have resulted in a model (Fig 4) showing high osmolarity, within the range of 100 to 200 mM sucrose and 100 mM NaCl, causing activation of ACG with subsequent increases in levels of cAMP. This newly formed cAMP freely binds to the regulatory subunit of PKA, thus causing it to become unbound from the catalytic subunit. The steps following this point are unclear, however it is thought that a downstream kinase may in turn phosphorylate monomeric actin, in conjunction with the downstream effects mediated by DHKB activation (Clements, 1998), as mentioned previously.
Figure 4. Signal transduction pathway for osmoregulation of spore germination.

The presence of osmotic pressure activates 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 dissociate and become active preventing spore germination.

From van Es et al., 1996.
Figure 4.
In relation to osmotic pressure, 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., 1999). Studies have shown the sori of *D. discoideum* strains NC4, V12, SG1 and SG2 contain endogenous concentrations of ammonia exceeding 100 mM. Exogenously, 69 mM ammonium phosphate alone has been shown to maintain dormancy in the premature maturation mutants (SG1 and SG2) for approximately one week (Cotter et al., 1999). Osmotic pressure and discadenine working in conjunction with one another act to ensure the maintenance of spore dormancy in the sori of fruiting bodies.

### III. Spore Germination and Regulatory Mechanisms in the Asexual Life Cycle of *Dictyostelium discoideum*.

Germination of dormant spores consists of four main stages (Fig 5); activation of dormant spores, post activation lag, swelling of dormant spores, and emergence of nascent myxamoebae (Cotter, 1981; Cotter and Raper, 1968; Cotter et al., 1992). Germination can be viewed morphologically by both phase (Fig 6) and electron (Fig 7) microscopy. Prior to and for a short time following activation, commonly referred to as the post activation lag stage, the dormant spore appears phase bright and oblong in shape. The spore then enters the swelling stage approximately 90 minutes post activation. The hallmark of this stage is the appearance of a protrusion typically located on one side of the swollen spore. After 2.5 hours the nascent amoeba begin to emerge from the cellulose capsule, thus giving rise to a fully emerged amoeba by approximately 3 hours.

Germination 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 occur during the lag phase prior to swelling (Cotter, 1977).
Figure 5. Germination kinetics of *Dictyostelium discoideum*. The mean and standard deviation are shown for each 30 minute period, where (O) represent swollen spores in the spore population and (●) represent the nascent amoebae in the spore population. The diagrammatic sequence of germination includes unswollen spores in the lag phase at 30 minutes after activation, early spore swelling at 1 hour, middle spore swelling at 1.5 hours, late spore swelling at 2 hours, amoebae emergence at 2.5 hours, and post emergence after 2.5 hours. From Cotter and Raper, 1968b.
Figure 5.
Figure 6. Stages of *Dictyostelium discoideum* spore germination observed by phase contrast microscopy. There are eight distinctive stages: (A) phase bright dormant encapsulated spore; (B) early spore swelling characterized by the beginnings of phase darkening; (C & D) mid-spore swelling characterized by the addition of a small protrusion; (E) late spore swelling characterized by a phase dark image and an oval shape; (F & G) amoebae emerging from the spore capsule; (H) remaining empty spore capsules. From Cotter and Raper, 1968a.
Figure 7. Stages of *Dictyostelium discoideum* spore germination observed by electron microscopy. *D. discoideum* spores were activated and allowed to germinate. This figure presents electron micrographs of thin sections (bar = 1 μm) of the organism at various stages of germination: (A) dormant spore; (B) swollen spore, 90 minutes after activation; (C) emerging amoebae, 2.5 hours after activation; (D) emerged amoebae, 3 hours after activation. From Kimmel, 1988.
The wild type strains, NC4 and V12, along with their premature maturation daughter mutants, SG1 and SG2, exhibit the same morphological changes throughout germination (Cotter and Dahlberg, 1977; Dahlberg, and Cotter, 1978). As demonstrated in the laboratory, wild type strains of *D. discoideum* spores can acquire spontaneous germination characteristics once aged 10-14 days. This may possibly be due to late spore maturation genes (Cotter and Glaves, 1989). The premature mutants, SG1 and SG2 also have the ability to spontaneously germinate upon formation of the mature fruiting body and release autoactivator thus stimulating spore germination in the remainder of the population (Dahlberg and Cotter, 1978). It has also been shown that 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).

Over the years a number of physical and chemical treatments have been described which are capable of activating the dormant spore: these include heat shock for 30 minutes at 45 °C (Cotter *et al*., 1976), incubation in 1% peptone (Cotter and Raper, 1966), 20% DMSO (Cotter *et al*., 1976), 8 M urea (Cotter and O’Connell, 1976), and a racemic mixture of the hydrophobic amino acids tryptophan, phenylalanine, and methionine (Cotter and Raper, 1966). Most of these treatments have destructive effects on some of the proteins and/or organelles within the cell. For example, 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).

IV. **Actin Cytoskeleton of Dictyostelium discoideum.**

Regulated actin cytoskeleton changes occur rapidly in most stages of the *D. discoideum* life cycle. Examples include cell mobility, cell morphology, phagocytosis, cytokinesis and vesicle trafficking. These changes involve both actin polymerization and actin cross linking which are regulated by actin binding proteins. Actin binding proteins play a vital role 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 capping proteins, monomer-sequestering proteins, cross-linking proteins, filament-severing proteins and membrane-binding proteins (Fig 8).

Microfilaments called F-actin or actin filaments are composed of the protein actin. Each actin molecule (globular or G-Actin) consists of two subdomains. The subunits become organized into strands that form a double helix in the presence of adenosine triphosphate (ATP), thus forming an actin filament (Karp, 1996). The associations between actin and the force generating myosin proteins are critical to the cytoskeleton. The two classes of myosin are the unconventional or myosin I class and the conventional or myosin II class. Myosin II is usually associated with cytokinesis, whereas myosin I has mainly been implicated in membraneous organelles, locomotion, and vesicular movement along the actin filaments (Karp, 1996). The objective of this thesis is to focus mainly on the regulation and role of actin, however, it can be assumed that myosin proteins are involved in the actin cytoskeleton.

In the past, 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). 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). Also, in the true slime mold, Physarum polycephalum, phosphorylation has been shown to occur on threonine residues 202 and 203 in a complex with the capping protein fragmin (De Corte et al., 1996).
Figure 8. Actin binding proteins in *Dictyostelium 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. From Noegel *et al.*, 1996.
Figure 8.
Actin genes are highly conserved in eukaryotes making the unicellular slime mold, *D. discoideum*, a model system for studies of this protein. It has been shown in *D. discoideum* that actin filaments exist in both the cytoplasm and the nucleus (Fig 9) (Fukui, 1978; Kishi et al., 1994; Sameshima et al., 1994; Sameshima et al., 2000). 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). Studies show that actin phosphorylation in *D. discoideum* is located at the tyrosine-53 residue following inhibition of oxidative phosphorylation in vegetative cells (Jungbluth et al., 1995). It has also been shown that dormant spores of *D. discoideum* 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).

V. *Dictyostelium discoideum* Mutant Strains.

The majority of researchers who are interested in actin tyrosine phosphorylation understand how this process occurs in normal developing *Dictyostelium* cells, however there is not much known about various *Dictyostelium* mutant cells. The object of this paper is to concentrate on tyrosine phosphorylation of actin in aged spores of various *Dictyostelium* mutants to determine whether they exhibit similar or different patterns of tyrosine phosphorylation compared to the wild types, NC4, V12, and JH10. The following *Dictyostelium* mutants which will be the focus for this thesis include, the premature
Figure 9. Actin rods. Actin plays a very important role in regulating spores in the dormant state. This figure illustrates how actin rods give the dormant spore rigidity, shape, and the ability to remain inert until favorable conditions allow it to germinate.

From Sameshima et al., 2000.
maturation daughter mutants (SG1 and SG2), Ax2, Ax3, CN', RegA', SpiA', SplA', Acm', and the acd' [PKA-C] overexpressor.

Dictyostelium cells express a G-protein-coupled adenylyl cyclase, ACA, during aggregation and an atypical adenylyl cyclase, ACG, in mature spores (van Es et al., 1996). The ACA enzyme is activated through G-protein-coupled surface cAMP receptors and is responsible for producing the oscillatory cAMP signals that regulate cell movement and differentiation. The two major pathways (cAMP and Ca^{2+}) by which G-protein linked cell surface receptors generate small intracellular mediators is diagrammed in Fig 10. The germination ACG enzyme contains a single transmembrane domain and is only expressed in spores.

It was determined by Saskia van Es and her coworkers in 1996, that ACG mediates inhibition of spore germination by high osmolarity. Whereas inactivation of the acd gene by homologous recombination generates spores that have lost the ability to respond to high osmolarity. It was also shown that rdecC (rapid developing) mutants, which display unrestrained protein kinase A (PKA) activity and a cell line, which overexpresses PKA under a prespore specific promoter, germinate very poorly, both at high and low osmolarity. Suggesting that PKA is the target for cAMP in the control of spore dormancy. Refer to Fig 4 for the signal transduction pathway for osmoregulation of spore germination.

The acd' [PKA-C] mutant overexpresses the catalytic subunit of PKA. It is known that PKA activity is important for preaggregation development and spore/stalk cell differentiation. The acd' [PKA-C] overexpressor can undergo normal development, however, at low amoebae densities, they are unable to aggregate. After cells have aggregated at high cell densities, the prestalk cells sort to the apex and can be seen later in the anterior of finger-like structures. These fingers fall over and migrate phototactically like wild-type slugs. All of these developmental events are carried out without any measurable adenylyl cyclase activity or accumulation of cAMP (Kuspa, 1997). Thus, as long as PKA is active, cell type divergence and sorting, slug migration, morphogenesis and terminal differentiation can proceed in the apparent absence of cAMP.
Figure 10. Two major pathways by which G-protein linked cell surface receptors generate small intracellular mediators. In both cases the binding of an extracellular ligand alters the conformation of the cytoplasmic domain of the receptor, causing it to bind to a G-protein that activates (or inactivates) a plasma membrane enzyme. In the cyclic AMP (cAMP) pathway the enzyme directly produces a soluble mediator (inositol trisphosphate) that releases Ca\(^{2+}\) from the endoplasmic reticulum. Like other small intracellular mediators, both cAMP and Ca\(^{2+}\) relay the signal by acting as allosteric effectors. They bind to specific proteins in the cell, thus altering their confirmation and thereby their activity. From Alberts et al., 1994.
The control of cAMP synthesis and PKA activity is required for chemotactic aggregation (Meima et al., 1997). An extracellular signal regulated kinase 2 (ERK2) is essential for the production of cAMP during aggregation and is thought to be required for the activation of ACA, and thus indirectly is required for PKA activation through intracellular cAMP. Aggregation, cAMP production, and ACA activation are all restored to ERK2 null cells when the phosphodiesterase RegA is inactivated by gene disruption. This suggests that ERK2 functions by inhibiting the cAMP-PDE activity of RegA (Mann, 1997).

There is a common genetic pathway that involves rdeA, rdeC, and regA, which controls the maturation of both stalk and spore cells in Dictyostelium (Traynor, 1997). The function of the rdeA gene is an intermediate in the phospho-relay of histidine kinase (Thomason et al., 1999), whereas the rdeC gene encodes the regulatory subunit of PKA, and regA encodes a novel hybrid protein consisting of an N-terminal region homologous to the response regulators of two component systems together with a C-terminal cAMP phosphodiesterase domain. In common with other response regulators, expressed regA can accept phosphate from acetyl phosphate.

The RegA– mutant is considered to be a negative regulator of PKA. It is deficient in an intracellular cAMP phosphodiesterase, thus leading to elevated levels of intracellular cAMP, which results in an overexpression of PKA. RegA– spores show strongly retarded germination, suggesting that in addition to ACG, regA may also be an important factor in the control of spore germination.

The CN– mutant is reported to be deficient in calcineurin (CN) or protein phosphatase 2B (PP2B) (Lydan et al., unpublished work). Normally calcineurin binds specifically to calmodulin and calcium (Lydan et al., unpublished work). Unfortunately there is not a lot known about this mutant because it is still relatively new in the field of Dictyostelium research. Previous studies show that it germinates and develops normally in comparison to the wild type, NC4.
The *spiA* gene of *Dictyostelium* is expressed specifically in prespore cells and spores during culmination, the final stage of development during which prespore cells undergo terminal differentiation to form spores and a stalk. Homologous recombination was used to delete this gene (Richardson and Loomis, 1992). The SpiA⁻ mutant strain develops normally and produces spores that are indistinguishable from those of wild type strains by transmission and electron microscopy. Mutant spores only display normal viability if collected and assayed as soon as possible after the completion of their development. It is a known fact that as SpiA⁻ spores age, they lose their viability more rapidly than those of the SpiA⁺ parent strain. Reinserting an intact copy of the *spiA* gene into a SpiA⁻ strain restores the stability of its spores. The product of the *spiA* gene, Dd31, was identified on Western blots as a 30-kDa protein using an antibody raised against a fusion protein containing a portion of the coding sequence (Richardson and Loomis, 1992).

The *splA* gene is expressed throughout development with a peak during the mound stage of morphogenesis. It is considered a novel dual specificity kinase that regulates the differentiation of spore cells. Strains in which the *splA* gene has been disrupted completed fruiting body formation, however the spores spontaneously lyse before completing their differentiation (Nuckolls *et al.*, 1996).

In general, mutant analysis will provide an understanding of the molecular events in sporulation, dormancy maintenance, and germination. Thus each mutant can be phenotypically characterized, germinated, and analyzed for actin tyrosine phosphorylation by Western Blot analysis using an anti-phosphotyrosine antibody.
VI. Spore Coat of Dictyostelium discoideum.

Recently, ultrastructural studies have provided some understanding of the nature of the spore cell wall. The cell wall of the spore appears to consist of four morphologically distinct layers of different thicknesses and electron densities. The composition of the spore coat has been determined from coats shed upon germination and purified to homogeneity on density gradients. Its dry weight is equally divided into protein and cellulose. Differentiated Dictyostelium spores synthesize a tough spore coat at their cell surfaces. Assembly seems to be based on the infusion of cellulose from the plasma membrane into a mix of secreted proteins awaiting at the cell surface, resulting in formation of a trilaminar structure with layers of protein sandwiched around the cellulose core (West and Erdos, 1990).

As determined by immunogold localization studies on sections of washed spores, the outer layer contains the interspore matrix, only seen in unwashed spores. Followed by the outer, middle and inner layers of the spore coat, in which the inner layer of the spore coat is surrounded by the plasma membrane (Figure 11). Cellulose is only found in the middle spore coat layer. Dictyostelium contains at least two different types of cellulose, α and β, distinguishable by their solubility properties (Roseness and Wright, 1974).

It is thought that there are weak associations between the plasma membrane and the spore coat, in which these may be sufficient to provide polarity to the coat during its morphogenesis. Past studies (Cotter et al., 1969) suggest the two outer layers split and can retract during the swelling phase of germination, exposing the outer surface of the inner layer, which remains continuous over the surface of the cell. Completion of germination disrupts the inner layer resulting in its shedding, thus causing lysis of the spore cell wall and allowing the emergence of the viable amoeba.
Figure 11. Trilaminar organization of the spore coat. The plasma membrane (bottom) and the interspore matrix (top) surround the three layers of the spore coat (inner, middle, and outer). Cellulose is found in the middle layer of the spore coat. From West and Erdos, 1990.
Figure 11.
VII. Cellulose.

Cellulose is one of the most important primary plant products. A distinguishing feature of plant cells is the presence of a cell wall, which is required not only for its structural traits but also for its many functional properties. Plant cell walls are not homogenous; they are a complex mixture of polysaccharides and proteins. The major polysaccharide is cellulose, an aggregate of β-1,4-linked glucan chains (Fig 12) (Brown, 1996). Bundles of these glucan chains (Fig 13) associate via hydrogen bonding to form an insoluble ultramicroscopic thread known as cellulose microfibrils (Fig 14d) (Brown, 1978).

Cellulose synthesis has reached its greatest diversification among eukaryotic cells. Non-photosynthetic cells can synthesize cellulose. These include certain fungi, amoeba cysts, and the cellular slime molds, of which D. discoideum is a famous example. Although Dictyostelium amoebae do not make cellulose while they are growing, developing cells accumulate cellulose after the mound stage. Cellulose microfibrils are found in the extracellular matrix that surrounds the slugs as well as in the trails they leave behind (Fig 14a & 14b). During culmination, cellulose is deposited in the stalk tube and in the spore coats and stalk cell walls (Fig 14c & 14h) (Blanton, 1993; Blanton, 1997; and Blanton, 2000). As mentioned previously, cellulose is also found in the spore coat. During spore germination, degradation of the cellulose in the spore’s cell wall by cellulase is required to allow the amoeba to emerge (Ramalingam et al., 1992). The presence and location of cellulose in different stages of the life cycle of Dictyostelium can be demonstrated by the use of the fluorescent cellulose dye, Congo Red.

Congo Red is a fluorescent dye used to stain amyloid and cellulose (Fig 15). It is believed that it hydrogen bonds to the beta pleated sheet structure of the amyloid protein. At this time the mechanism of how it stains cellulose is unknown. Congo Red is also known as Direct Red or Cotton Red and stains amyloid and cellulose red in colour (Stainsfile, 2000).
Figure 12. The structural formula for the β-1,4-linked glucan polymer chain (cellulose). The repeating unit, cellobiose is indicated in brackets. From Brown, 1996.
Figure 12.
Figure 13. Diagrammatic representation of glucan chains (single lines) as they associate to form an extended chain microfibril. Regions of uniform crystallinity are depicted by straight lines. Paracrystalline regions are suggested by wavy lines. From Brown, 1978.
Figure 13.
Figure 14. *Dictyostelium discoideum* extracellular matrices that contain cellulose. The following images depicted are: (a) surface sheath forming on the surface of a consolidating aggregate; (b) slug (cellulose slime trail left behind slug); (c) stalk, consisting of a cellulosic stalk tube and stalk tube surrounding the stalk cells with cellulosic cell walls; (d) cellulose microfibrils of the stalk tube and stalk cell wall; (e) culmination; (f) stalk cells and spore cell walls found in the fruiting body; (g) fruiting body head consisting of thousands of tiny dormant spores; (h) spores that contain cellulose in the middle layers of their cell walls. From various sources: Alberts, 1994; Blanton, 1997; Karp, 1996; and Sameshima, 1994.
Figure 15. Structural formula for the fluorescent dye, Congo Red. From Stainsfile, 2000.
Figure 15.
By definition dyes are aromatic organic compounds, and as such are based fundamentally on the structure of benzene (meaning they must have at least one aryl ring (phenyl) or benzene ring). The delocalized electrons in this benzene ring are the fundamental cause of the absorbance of the electromagnetic radiation in organic compounds, and consequently the appearance of colour in dyes. Benzene absorbs electromagnetic radiation about 200 nm (Stainsfile, 2000). However the perception of colour is an ability of some animals, including humans, to detect some wavelengths of electromagnetic radiation (light) differently from other wavelengths. Normal daylight, or white light, is a mixture of all the wavelengths to which we can respond and some to which we cannot, in particular the infrared and ultra-violet rays. We respond to wavelengths between 400-700 nm. Thus, when an object absorbs some of the radiation from within that range we see the waves that are left over, and the object appears coloured. However, since benzene is outside the range that humans can perceive, therefore it appears to be colourless (Pawley, 1995).

When some of the wavelengths found in white light are absorbed, we then see what is left over as coloured light. The colour that we see is referred to as the complementary colour of the colour that was removed. For instance, if the red rays are removed from white light, the colour we detect is blue-green. Thus blue-green is complementary to red, and red is complementary to blue-green. In the case of Congo Red, the opposite holds true. Where the excitation spectrum of the compound being used is 596 nm and the emission spectrum of the fluorophore is 615 nm, thus the colour detected is red (Pawley, 1995).

Congo Red also contains the presence of a chromophore. By definition these chromophores are atomic configurations which can alter the energy in delocalized systems (Stainsfile, 2000). Keeping in mind that chromophores do not make dyes coloured, rather they function by altering the energy in the delocalized electron cloud of the dye, and this alteration results in the compound absorbing radiation from within the visible range instead of outside it.
Chromophores are composed of atoms joined in a sequence of alternating single and double bonds. Double bonds in organic compounds can be of two types. If the atoms of the double bonds are not adjacent, they are termed isolated double bonds, and exist independently of other double bonds in the same molecule. If adjacent atoms have double bonds they are termed conjugated double bonds and the bonds interact with each other (Stainsfile, 2000). In the case of Congo Red, it contains isolated double bonds and has a chromophoric configuration of \(-\text{N} = \text{N}-\) in its structural formula.

As with most dyes, there is a gradual decay and the electrons return to the ground state. Even though we do not see anything, nonetheless, something may happen that we do not see. Possibly there is an increase in temperature, or some chemical changes occur which disrupt the dye's structure and cause it to lose colour or fade (Pawley, 1995). This is a minor problem seen with the use of some fluorescent dyes but can be overcome by the use of anti-fade reagents available on the market today.

VIII. Cellulose Synthesis of *Dictyostelium discoideum*.

Organisms that synthesize cellulose can be found amongst the bacteria, protistans, fungi, and animals, but it is in plants that the importance of cellulose in function (as a major structural component of plant cell walls) and economic use (as wood and fiber) can be best appreciated. The structure of cellulose and its biosynthesis have been the subjects of intense investigation for several years. The synthesis of cellulose by cellular slime molds has been known since their initial discovery (Brefeld, 1869). Cellular slime mold vegetative amoebae do not make cellulose. In some species, the amoebae can form microcysts, of which the walls contain cellulose (Raper, 1984). In species that do not form microcysts, cellulose synthesis is normally an activity of multicellular development. It was also found in 1968, by Harrington and Raper that cellulose is also found in the wall layers of macrocysts.
In *D. discoideum*, cellulose is first detected cytologically during late aggregation (Harrington and Raper, 1968). It remains at low levels until culmination, when the amount of cellulose increases dramatically. Cellulose accounts for approximately 3-4% of the dry weight of mature culminants (Loomis, 1975; Sussman and Sussman, 1969). Cellulose is also found in the surface sheath (cellulose microfibrils) that surrounds the multicellular structures, in the slime sheath left behind by migrating slugs, in the stalk tube that surrounds the stalk cell population, in the stalk cells, and in the spore cell walls (Figure 14).

It is not known which cells contribute cellulose to the surface sheath or the slime trail. Cytological (Bonner, 1955; Harrington and Raper, 1968), ultrastructural (George, 1972), and autoradiographic (George, 1969) evidence suggests that at least a sub-population of the prestalk cells is responsible for stalk tube cellulose synthesis. Stalk cell wall and spore cell wall cellulose is synthesized by the individual prestalk and prespore cells. Therefore, all cells will have contributed cellulose to at least one structure by the conclusion of development. The presence of cellulose in the stalks of *D. discoideum* was first reported by Raper and Fennell in 1952, and was then confirmed by X-ray diffraction studies in 1957, by Gezelius and Ranby, as well as by electron microscope examination in 1956 by Muhlethaler.

By analogy with plants, one would assume that a significant role of cellulose would be to confer structural strength to sheaths, stalks, and spore walls, but it is likely that there are additional roles for cellulose. The discovery of cellulose synthesis mutants has allowed researchers to provide evidence that cellulose is important in development. Cellulose is critical in maintaining the integrity of slugs and in slug motility. Mutants that were incapable of synthesizing cellulose (lacked UDPG-pyrophosphorylase) were poorly motile and could not maintain their integrity over time, thus ceasing at culmination and failing to produce spores, stalk tube, or stalk cells (Dimond, 1976).
Cellulose containing structures have been determined to play key roles in developmental events. For instance the dimensions of the stalk tube determine the final form of the sorocarp. The formation of the surface sheath on tight aggregates determines the size of the mound. The surface sheath and slug slime sheath are important in slug motility, determination of slug polarity, response to external stimuli, and cell differentiation. The integrity of the surface sheath may protect the multicellular structures from predators. However in each of these instances there is no evidence of cellulose doing anything other than providing strength (Blanton, 1997).

There is however good evidence that suggests cellulose provides a framework for the assembly of extracellular matrix proteins. A number of proteins are released from the slime sheath by cellulase treatment, suggesting a close association with cellulose (Grant and Williams, 1983). Among these is a family of oligometric proteins called the sheathins, which co-localize with cellulose in the cell print zones in the slug sheath (Fig 16). The sheathins are glycoproteins whose O-linked glycosylation is dependent upon the modB locus. In modB mutants, the sheathins and cellulose are deposited normally, but the slugs are altered in their morphology and migratory behavior (Zhou., 1995). Cellulose-glycoprotein interactions are, therefore important in the structuring of the spore coat and the slug slime sheath. It is likely that similar interactions are involved in structuring the surface sheath, the stalk tube, and the stalk cell walls.

*D. discoideum* is just beginning to contribute fundamental insights into the cellular control of cellulose biosynthesis, the role of cellulose in development, and the role of cellulose in extracellular matrices. Regardless of whether specific mechanisms may or may not be applicable to cellulose synthesis in plants, certainly the ideas and approaches developed with *D. discoideum* will provide new approaches and insight into the mechanisms of plant cellulose biogenesis.
Figure 16. Appearance of Nessler-stained "cell prints" of a migrating slug of Dictyostelium discoideum. The two photos outlined depict: (A) cell prints on the surface of a migrating slug of D. discoideum; (B) globules within the boundaries of the cell prints. From Feit, 1994.
IX. Objectives of This Investigation.

From past work, it is clear that further investigation of the role of actin tyrosine phosphorylation in mutants of *D. discoideum* is required. The majority of researchers who are interested in actin tyrosine phosphorylation understand how this process occurs in normal developing *Dictyostelium* cells, however there is not much known about various *Dictyostelium* mutant cells. The first main objective of this paper is to concentrate on tyrosine phosphorylation of actin in various aged spores of *Dictyostelium* mutants to determine whether they exhibit similar or different patterns of tyrosine phosphorylation compared to the wild type NC4, V12, and JH10. The following *Dictyostelium* mutants that will be of interest, include the premature maturation daughter mutants (SG1 and SG2), Ax2, Ax3, CN, RegA, SpiA, and SplA.

A. Determine the actin tyrosine phosphorylation patterns of aged *D. discoideum* mutants during the sporulation process. Sample collections started with the late culmination stage (22 hours of development), 24 hours, 36 hours, 48 hours, 72 hours, 4 days, 5 days, 7 days, 10 days, 12 days, until they reach the age of 14 days. Levels of actin tyrosine phosphorylation were studied in various mutants SG1, SG2, Ax2, Ax3, CN, RegA, and SpiA, and were compared to those of wild type NC4, V12, and JH10 spores by Western blot analysis.

B. Determine the individual spore morphology of various *D. discoideum* strains. Two day-old dormant spores were used to observe the spore morphology of *D. discoideum* strains (wild type NC4, V12, JH10, and mutant strains SG1, SG2, Ax2, Ax3, CN, RegA, SpiA, SplA, Aca, and the *aca* [PKA-C] overexpressor). As a preliminary investigation it was important to study the spore morphology of each *D. discoideum* strain to better understand how the shape of the spore reflects the cytoskeleton structure and heat resistant properties.
C. Determine how the shape of the spore reflects the cytoskeleton, which in turn reflects the heat-resistant properties of the spore by examining the change of shape via temperature change. Actin plays a very important role in regulating spores in the dormant state. Actin rods aid in giving the dormant spore rigidity, shape, and the ability to remain inert until favorable conditions allow it to germinate. Cultures were grown at room temperature (21-23 °C) and 15 °C for 10 days and changes in actin tyrosine phosphorylation and spore shape and size were compared for the *D. discoideum* strains NC4, SG1, SG2, Ax3, and RegA⁺ by Western blot analysis and light microscopy.

D. Determine how the actin tyrosine phosphorylation patterns of aged *D. discoideum* strains (1, 5, and 10 days) reflect the heat-resistant properties of the spore by examining the change of shape via temperature change. Cultures were grown at room temperature (21 °C – 23 °C) and 15 °C and samples were collected at 1, 5, and 10 days. Levels of actin tyrosine phosphorylation were compared for the *D. discoideum* strains NC4, SG1, SG2, Ax3, and RegA⁺ by Western blot analysis.

The second main objective of this thesis is to use a confocal microscope with the aid of the fluorescent dye, Congo Red under an argon-krypton laser to study the cellulose contents/patterns demonstrated in the cellular slime mold, *D. discoideum*. As with every experiment, controls are needed to ensure that an experiment is correctly performed. For the purpose of this thesis the same controls were used for all experiments in this section. Understanding and realizing that cellulose is ubiquitous in nature made it very difficult to find a negative control, but yet on the other hand, easy to find a positive control. The positive control used was 100% cotton fibers that were observed and stained positive (red in colour) for Congo Red. As stated earlier it is known that *Dictyostelium* vegetative amoebae do not make cellulose (Brefeld, 1869), thus it is reasonable to use this as a negative control for each of the experiments performed.
E. **Determine the presence or absence of cellulose in the spore cell wall.** The following *D. discoideum* strains analyzed were: wild type NC4, V12, JH10, and mutant strains SG1, SG2, Ax2, Ax3, CN', RegA', SpiA', SplA', Acg', and the *aca' [PKA-C]* overexpressor.

F. **Determine the presence or absence of cellulose in the spore cell wall during the germination process.** The following *D. discoideum* strains analyzed were: wild type NC4, V12, JH10 and mutant strains SG1, SG2, Ax2, Ax3, CN', RegA', SpiA', SplA', Acg', and the *aca' [PKA-C]* overexpressor.

G. **Determine whether there is a difference in dormant spore cell wall splitting (natural vs. mechanical cell disruption).** Natural cell disruption refers to allowing spores to enter the germination process on there own, where as mechanical cell disruption refers to grinding spores with the aid of small glass beads. This experiment was carried out on the *D. discoideum* strains NC4, SG1, and SG2.

H. **Determine if cellulose is present throughout the spore or if it is only present in the spore cell wall compartment.** This was accomplished by performing a z-series (several submicron sections through an individual spore from the bottom layers of the spore to the top). The following *D. discoideum* strains analyzed were: wild type NC4, V12, JH10, and mutant strains SG1, SG2, Ax2, Ax3, CN', RegA', SpiA', SplA', Acg', and the *aca' [PKA-C]* overexpressor.
MATERIALS AND METHODS

I. Strains Utilized.

Numerous strains of the cellular slime mold, *D. discoideum* were used in this study. Aging studies were performed using the wild type NC4 (diploid), V12, JH10, and mutant strains SG1 and SG2 (premature maturation mutants), Ax2, Ax3, CN', RegA', SpiA', and SplA'. Morphology, germination, and cellulose studies include the strains: wild type NC4 (diploid), V12, JH10, and mutant strains SG1 and SG2 (premature maturation mutants), Ax2, Ax3, CN', RegA', SpiA', and SplA', Acg', and aca' [PKA-C]. The advantage of using spores from two mutant spontaneous germinator strains (SG1 and SG2) provided an advantage by monitoring changes of proteins of interest more discretely throughout germination. As mentioned earlier, both mutants have the capability to undergo spontaneous germination, however, the lag time post activation for spores of SG2 is significantly decreased compared to the aged parental wild type V12.

II. Media Utilized.

Three different types of media were used in this study, standard medium/2 (SM/2), glucose salts (GS), and 1% peptone broth. All cultures were maintained in the laboratory on either standard medium/2 (SM/2) or glucose salts (GS). Standard medium/2 contained the following components: 5.0 g glucose (Sigma), 5.0 g bactopeptone (Difco), 0.5 g yeast extract (Difco), 1.1 g KH₂PO₄ (Sigma), 0.5 g K₂HPO₄ (Sigma), 0.5 g MgSO₄ (Sigma), and 15.0 g agar (Difco) in a final volume of 1L of ddH₂O. Whereas glucose salts (GS) contained the following components: 1.0 g NH₄Cl (Sigma), 3.0 g KH₂PO₄ (Sigma), 6.0 g Na₂PO₄ (Sigma), 0.13 g MgSO₄ (Sigma), and 20.0 g agar (Difco) in a final volume of 1L of ddH₂O.

Germination studies were carried out in 1% peptone broth, which contained the following components: 10.0 g bactopeptone (Difco) in a final volume of 1L of 10 mM potassium phosphate buffer (KPi). All media were then placed on a stirrer for
approximately 5 minutes to ensure proper mixing, followed by an administration of concentrated KOH to bring the final pH up to a value of 6.5. The medium was then autoclaved and subsequently poured (15 mL) into sterile petri dishes (Pheonix) and stored at 4 °C until further use (Cotter and Raper, 1968a).

III. Culturing of Strains.

All strains of *D. discoideum* for production of spores were cultured as per standard plating protocol (Cotter et al., 1999). Double distilled water was briefly boiled for approximately 3 minutes to ensure sterility. An appropriate amount was aliquoted into a plastic, sterile, 50 mL conical tube (Fisher) and allowed to cool to room temperature. To obtain sufficient samples for experiments, spores from a stock culture (five to ten sori) were aseptically transferred to the conical tube containing the sterile ddH2O. To the same solution a bacterial inoculum (*Klebsiella aerogenes* (SM/2) or *Escherichia coli* (GS)) was added through the use of a sterile pasture pipette by running the bulb end of the pipette over the agar of the surface on which the bacteria were grown. The spore suspension was vortexed and 1.5 mL of the solution was added onto either SM/2 or GS agar plates. The plates were gently shaken to facilitate an even spread of the spore solution and then incubated at room temperature in the presence of light. After 24 hours following the initial inoculation, the plates were again shaken to further promote an even distribution of the spores and bacteria. They are then set aside for approximately 3-4 days after the initial inoculation to allow for development of a new generation of spores.

IV. Harvesting of Spores.

Spores were harvested by collecting the sori 1 mm above the agar surfaces with a glass microscope slide at a 45 ° angle and the petri plate was rotated. The spores were then rinsed in a 50 mL beaker containing a small volume (5 mL) of ddH2O. An IEC Clinical Centrifuge was used to centrifuge the solution at 4500 g for approximately 3
minutes to produce a pellet. The supernatant, that contained crude matrix material, was saved while the pellet was resuspended, washed and centrifuged twice in 5 mL of potassium phosphate buffer (KPi, pH 6.5). Washed spores were either subjected to experimental studies, or stored at -20°C in appropriately labeled 10 mL glass test tubes partially submerged into a small volume of 95% ethanol until ready for protein analysis.

V. Cellulose Studies.

The following outlines how the presence/location of cellulose was detected in strains of *D. discoideum* in the spore cell wall. *D. discoideum* strains were grown on SM2 media and fed *K. aerogenes* as a food source. Strains were allowed to grow and develop until fruiting, and were harvested on day 4 post fruiting in 5 mL of ddH2O. Spores were centrifuged at 3500 rpm for 3 minutes and the supernatant was carefully removed. The pellet of spores was resuspended in 1 mL of a 0.1% Congo Red dye solution (Congo Red was diluted in KPi). The mixture was then allowed to equilibrate for ½ hour on a rocker to allow for even absorption of the dye throughout the spores. The mixture was centrifuged at 3500 rpm for 3 minutes and the pellet was then resuspended in 1 mL of KPi. Live preparations of spores were put on microscope slide, coverslipped and viewed under oil immersion (10x/25 eyepiece X 60x/140 oil objective) on the confocal microscope. See section VI. in material and methods for spore germination conditions for cellulose studies. The location of cellulose within the spore was determined through the use of z-series. Performance of z-series on a confocal microscope produces several submicron sections through an individual spore from the bottom layers of the spore to the top (z-step size = 0.2-0.3 μm).

VI. Spore Germination Conditions for Cellulose Studies.

Spores were harvested on day 4 post fruiting by collecting sori from the agar surfaces with a glass microscope slide as stated in section IV: Harvesting of Spores. After careful washing of the spores, the spores were centrifuged and the pellet was resuspended in 1 mL of a 0.1 % Congo Red dye mixture at a concentration of 4 x 10^5
spores/mL (determined with the aid of a hemocytometer) (Cotter and Raper, 1968a). The mixture was allowed to equilibrate for 30 minutes on a rocker to allow for even absorption of the dye throughout the spores. The mixture was centrifuged at 4500 g for approximately 3 minutes and the pellet was then resuspended, washed and centrifuged in 5 mL of KPi (pH 6.5). Once the excess dye mixture was removed the spores were then resuspended in 5 mL of 1% peptone broth.

To allow sufficient oxygen exchange spore suspensions contained 6 mm magnetic stir bars and were kept at or below 4 mL and placed on a magnetic stirrer for the duration of the germination experiment (5-8 hours depending on the strain used). Live preparations of spores were placed on a microscope slide and viewed (10x/25 eyepiece X 60x/140 oil objective) on the confocal microscope under an argon-krypton laser. 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 spores were counted and recorded.

VII. Cell Breakage.

Samples for electrophoretic protein separation were collected at approximately $1 \times 10^8$ spores/test tube. Spores collected from all strains following experimental analysis were subjected to mechanical disruption by using a mixture of small (0.25-0.32 mm) and large (0.7-1.2 mm) glass beads (Sigma) in a 50:50 ratio. Frozen aliquots were resuspended in 60 μL lysis buffer (containing: 1% Triton X-100, 1 μg/mL leupeptin, and 1 mM phenylmethylsulfonyl fluoride (PMSF)), and 0.2 mL of glass beads. Spores were resuspended and vortexed for a total of 5 minutes at 30 second intervals to avoid excess heating and protein degradation. Each sample was kept on ice between vortexing of other samples. Phase contrast microscopy was utilized to ensure spore breakage of at least 80-90% of the original collected sample of $1 \times 10^8$ spores. The suspension was removed from the glass beads using a micropipette (Gilson) and transferred to a 1.5 mL microcentrifuge tube (Eppendorf) and pelleted in a microcentrifuge tube at 14 000g
(Beckman). The supernatants were transferred once again to a new microcentrifuge tube ready for protein analysis.

VIII. Protein Quantification and Sample Preparation.

The Bradford method (1976) was used to quantify the protein of the ground spore samples using the BioRad Protein Assay (BioRad Laboratories). Quantification took place in a 96 well microtitre plate (NUNC) in a final volume of 200 $\mu$L solution containing 155 $\mu$L 10 mM KPi, 40 $\mu$L BioRad protein dye, and 5 $\mu$L of sample. The mixture was gently mixed and allowed to develop for 10 minutes prior to spectrophotometric analysis at 595 nm using a Microplate Reader (Mandel Scientific Co.). Protein concentrations were determined by utilizing a standard curve that was generated with bovine serum albumin (BSA) (Sigma). Sample preparation involved adding 10 mM KPi and 5X sample buffer (2.5 mL $\beta$-mercaptoethanol (BME), 5.0 mL glycerol, 2.5 mL 0.5 M Tris, pH 6.8, 1.0 g sodium dodecyl sulfate (SDS), and 0.5 g bromophenol blue) and the appropriate volume of protein as determined using the BioRad Protein Assay prior to loading into their respective wells.

VI. SDS PAGE.

Proteins were analyzed using the Mini Protein II Electrophoresis System (BioRad) by the Laemmli method (1970). A 10 % gel was used that consisted of a 10 % separating gel (containing 4.0 mL ddH$_2$O, 2.5 mL 1.5 M Tris-Cl, pH 8.8, 3.35 mL 30 % acrylamide (BioRad), 100 $\mu$L 10 % SDS, which was thoroughly mixed prior to the addition of the polymerizing agents (50 $\mu$L 10 % ammonium persulfate (APS) and 10 $\mu$L N,N,N',N'-tetramethylethylenediamine (TEMED)). The separating gel was allowed to polymerize at room temperature for 30-45 minutes. After polymerization a 4 % stacking gel was poured above the separating gel (containing 5.9 mL ddH$_2$O, 2.5 mL 0.5 M Tris-Cl, pH 6.8, 1.5 mL 30 % acrylamide (BioRad), 100 $\mu$L 10 % SDS, which was thoroughly mixed prior to the addition of the polymerizing agents (50 $\mu$L 10 % APS and 10 $\mu$L
TEMED). A 10 well comb template was quickly inserted into the stacking gel and allowed to polymerize for 30-45 minutes at room temperature.

After polymerization of the stacking gel, the combs were removed and the wells were flooded with ddH₂O and then the wells were emptied of all ddH₂O. The prepared samples and the prestained colour molecular weight markers (Sigma) were boiled for 3 minutes prior to loading onto the 10 % SDS polyacrylamide gels. All samples loaded were prepared to contain a final protein concentration of 0.1 μg/μL, thus a loading volume of 10 μL per well would equal a total protein concentration of 1.0 μg per lane. Sample preparations were loaded at 10 μL aliquots per lane, along with 5 μL of prestained protein standards (Sigma) in a single well. Following an overlay with 1X running buffer (14.4 g glycine, 3.0 g Tris Base, 1.0 g SDS in 1L ddH₂O) the protein gels were subjected to an electric field at 200 V/3mA for a duration of 45 minutes to an hour or until the dye front reached the bottom.

VII. Western Blot Analysis.

Once the proteins were separated, the gels were removed from the Mini Protean II apparatus and temporarily placed into a 1X blotting buffer solution (containing: 3.3 g Tris Base, 14.5 g glycine, 20 % methanol in 1L volume with ddH₂O at a pH of 8.3). A Mini Trans-Blot Electrophoretic Transfer Cell (BioRad) was used to transfer separated proteins from the acrylamide gel onto a polyvinylidene fluoride (PVDF) microporous membrane (Millipore) using ice cold blotting buffer. Prior to transferring the PVDF microporous membrane, it was activated by submerging it in 100 % methanol for 15 seconds. Protein transfer occurred at 130 V/400 mA for a period of 1 hour and 15 minutes using the BioRad Power Supply, Model 250/25.

After the PVDF microporous membranes were removed from the transfer chamber the membranes were blocked with 2 g dry skim milk powder (Carnation) in 250 mL Tris-Tween-Saline-Buffer Solution (TTBS) (contains: 1.6 g Tris, 8.2 g NaCl, 0.1 % polyoxyethylene-sorbitan monolaurate (Tween 20), pH 7.4) for 1 hour at room
temperature. Membranes were then probed for phosphotyrosine containing proteins using the recombinant antiphosphotyrosine antibody RC20-E120AP (Transduction Laboratories) preconjugated with alkaline phosphatase at a 1:2500 dilution in blocking buffer at room temperature for 1 hour. To prevent banding patterns appearing as a result of nonspecific binding, membranes were washed for 15-20 minutes in fresh TTBS or ddH₂O following probing with the antibody. Visualization of the phosphotyrosine containing proteins was done with the chromogenic substrates for alkaline phosphatase (containing: 1.65 mg/mL 5-bromo-3-chloro-indoylphosphate (BCIP) and 12.7 mg/mL nitro blue tetrazolium (NBT) (Gibco) in alkaline phosphatase buffer containing 0.1 M Tris-HCl, 0.1 M NaCl, and 50 mM MgCl₂ in 1 L of ddH₂O, pH 9.5) for 10-20 minutes. They are used together at a 1:1 molar ratio under alkaline conditions to obtain optimal sensitivity. A reaction initiated by cleavage of the phosphate group from the BCIP by alkaline phosphatase yields a blue color and reduces NBT to yield an insoluble purple precipitate. Membranes were allowed to air dry for 4 hours and then scanned for recording purposes. Densitometry was performed on the tyrosine phosphorylated developed membranes using a Kodak Digital Imaging System.

All experiments involving Western blot analysis were performed three times and the densitometry results for each blot (percent mean intensity of individual bands) was averaged and graphed with standard error bars accordingly. Due to space constraints only one blot (best of the three) is shown in the results section for Figures 17-26, 29, and 31 (keeping in mind the banding patterns of the chosen blot may not always directly correspond to the graph included).
RESULTS

I. Characterization of Actin Tyrosine Phosphorylation Patterns of Aged *D. discoideum* Strains During the Sporulation Process.

Upon removal of nutrients, *D. discoideum* amoebae differentiate into dormant spores, which survive starvation stress. Past experiments have shown that high levels of actin tyrosine phosphorylation, specific to the spore stage may be required for maintaining dormancy to withstand starvation stress. The rapid dephosphorylation of actin leads to a reactivated dynamic actin system which participates in spore swelling, vesicle movement, and mitochondrial shape changes during the spore germination process (Kishi et al., 1998). Upon emergence of amoebae little or no actin tyrosine phosphorylation is present (Gauthier et al., 1997; Kishi et al., 1998). However, the phosphorylation levels increase at the end of the culmination stage when spores have matured morphologically and physiologically, and reach maximum levels after an additional 12 hours of development. The levels are stable for 20 days following spor maturation, and decline to undetectable levels within the next 10 days (Kishi et al., 1998). Therefore, it is of great interest to investigate whether the same holds true for various *D. discoideum* mutant strains, SG1, SG2, Ax2, Ax3, CN−, RegA−, and SpiA− in comparison to wild type NC4, V12, and JH10 strains.

Sample collections started with the late culmination stage (22 hours of development), 24 hours, 36 hours, 48 hours, 72 hours, 4 days, 5 days, 7 days, 10 days, 12 days, until they reach the age of 14 days. All samples were processed for SDS page and 10 μL was loaded to each well at a final protein concentration of 0.1 μg/μL, thus giving a total protein concentration of 1.0 μg per lane. Western blot analysis was performed and membranes were probed for phosphotyrosine containing proteins using a recombinant antiphosphotyrosine antibody preconjugated with alkaline phosphatase. The membranes were allowed to dry and then scanned for recording purposes, densitometry was then performed on the tyrosine phosphorylated developed membranes. All strains analyzed were treated equally and followed the above procedure.
As depicted in Figure 17B, the main events associated with the developmental life cycle are vegetative growth (following spore germination), aggregation, formation of the multicellular pseudoplasmodium (slug), culmination, and fruiting body development. As expected none of the *D. discoideum* strains analyzed showed tyrosine phosphorylation of actin in the late culmination stage (22 hours of development). For the majority of the *D. discoideum* strains tyrosine phosphorylation levels of actin increased at 24 hours of development, excluding Ax2 (showed small levels, Fig 22B), and Ax3 (showed minute amounts, Fig 23B). The wild type, NC4 showed initial levels of actin tyrosine phosphorylation at 24 hours, which peaked at 72 hours, and started slowly decreasing there after (Fig 17A). The Western blot analysis for NC4 (Fig 17B) showed an interesting band at 36 hours that was determined to be 31 kDa (indicated by arrow). The V12 strain showed similar actin tyrosine phosphorylation patterns to the wild type, NC4. The highest levels were reached on the forth day of development and started decreasing there after (Fig 18). Figure 19 represents JH10 and reveals high levels of phosphorylation on the fifth day (Fig 19A) and then slowly plateaus throughout the remainder of its development.

The spontaneous germinators, SG1 (Fig 20) and SG2 (Fig 21) show almost identical phosphorylation patterns to each other and their parent strains NC4 and V12 respectively. They both show their highest levels of phosphorylation on the forth day, however, SG1 tends to demonstrate lower levels of phosphorylation throughout the remainder of its development compared to SG2. The axenic strains, Ax2 (Fig 22) and Ax3 (Fig 23) are the most unique for their phosphorylation levels compared to the other strains analyzed. Ax2 shows very little actin tyrosine phosphorylation at 24 hours and Ax3 shows minute quantities, if any at all. Ax2 tends to have the highest levels of phosphorylation on the seventh day, which then takes a dramatic drop in levels there after (Fig 22A). Where as Ax3 shows it’s highest levels of phosphorylation on the sixth day and then gradually drops to low levels throughout the rest of its development (Fig 23A).
Figure 17. Western blot analysis for levels of actin tyrosine phosphorylation throughout the aging of *Dictyostelium discoideum*, wild type strain NC4. Panel A represents the densitometric analysis of corresponding Western blot (Panel B) for levels of actin tyrosine phosphorylation during NC4 aging process of sporulation for 22h, 24h, 36h, 48h, 72h, 4d, 5d, 7d, 10d, 12d, and 14d. For Panel A the following holds true: 22h of development is represented by 0d for time (days); 24h = 1d; 36h = 1.5d; 48h = 2d; 72 = 3d; 4d = 4d; etc. Panel B illustrates Western blot analysis using anti-phosphotyrosine antibodies, which revealed 43 kDa proteins containing phophotyrosyl residues.
Figure 17.
Figure 18. Western blot analysis for levels of actin tyrosine phosphorylation throughout the aging of *Dictyostelium discoideum*, V12 strain. Panel A represents the densitometric analysis of corresponding Western blot (Panel B) for levels of actin tyrosine phosphorylation during V12 aging process of sporulation for 22h, 24h, 36h, 48h, 72h, 4d, 5d, 7d, 10d, 12d, and 14d. For Panel A the following holds true: 22h of development is represented by 0d for time (days); 24h = 1d; 36h = 1.5d; 48h = 2d; 72 = 3d; 4d = 4d; etc. Panel B illustrates Western blot analysis using anti-phosphotyrosine antibodies, which revealed 43 kDa proteins containing phosphotyrosyl residues.
Figure 18.
Figure 19. Western blot analysis for levels of actin tyrosine phosphorylation throughout the aging of *Dictyostelium discoideum*, JH10 strain. Panel A represents the densitometric analysis of corresponding Western blot (Panel B) for levels of actin tyrosine phosphorylation during JH10 aging process of sporulation for 22h, 24h, 36h, 48h, 72h, 4d, 5d, 7d, 10d, 12d, and 14d. For Panel A the following holds true: 22h of development is represented by 0d for time (days); 24h = 1d; 36h = 1.5d; 48h = 2d; 72 = 3d; 4d = 4d; etc. Panel B illustrates Western blot analysis using anti-phosphotyrosine antibodies, which revealed 43 kDa proteins containing phosphotyrosyl residues.
Figure 19.
Figure 20. Western blot analysis for levels of actin tyrosine phosphorylation throughout the aging of *Dictyostelium discoideum*, spontaneous germinator strain SG1. Panel A represents the densitometric analysis of corresponding Western blot (Panel B) for levels of actin tyrosine phosphorylation during SG1 aging process of sporulation for 22h, 24h, 36h, 48h, 72h, 4d, 5d, 7d, 10d, 12d, and 14d. For Panel A the following holds true: 22h of development is represented by 0d for time (days); 24h = 1d; 36h = 1.5d; 48h = 2d; 72 = 3d; 4d = 4d; etc. Panel B illustrates Western blot analysis using anti-phosphotyrosine antibodies, which revealed 43 kDa proteins containing phosphotyrosyl residues.
Figure 20.
Figure 21. Western blot analysis for levels of actin tyrosine phosphorylation throughout the aging of Dictyostelium discoideum, spontaneous germinator strain SG2. Panel A represents the densitometric analysis of corresponding Western blot (Panel B) for levels of actin tyrosine phosphorylation during SG2 aging process of sporulation for 22h, 24h, 36h, 48h, 72h, 4d, 5d, 7d, 10d, 12d, and 14d. For Panel A the following holds true: 22h of development is represented by 0d for time (days); 24h = 1d; 36h = 1.5d; 48h = 2d; 72 = 3d; 4d = 4d; etc. Panel B illustrates Western blot analysis using anti-phosphotyrosine antibodies, which revealed 43 kDa proteins containing phosphotyrosyl residues.
Figure 21.
Figure 22. Western blot analysis for levels of actin tyrosine phosphorylation throughout the aging of *Dictyostelium discoideum*, axenic strain Ax2. Panel A represents the densitometric analysis of corresponding Western blot (Panel B) for levels of actin tyrosine phosphorylation during Ax2 aging process of sporulation for 22h, 24h, 36h, 48h, 72h, 4d, 5d, 7d, 10d, 12d, and 14d. For Panel A the following holds true: 22h of development is represented by 0d for time (days); 24h = 1d; 36h = 1.5d; 48h = 2d; 72 = 3d; 4d = 4d; etc. Panel B illustrates Western blot analysis using anti-phosphotyrosine antibodies, which revealed 43 kDa proteins containing phophotyrosyl residues.
Figure 22.
Figure 23. Western blot analysis for levels of actin tyrosine phosphorylation throughout the aging of *Dictyostelium discoideum*, axenic strain Ax3. Panel A represents the densitometric analysis of corresponding Western blot (Panel B) for levels of actin tyrosine phosphorylation during Ax3 aging process of sporulation for 22h, 24h, 36h, 48h, 72h, 4d, 5d, 7d, 10d, 12d, and 14d. For Panel A the following holds true: 22h of development is represented by 0d for time (days); 24h = 1d; 36h = 1.5d; 48h = 2d; 72 = 3d; 4d = 4d; etc. Panel B illustrates Western blot analysis using anti-phosphotyrosine antibodies, which revealed 43 kDa proteins containing phosphotyrosyl residues.
Figure 23.
The mutant CN\(^-\) lacks calcineurin or protein phosphatase 2B (PP2B) activity, which binds specifically to calcium and calmodulin (Lydan et al., unpublished results). This strain reveals its highest levels of phosphorylation on the forth day and then slowly decreases over time (Fig 24). The phosphodiesterase deletion mutant, RegA\(^-\) also exposes interesting results. RegA\(^-\) reaches its highest phosphorylation levels on the fifth day of development and plateaus off slowly decreasing over the 14 days (Fig 25A). Like the wild type, NC4, this mutant also demonstrates an extra band (indicated by the arrow) at 22 hours of development that was determined to be 64 kDa (Fig 25B). The SpiA- mutant strain develops normally and produces spores that are indistinguishable from wild type spores. This mutant shown in Fig 26 reveals its highest level of phosphorylation on the forth day and slowly decreases there after in development (Fig 26A).
Figure 24. Western blot analysis for levels of actin tyrosine phosphorylation throughout the aging of \textit{Dictyostelium discoideum}, strain CN\textsuperscript{–}. Panel A represents the densitometric analysis of corresponding Western blot (Panel B) for levels of actin tyrosine phosphorylation during CN\textsuperscript{–} aging process of sporulation for 22h, 24h, 36h, 48h, 72h, 4d, 5d, 7d, 10d, 12d, and 14d. For Panel A the following holds true: 22h of development is represented by 0d for time (days); 24h = 1d; 36h = 1.5d; 48h = 2d; 72 = 3d; 4d = 4d; etc. Panel B illustrates Western blot analysis using anti-phosphotyrosine antibodies, which revealed 43 kDa proteins containing phophotyrosyl residues.
Figure 24.
Figure 25. Western blot analysis for levels of actin tyrosine phosphorylation throughout the aging of *Dictyostelium discoideum*, RegA⁻ strain. Panel A represents the densitometric analysis of corresponding Western blot (Panel B) for levels of actin tyrosine phosphorylation during RegA⁻ aging process of sporulation for 22h, 24h, 36h, 48h, 72h, 4d, 5d, 7d, 10d, 12d, and 14d. For Panel A the following holds true: 22h of development is represented by 0d for time (days); 24h = 1d; 36h = 1.5d; 48h = 2d; 72 = 3d; 4d = 4d; etc. Panel B illustrates Western blot analysis using anti-phosphotyrosine antibodies, which revealed 43 kDa proteins containing phophotyrosyl residues.
Figure 25.
Figure 26. Western blot analysis for levels of actin tyrosine phosphorylation throughout the aging of Dictyostelium discoideum, SpiA- strain. Panel A represents the densitometric analysis of corresponding Western blot (Panel B) for levels of actin tyrosine phosphorylation during SpiA- aging process of sporulation for 22h, 24h, 36h, 48h, 72h, 4d, 5d, 7d, 10d, 12d, and 14d. For Panel A the following holds true: 22h of development is represented by 0d for time (days); 24h = 1d; 36h = 1.5d; 48h = 2d; 72 = 3d; 4d = 4d; etc. Panel B illustrates Western blot analysis using anti-phosphotyrosine antibodies, which revealed 43 kDa proteins containing phosphotyrosyl residues.
Figure 26.
II. Individual Spore Morphology of *D. discoideum* Strains.

As a preliminary investigation it is important to study the spore morphology of each *D. discoideum* strain to better understand how the shape of the spore reflects the cytoskeleton structure and cold resistant properties of this model organism. The following *D. discoideum* strains were observed for their cell morphology, including their length and width (diameter) measured in micrometers (μm), and their spore shape and size. Strains were collected as 2 day-old dormant spores and were viewed under a light microscope at the 60 x objective and results were recorded.

The following *D. discoideum* strains were observed: wild type NC4, V12, JH10, and mutant strains SG1, SG2, Ax2, Ax3, CN', RegA', SpiA', SplA', Asc', and the *aca* [PKA-C] overexpressor. The following Panels in Figure 27 are arranged from A-M. Where Panel A is wild type, NC4: parent of SG1, 9-13 μm length x 4-5 μm width, elliptical or banana in shape and long in size. Panel B is V12: parent of SG2, 9-13 μm length x 4-5 μm width, elliptical and long in shape and size. Panel C is JH10: parent of SplA', 6-7 μm length x 4-5 μm width, short and oval in shape and size. Panel D is spontaneous germinator, SG1: 8-11 μm length x 3-4 μm width, elliptical and long shape and size. Panel E is spontaneous germinator, SG2: 8-11 μm length x 3-4 μm width, elliptical and long shape and size. Panel F is axenic strain, Ax2: 5-6 μm length x 3-4 μm width, oval and short in shape and size. Panel G is axenic strain, Ax3: parent strain of RegA-, 5-6 μm length x 3-4 μm width, oval and short in shape and size. Panel H is CN': 6-7 μm length x 3-4 μm width, oval and short in shape and size. Panel I is RegA': 6-7 μm length x 3-4 μm width, oval and small in shape and size. Panel J is SpiA': 6-7 μm length x 3-4 μm width, oval and small in shape and size. Panel K is SplA': 6-7 μm length x 3-4 μm width, round and small in shape and size. Panel L is Asc': 5-6 μm length x 3-4 μm width, oval and small in shape and size. Panel M is *aca* [PKA-C] overexpressor: 6-7 μm length x 5-6 μm width, round and small in shape and size.
Figure 27. Spore Morphology of *Dictyostelium discoideum* strains. The following Panels A-M are photographs of dormant two-day old dormant spores of various *D. discoideum* strains wild type NC4(A), V12(B), JH10(C), and mutant strains SG1(D), SG2(E), Ax2(F), Ax3(G), CN'(H), RegA'(I), SpiA'(J), SplA'(K), Acg' (L), and the *aca*' [PKA-C] overexpressor (M). Note the photographs merely show shape and do not reflect the size of the spore.
Figure 27.
III. Comparison of Actin Tyrosine Phosphorylation Patterns and Spore Shape Changes of *D. discoideum* via Temperature Change.

Actin plays a very important role in regulating spores in the dormant state. Actin rods (Fig 9) aid in giving the dormant spore rigidity, shape, and the ability to remain inert until favorable conditions allow it to germinate. Cultures are grown at room temperature (RT) (21-23 °C) and 15 °C for 10 days and changes in actin tyrosine phosphorylation and spore shape and size are compared for the *D. discoideum* strains NC4, SG1, SG2, Ax3, and RegA by Western blot analysis and light microscopy.

Sample collections started with dormant spores on 1 day, 5 days, and 10 days. All samples were processed for SDS page and 10 μL was loaded to each well at a final protein concentration of 0.1 μg/μL, thus giving a total protein concentration of 1.0 μg per lane. Western blot analysis was performed and membranes were probed for phosphotyrosine containing proteins using a recombinant antiphosphotyrosine antibody preconjugated with alkaline phosphatase. The membranes were allowed to dry and then scanned for recording purposes, densitometry was then performed on the tyrosine phosphorylated developed membranes. All strains analyzed were treated equally and followed the above procedure.

Figure 28 depicts photographs of 10 day-old dormant spores that have been grown at RT and 15 °C for wild type NC4, and the spontaneous germinators, SG1 and SG2. Each strain was observed and data was recorded for the spore length and width (diameter) in micrometers (μm), and spore shape and size for both RT and 15 °C cultures. The following Panels in Figure 28 are arranged from A-F. Where Panel A is a photograph of wild type, NC4 (9-13 μm length x 4-5 μm width) grown at room temperature. Panel B is a photograph of wild type, NC4 (10-14 μm length x 4-5 μm width) grown at 15 °C. Panel C is a photograph of SG1 (8-11 μm length x 3-4 μm width) grown at room temperature. Panel D is a photograph of SG1 strain (9-13 μm length x 4-5 μm width) grown at 15 °C. Panel E is a photograph of SG2 strain (8-11 μm...
Figure 28. Comparison of spore shape changes of *D. discoideum* strains, wild type NC4, SG1, and SG2 grown at room temperature and 15 °C. The following Panels A, C, and E are photographs of dormant ten-day old spores of *D. discoideum* strains NC4(A), SG1(C), and SG2(E) at room temperature and Panels B, D, and F are photographs of dormant ten-day old spores of *D. discoideum* strains NC4(B), SG1(D), and SG2(F) at 15 °C. Note the photographs merely show shape and do not reflect the size of the spore.
Figure 28.
length x 3-4 μm width) grown at room temperature. Panel F is a photograph of SG2 strain (10-12 μm length x 4-5 μm width) grown at 15 °C.

Figure 29 outlines the Western blot analysis levels of actin tyrosine phosphorylation for the D. discoideum strains NC4, SG1, and SG2 at RT and 15 °C. Figure 29 A and B represent the densitometry levels of actin tyrosine phosphorylation for RT and 15 °C respectively. Figure 29 C, D, and E represent 1 day, 5 day, and 10 day-old dormant spores respectively, grown at RT and 15 °C. The results show that at RT (Fig 29A) the levels of phosphorylation for NC4, SG1, and SG2 are overall higher than those seen at 15 °C (Fig 29B).

Figure 30 depicts photographs of 10 day-old dormant spores that have been grown at RT and 15 °C for wild type NC4, Ax3, and RegA-. Each strain was observed and data was recorded for the spore length and width (diameter) in micrometers (μm), and spore shape and size for both RT and 15 °C cultures. The following Panels in Figure 30 are arranged from A-F. Where Panel A is a photograph of wild type, NC4 (9-13 μm length x 4-5 μm width) grown at room temperature. Panel B is a photograph of wild type, NC4 (10-14 μm length x 4-5 μm width) grown at 15 °C. Panel C is a photograph of Ax3 (5-6 μm length x 3-4 μm width) grown at room temperature. Panel D is a photograph of Ax3 (6-8 μm length x 4-5 μm width) grown at 15 °C. Panel E is a photograph of RegA- (6-7 μm length x 3-4 μm width) grown at room temperature. Panel F is a photograph of RegA- (7-9 μm length x 4-5 μm width) grown at 15 °C.

Figure 31 outlines the Western blot analysis levels of actin tyrosine phosphorylation for the D. discoideum strains NC4, Ax3, and RegA- at RT and 15 °C. These results are very similar to those found in Figure 29. Figure 31 A and B represent the densitometry levels of actin tyrosine phosphorylation for RT and 15 °C respectively. Whereas, Figure 31 C, D, and E represent 1 day, 5 day, and 10 day-old dormant spores respectively, grown at RT and 15 °C. The results show that at RT (Fig 31A) the levels of
phosphorylation for NC4, Ax3, and RegA' are overall higher than those seen at 15 °C (Fig 31B).
Figure 29. Western blot analysis for levels of actin tyrosine phosphorylation throughout the aging of Dictyostelium discoideum strains NC4, SG1, and SG2 by temperature change. Panel A represents the densitometric analysis of corresponding room temperature (RT) Western blot (Panel C, D, and E) for levels of actin tyrosine phosphorylation during NC4 (square), SG1 (circle), and SG2 (triangle) aging process of sporulation for 1 day, 5 days, and 10 days. Panel B represents the densitometric analysis of corresponding 15 °C Western blot (Panel C, D, and E) for levels of actin tyrosine phosphorylation during NC4 (square), SG1 (circle), and SG2 (triangle) aging process of sporulation for 1 day, 5 days, and 10 days. Panel C illustrates Western blot analysis using anti-phosphotyrosine antibodies, which revealed 43 kDa proteins containing phophotyrosyl residues for 1 day NC4, SG1, and SG2 spores at RT and 15 °C. Panel D illustrates Western blot analysis using anti-phosphotyrosine antibodies, which revealed 43 kDa proteins containing phophotyrosyl residues for 5 day NC4, SG1, and SG2 spores at RT and 15 °C. Panel E illustrates Western blot analysis using anti-phosphotyrosine antibodies, which revealed 43 kDa proteins containing phophotyrosyl residues for 10 day NC4, SG1, and SG2 spores at RT and 15 °C.
Figure 29.
Figure 30. Comparison of spore shape changes of *D. discoideum* strains, wild type NC4, Ax3, and RegA\(^{-}\) grown at room temperature versus 15 °C. The following Panels A, C, and E are photographs of dormant ten-day old spores of *D. discoideum* strains NC4(A), Ax3(C), and RegA\(^{-}\)(E) at room temperature and Panels B, D, and F are photographs of dormant ten-day old spores of *D. discoideum* strains NC4(B), Ax3(D), and RegA\(^{-}\)(F) at 15 °C. Note the photographs merely show shape and do not reflect the size of the spore.
Figure 30.
Figure 31. Western blot analysis for levels of actin tyrosine phosphorylation throughout the aging of *Dictyostelium discoideum* strains NC4, Ax3, and RegA^- by temperature change. **Panel A** represents the densitometric analysis of corresponding room temperature (RT) Western blot (Panel C, D, and E) for levels of actin tyrosine phosphorylation during NC4 (square), Ax3 (circle), and RegA^- (triangle) aging process of sporulation for 1 day, 5 days, and 10 days. **Panel B** represents the densitometric analysis of corresponding 15 °C Western blot (Panel C, D, and E) for levels of actin tyrosine phosphorylation during NC4 (square), Ax3 (circle), and RegA^- (triangle) aging process of sporulation for 1 day, 5 days, and 10 days. **Panel C** illustrates Western blot analysis using anti-phosphotyrosine antibodies, which revealed 43 kDa proteins containing phophotyrosyl residues for 1 day NC4, Ax3, and RegA^- spores at RT and 15 °C. **Panel D** illustrates Western blot analysis using anti-phosphotyrosine antibodies, which revealed 43 kDa proteins containing phophotyrosyl residues for 5 day NC4, Ax3, and RegA^- spores at RT and 15 °C. **Panel E** illustrates Western blot analysis using anti-phosphotyrosine antibodies, which revealed 43 kDa proteins containing phophotyrosyl residues for 10 day NC4, Ax3, and RegA^- spores at RT and 15 °C.
Figure 31.
IV. Presence of Cellulose in the Spore Cell Wall.

The cell wall of the spore appears to consist of four morphological distinct layers of different thickness and electron densities. Immunogold localization studies done on sections of washed spores show that the outer layer contains the interspore matrix (only seen in unwashed spores), followed by the outer, middle and inner layers of the spore coat, where the inner layer is surrounded by the plasma membrane (Fig 11). Cellulose is only found in the middle spore coat layer (Roseness and Wright, 1974). Cellulose plays a critical role in conferring structural strength to slugs, sheaths, stalks, and spore walls in the cellular slime mold, *D. discoideum*, therefore making it an important subject to study.

Table 1 outlines the cell morphology (length and width (diameter) in micrometers (μm); and spore shape and size) and the presence or absence of cellulose found in various strains of *D. discoideum*. The presence of cellulose was analyzed by using a fluorescent dye, 0.1% Congo Red, by confocal microscopy, viewed under oil immersion at the 60 x objective, under an argon-krypton laser. All of the 4 day-old, *D. discoideum* dormant spore strains analyzed were found to contain cellulose in the middle spore coat cell wall layer.

Figures 32 through 36 are photographs demonstrating the presence of cellulose in the middle spore coat cell wall layer of the *D. discoideum* strains, NC4 (Fig 32B), SG1 (Fig 32D), SG2 (Fig 32F), Ax2 (Fig 33B), Ax3 (Fig 33D), V12 (Fig 34B), JH10 (Fig 34D), CN− (Fig 34F), SpiA− (Fig 35B), SpiA− (Fig 35D), RegA− (Fig 36B), Acg− (Fig 36D), and the acrP [PKA-C] overexpressor (Fig 36F).
Table 1. Cell morphology and the presence of cellulose in various 4 day-old dormant *Dictyostelium discoideum* spores.

<table>
<thead>
<tr>
<th><em>Dictyostelium</em> Strain Examined</th>
<th>Length (μm) x Diameter (μm)</th>
<th>Spore Shape &amp; Length</th>
<th>Presence or Absence of Cellulose *</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC4</td>
<td>9-13 x 4-5</td>
<td>Oval &amp; Long</td>
<td>++</td>
</tr>
<tr>
<td>SG1</td>
<td>8-11 x 3-4</td>
<td>Oval &amp; Long</td>
<td>++</td>
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<tr>
<td>SG2</td>
<td>8-11 x 3-4</td>
<td>Oval &amp; Long</td>
<td>++</td>
</tr>
<tr>
<td>Ax2</td>
<td>5-6 x 3-4</td>
<td>Oval &amp; Short</td>
<td>++</td>
</tr>
<tr>
<td>Ax3</td>
<td>5-6 x 3-4</td>
<td>Oval &amp; Short</td>
<td>++</td>
</tr>
<tr>
<td>V12</td>
<td>9-12 x 4-5</td>
<td>Oval &amp; Long</td>
<td>++</td>
</tr>
<tr>
<td>JH10</td>
<td>6-7 x 5</td>
<td>Oval &amp; Short</td>
<td>++</td>
</tr>
<tr>
<td>CN</td>
<td>5-6 x 3-4</td>
<td>Oval &amp; Short</td>
<td>++</td>
</tr>
<tr>
<td><em>SpiA</em></td>
<td>6-7 x 4</td>
<td>Oval &amp; Short</td>
<td>++</td>
</tr>
<tr>
<td><em>SpiA</em></td>
<td>6-7 x 5</td>
<td>Round</td>
<td>+</td>
</tr>
<tr>
<td><em>RegA</em></td>
<td>6-7 x 3-4</td>
<td>Oval &amp; Short</td>
<td>++</td>
</tr>
<tr>
<td><em>acg</em></td>
<td>6-7 x 3-4</td>
<td>Oval &amp; Short</td>
<td>++</td>
</tr>
<tr>
<td><em>aca</em> [PKA-C] overexpressor</td>
<td>5 x 3-4</td>
<td>Round</td>
<td>++</td>
</tr>
</tbody>
</table>

* Congo Red staining is based - = negative for Congo Red staining (cellulose absent); (+/-) = small amounts of Congo Red stain observed; to (++) = strong Congo Red staining observed (cellulose present).
Figure 32. Confocal analysis with the aid of Congo Red for cellulose presence in dormant spores of *Dictyostelium discoideum*, wild type NC4, and spontaneous germinators SG1 and SG2. Panel A is a photograph of a dormant four-day old wild type, NC4 strain (control), whereas Panel B represents the confocal image stained with Congo Red under an argon-krypton laser. Panel C is a photograph of a dormant four-day old spontaneous germinator, SG1 strain (control), whereas Panel D represents the confocal image stained with Congo Red under an argon-krypton laser. Panel E is a photograph of a dormant four-day old spontaneous germinator, SG2 strain (control), whereas Panel F represents the confocal image stained with Congo Red under an argon-krypton laser.
Figure 33. Confocal analysis with the aid of Congo Red for cellulose presence in dormant spores of *Dictyostelium discoideum*, axenic strains Ax2 and Ax3. Panel A is a photograph of a dormant four-day old axenic Ax2 strain (control), whereas Panel B represents the confocal image stained with Congo Red under an argon-krypton laser. Panel C is a photograph of a dormant four-day old axenic Ax3 strain (control), whereas Panel D represents the confocal image stained with Congo Red under an argon-krypton laser.
Figure 34. Confocal analysis with the aid of Congo Red for cellulose presence in dormant spores of *Dictyostelium discoideum*, strains V12, JH10, and CN⁻. **Panel A** is a photograph of a dormant four-day old V12 strain (control), whereas **Panel B** represents the confocal image stained with Congo Red under an argon-krypton laser. **Panel C** is a photograph of a dormant four-day old JH10 strain (control), whereas **Panel D** represents the confocal image stained with Congo Red under an argon-krypton laser. **Panel E** is a photograph of a dormant four-day old CN⁻ strain (control), whereas **Panel F** represents the confocal image stained with Congo Red under an argon-krypton laser.
Figure 35. Confocal analysis with the aid of Congo Red for cellulose presence in spores of *Dictyostelium discoideum*, strains SpiA<sup>-</sup> and SpiA<sup>-</sup>. Panel A is a photograph of a dormant four-day old SpiA<sup>-</sup> strain (control), whereas Panel B represents the confocal image stained with Congo Red under an argon-krypton laser. Panel C is a photograph of an approximately four-day old amoeba SpiA<sup>-</sup> strain (control), whereas Panel D represents the confocal image stained with Congo Red under an argon-krypton laser.
Figure 36. Confocal analysis with the aid of Congo Red for cellulose presence in spores of *Dictyostelium discoideum*, strains RegA⁻, Acg⁻, and aca⁻ [PKA-C] overexpressor. Panel A is a photograph of a dormant four-day old RegA⁻ strain (control), whereas Panel B represents the confocal image stained with Congo Red under an argon-krypton laser. Panel C is a photograph of a dormant four-day old Acg⁻ strain (control), whereas Panel D represents the confocal image stained with Congo Red under an argon-krypton laser. Panel E is a photograph of an approximately four-day old aca⁻ [PKA-C] overexpressor strain (control), whereas Panel F represents the confocal image stained with Congo Red under an argon-krypton laser.
V. Presence of Cellulose in the Spore Coat Cell Wall During Germination.

Germination of dormant spores consists of four main stages (Fig 5): activation of dormant spores, post activation lag, swelling of dormant spores, and emergence of nascent myxamoebae (Cotter, 1981; Cotter and Raper, 1968; Cotter et al., 1992). Prior to and for a short time following activation, commonly referred to as the post activation lag stage, the dormant spore appears phase bright and oblong in shape. The spore then enters the swelling stage approximately 90 minutes post activation. The hallmark of this stage is the appearance of a protrusion typically located on one side of the swollen spore. After 2.5 hours the nascent amoebae begin to emerge from the cellulose capsule, thus giving rise to a fully emerged amoebae by approximately 3 hours (Fig 5).

Spores were harvested on day 4 post fruiting and stained with a 0.1 % Congo Red dye solution at a concentration of $4 \times 10^5$ spores/mL. After removal of excess dye, the spores were resuspended in 1% peptone broth and were allowed to germinate for 5-8 hours depending on the strain used. Live preparations of spores were placed on a microscope slide and viewed under oil immersion (60 x objective) on the confocal microscope under an argon-krypton laser.

Table 2 outlines the cell morphology (length and width (diameter) in micrometers ($\mu$m); and spore shape and size) and the presence or absence of cellulose found in various germinating strains of D. discoideum. Cellulose was found to be present in dormant spores, partially in swollen spores, and in empty spore capsules. It was demonstrated through confocal microscopy with the aid of Congo Red (data not shown) and fluorescent microscopy with the aid of the fluorescent dye, calcofluor that cellulose was absent in all amoebae, see Appendix, Fig 52.

Figures 37 through 41 are photographs demonstrating the presence of cellulose in the spore coat cell wall layer of the germinating D. discoideum strains, NC4 (Fig 37B), SG1 (Fig 37D), SG2 (Fig 37F), Ax2 (Fig 38B), Ax3 (Fig 38D), V12 (Fig 39B), JH10 (Fig 39D), CN (Fig 39F), SpiA (Fig 40B), SpiA (Fig 40D), RegA (Fig 41B), Acg (Fig 41D), and the aca [PKA-C] overexpressor (Fig 41F).
Table 2. Cell morphology and the presence of cellulose in various *Dictyostelium discoideum* spore strains during the germination process.

<table>
<thead>
<tr>
<th>Dicyostelium Strain</th>
<th>Cell Morphology</th>
<th>Cellulose Presence or Absence Observed During Germination *</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Length (μm) x Diameter (μm)</td>
<td>Spore Shape &amp; Length</td>
</tr>
<tr>
<td>NC4</td>
<td>9-13 x 4-5</td>
<td>Oval &amp; Long</td>
</tr>
<tr>
<td>SG1</td>
<td>8-11 x 3-4</td>
<td>Oval &amp; Long</td>
</tr>
<tr>
<td>SG2</td>
<td>8-11 x 3-4</td>
<td>Oval &amp; Long</td>
</tr>
<tr>
<td>Ax2</td>
<td>5-6 x 3-4</td>
<td>Oval &amp; Short</td>
</tr>
<tr>
<td>Ax3</td>
<td>5-6 x 3-4</td>
<td>Oval &amp; Short</td>
</tr>
<tr>
<td>V12</td>
<td>9-12 x 4-5</td>
<td>Oval &amp; Long</td>
</tr>
<tr>
<td>JH10</td>
<td>6-7 x 5</td>
<td>Oval &amp; Short</td>
</tr>
<tr>
<td>CN</td>
<td>5-6 x 3-4</td>
<td>Oval &amp; Short</td>
</tr>
<tr>
<td><em>SpIA</em></td>
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<td><em>SpIB</em></td>
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<td>Round</td>
</tr>
<tr>
<td><em>RegA</em></td>
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<td><em>acg</em></td>
<td>6-7 x 3-4</td>
<td>Oval &amp; Short</td>
</tr>
<tr>
<td><em>aca</em></td>
<td>5 x 3-4</td>
<td>Round</td>
</tr>
<tr>
<td>[PKA-C] overexpressor</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Congo Red staining is based (−) = negative for Congo Red staining (cellulose absent); (+/-) = small amounts of Congo Red stain observed; to (++) = strong Congo Red staining observed (cellulose present).
Figure 37. Confocal analysis with the aid of Congo Red for cellulose presence in germinating spores of *Dictyostelium discoideum*, wild type NC4, and spontaneous germinators SG1 and SG2. Panel A is a photograph of a swollen (indicated by arrows) wild type, NC4 strain (control), whereas Panel B represents the confocal image stained with Congo Red under an argon-krypton laser. Panel C is a photograph of a swollen (indicated by the arrow) spontaneous germinator, SG1 strain (control), whereas Panel D represents the confocal image stained with Congo Red under an argon-krypton laser. Panel E is a photograph of a swollen (indicated by the arrow) spontaneous germinator, SG2 strain (control), whereas Panel F represents the confocal image stained with Congo Red under an argon-krypton laser.
Figure 38. Confocal analysis with the aid of Congo Red for cellulose presence in germinating spores of Dictyostelium discoideum, axenic strains Ax2 and Ax3. Panel A is a photograph of a swollen (indicated by the arrow) axenic Ax2 strain (control), whereas Panel B represents the confocal image stained with Congo Red under an argon-krypton laser. Panel C is a photograph of a swollen (indicated by the arrow) axenic Ax3 strain (control), whereas Panel D represents the confocal image stained with Congo Red under an argon-krypton laser.
Figure 38.
Figure 39. Confocal analysis with the aid of Congo Red for cellulose presence in germinating spores of Dictyostelium discoideum, strains V12, JH10, and CN⁻. Panel A is a photograph of a swollen/emerged amoeba (indicated by the arrow) V12 strain (control), whereas Panel B represents the confocal image stained with Congo Red under an argon-krypton laser. Panel C is a photograph of a swollen/emerged amoeba (indicated by the arrow) JH10 strain (control), whereas Panel D represents the confocal image stained with Congo Red under an argon-krypton laser. Panel E is a photograph of a swollen/emerged amoebae (indicated by arrows) CN⁻ strain (control), whereas Panel F represents the confocal image stained with Congo Red under an argon-krypton laser.
Figure 40. Confocal analysis with the aid of Congo Red for cellulose presence in germinating spores of Dictyostelium discoideum, strains SpiA⁻ and SplA⁻. Panel A is a photograph of a swollen (indicated by the arrow) SpiA⁻ strain (control), whereas Panel B represents the confocal image stained with Congo Red under an argon-krypton laser. Panel C and E are photographs of swollen (possibly amoebae) SplA⁻ strain (control), whereas Panel D and F are the confocal images stained with Congo Red under an argon-krypton laser.
Figure 41. Confocal analysis with the aid of Congo Red for cellulose presence in germinating spores of *Dictyostelium discoideum*, strains RegA⁺, Acg⁺, and acα⁺ [PKA-C] overexpressor. Panel A is a photograph of a swollen/emerged amoeba (indicated by the arrow) RegA⁺ strain (control), whereas Panel B represents the confocal image stained with Congo Red under an argon-krypton laser. Panel C is a photograph of a swollen/emerged amoeba (indicated by the arrow) Acg⁺ strain (control), whereas Panel D represents the confocal image stained with Congo Red under an argon-krypton laser. Panel E is a photograph of a swollen (possibly an amoeba) acα⁺ [PKA-C] overexpressor strain (control), whereas Panel F represents the confocal image stained with Congo Red under an argon-krypton laser. Panel G is a photograph of an empty capsule with an emerged amoeba (indicated by the arrow) of the acα⁺ [PKA-C] overexpressor strain (control), whereas Panel H represents the confocal image stained with Congo Red under an argon-krypton laser.
Figure 41.
VI. The Effects of Spore Cell Wall Splitting via Natural and Mechanical Cell Wall Disruption.

This experiment was a comparative study to determine whether there is a difference in where the spore cell wall splits, for example the natural germination process (spore swelling) versus the mechanical disruption of the cell wall with the aid of small glass beads (spore splitting). This experiment was carried out on *D. discoideum* strains NC4, SG1, and SG2 as per materials and methods (section V. & VI.), the cell morphology was observed and recorded as follows.

Table 3 outlines the cell morphology (length and width (diameter) in micrometers (μm); and spore shape and size) and shows the effects of natural versus mechanical spore coat splitting on the *D. discoideum* strains NC4, SG1, and SG2. All of the 4 day-old, *D. discoideum* dormant spore strains analyzed were found to contain cellulose in the middle spore coat cell wall layer during both spore coat splitting processes; however mechanical spore coat splitting produced stronger Congo Red staining spores with no established spore coat splitting patterns, whereas natural spore coat splitting produced weaker Congo Red staining spores and demonstrated the same pattern of spore coat splitting.

Figures 42 and 43 are photographs demonstrating the effects of mechanical spore cell wall splitting on cellulose in the middle spore coat cell wall layer of *D. discoideum* strains, NC4 (Fig 42B, 43A), SG1 (Fig 42D, 43B), and SG2 (Fig 42F, 43C); where Figure 42 represents individual cell morphology and Figure 43 represents z-series performed on those spores examined. Figures 44 and 45 are the mirror image of the above, except they demonstrate the effects of natural spore cell wall splitting on cellulose in the middle spore coat cell wall layer of *D. discoideum* strains, NC4 (Fig 44B, 45A), SG1 (Fig 44D, 45B), and SG2 (Fig 44F, 45C); where Figure 44 represents individual cell morphology and Figure 45 represents z-series performed on those spores examined.
Table 3. Cell morphology and the presence of cellulose in various *Dictyostelium discoideum* strains, NC4, SG1, and SG2 during spore cell wall splitting (natural vs. mechanical).

<table>
<thead>
<tr>
<th>Dictyostelium Strain</th>
<th>Cell Morphology</th>
<th>Presence or Absence of Cellulose *</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Length (µm) x Diameter (µm)</td>
<td>Spore Shape &amp; Length</td>
</tr>
<tr>
<td>NC4</td>
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<td>SG1</td>
<td>8-11 x 3-4</td>
<td>Oval &amp; Long</td>
</tr>
<tr>
<td>SG2</td>
<td>8-11 x 3-4</td>
<td>Oval &amp; Long</td>
</tr>
</tbody>
</table>

* Congo Red staining is based (-) = negative for Congo Red staining (cellulose absent); (+/-) = small amounts of Congo Red stain observed; to (+++) = strong Congo Red staining observed (cellulose present).
Figure 42. Confocal analysis with the aid of Congo Red for cellulose presence in spores of *Dictyostelium discoideum*, strains NC4, SG1, and SG2 during mechanical spore cell wall splitting. **Panel A** is a photograph of a dormant wild type, NC4 strain (control) showing large tears in the spore cell wall (indicated by arrows), whereas **Panel B** represents the confocal image stained with Congo Red under an argon-krypton laser. **Panel C** is a photograph of a dormant spontaneous germinator, SG1 strain (control) showing large tears in the spore cell wall (indicated by arrows), whereas **Panel D** represents the confocal image stained with Congo Red under an argon-krypton laser. **Panel E** is a photograph of a dormant spontaneous germinator, SG2 strain (control) also showing large tears in the spore cell wall (indicated by arrows), whereas **Panel F** represents the confocal image stained with Congo Red under an argon-krypton laser.
Figure 43. Confocal analysis (z-series) with the aid of Congo Red for cellulose presence in spores of *Dictyostelium discoideum*, strains NC4, SG1, and SG2 during mechanical spore cell wall splitting. The performance of a z-series illustrates several submicron sections through spores from the bottom layers (upper left-hand corner) of the spore to the top (lower right-hand corner). Panel A is a photograph of NC4 spores that were subjected to mechanically spore cell wall splitting with the aid of small glass beads and were then stained with Congo Red and observed under the confocal microscope with an argon-krypton laser. Panel B represents SG1 and Panel C represents SG2, both were treated the same as Panel A.
Figure 44. Confocal analysis with the aid of Congo Red for cellulose presence in spores of *Dictyostelium discoideum*, strains NC4, SG1, and SG2 during natural spore cell wall splitting. Panel A is a photograph of a dormant wild type, NC4 strain (control) showing slits in the spore cell wall (indicated by arrows), whereas Panel B represents the confocal image stained with Congo Red under an argon-krypton laser. Panel C is a photograph of a dormant spontaneous germinator, SG1 strain (control) showing slits in the spore cell wall (indicated by the arrow), whereas Panel D represents the confocal image stained with Congo Red under an argon-krypton laser. Panel E is a photograph of a dormant spontaneous germinator, SG2 strain (control) also showing slits in the spore cell wall (indicated by arrows), whereas Panel F represents the confocal image stained with Congo Red under an argon-krypton laser.
Figure 45. Confocal analysis (z-series) with the aid of Congo Red for cellulose presence in spores of Dictyostelium discoideum, strains NC4, SG1, and SG2 during natural spore cell wall splitting. The performance of a z-series illustrates several submicron sections through spores from the bottom layers (upper left-hand corner) of the spore to the top (lower right-hand corner). Panel A is a photograph of NC4 spores that were subjected to mechanically spore cell wall splitting with the aid of small glass beads and were then stained with Congo Red and observed under the confocal microscope with an argon-krypton laser. Panel B represents SG1 and Panel C represents SG2, both were treated the same as Panel A.
VII. Location of Cellulose within the *D. discoideum* Spore.

As mentioned earlier, cellulose is only found in the middle spore coat layer (Fig 11). This experiment was performed to determine if cellulose is present throughout the spore or if it is only present in the cell wall compartment, by performing z-series with the use of confocal microscopy. A z-series produces several tiny (microns) cuts sectioned through an individual spore from the bottom layers of the spore to the top. The following *D. discoideum* strains analyzed are: wild type NC4, V12, JH10, and mutant strains SG1, SG2, Ax2, Ax3, CN−, RegA−, SpiA−, and SplA− in comparison to wild type NC4, V12, and JH10 strains.

Table 4 outlines the cell morphology (length and width (diameter) in micrometers (μm); and spore shape and size) and shows whether cellulose is present throughout the spore or only present in the spore coat layers for the *D. discoideum* strains listed above. The results show that all of the *D. discoideum* strains examined have cellulose present in their spore coat layers. It was also noted that SplA− and the *aca−* [PKA-C)] overexpressor express small amount of Congo Red stain (cellulose) throughout the spore.

Figures 46 through 50 are black and white photographs demonstrating the location of cellulose in the spores of the *D. discoideum* strains, NC4 (Fig 46A), SG1 (Fig 46B), SG2 (Fig 46C), Ax2 (Fig 47A), Ax3 (Fig 47B), V12 (Fig 48A), JH10 (Fig 48B), CN− (Fig 48C), SpiA− (Fig 49A), SplA− (Fig 49B), RegA− (Fig 50A), Acg− (Fig 50B), and the *aca−* [PKA-C] overexpressor (Fig 50C). At this time colored z-series are unavailable.
Table 4. Cell morphology and the presence of cellulose in various *Dictyostelium discoideum* strains in the spore coat layers of the cell wall.

<table>
<thead>
<tr>
<th>Dictyostelium Strain</th>
<th>Length (μm) x Diameter (μm)</th>
<th>Spore Shape &amp; Length</th>
<th>Presence or Absence of Cellulose *</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Throughout Spore</td>
</tr>
<tr>
<td>NC4</td>
<td>9-13 x 4-5</td>
<td>Oval &amp; Long</td>
<td>-</td>
</tr>
<tr>
<td>SG1</td>
<td>8-11 x 3-4</td>
<td>Oval &amp; Long</td>
<td>-</td>
</tr>
<tr>
<td>SG2</td>
<td>8-11 x 3-4</td>
<td>Oval &amp; Long</td>
<td>-</td>
</tr>
<tr>
<td>Ax2</td>
<td>5-6 x 3-4</td>
<td>Oval &amp; Short</td>
<td>-</td>
</tr>
<tr>
<td>Ax3</td>
<td>5-6 x 3-4</td>
<td>Oval &amp; Short</td>
<td>-</td>
</tr>
<tr>
<td>V12</td>
<td>9-12 x 4-5</td>
<td>Oval &amp; Long</td>
<td>-</td>
</tr>
<tr>
<td>JH10</td>
<td>6-7 x 5</td>
<td>Oval &amp; Short</td>
<td>-</td>
</tr>
<tr>
<td>CN</td>
<td>5-6 x 3-4</td>
<td>Oval &amp; Short</td>
<td>-</td>
</tr>
<tr>
<td><em>spa</em>&lt;sup&gt;+&lt;/sup&gt;</td>
<td>6-7 x 4</td>
<td>Oval &amp; Short</td>
<td>-</td>
</tr>
<tr>
<td><em>spa</em>&lt;sup&gt;-&lt;/sup&gt;</td>
<td>6-7 x 5</td>
<td>Round</td>
<td>+/-</td>
</tr>
<tr>
<td><em>regA</em>&lt;sup&gt;-&lt;/sup&gt;</td>
<td>6-7 x 3-4</td>
<td>Oval &amp; Short</td>
<td>-</td>
</tr>
<tr>
<td><em>acr</em>&lt;sup&gt;-&lt;/sup&gt;</td>
<td>6-7 x 3-4</td>
<td>Oval &amp; Short</td>
<td>-</td>
</tr>
<tr>
<td><em>aca</em>&lt;sup&gt;-&lt;/sup&gt; [PKA-C overexpressor]</td>
<td>5 x 3-4</td>
<td>Round</td>
<td>+/-</td>
</tr>
</tbody>
</table>

* Congo Red staining is based (-) = negative for Congo Red staining (cellulose absent); (+/-) = small amounts of Congo Red stain observed; to (++) = strong Congo Red staining observed (cellulose present).
Figure 46. Confocal analysis with the aid of Congo Red for cellulose presence in dormant spore coat layers of *Dictyostelium discoideum*, strains NC4, SG1, and SG2. The performance of a z-series illustrates several submicron sections through spores from the bottom layers (upper left-hand corner) of the spore to the top (lower right-hand corner). Panel A is a photograph of a NC4 spore that was subjected to a z-series to illustrate that cellulose is present in the spore coat layers of NC4. Panel B represents SG1 and Panel C represents SG2, both were treated the same as Panel A and show the same cellulose patterns.
Figure 47. Confocal analysis with the aid of Congo Red for cellulose presence in dormant spore coat layers of *Dictyostelium discoideum*, axenic strains Ax2 and Ax3.

The performance of a z-series illustrates several submicron sections through spores from the bottom layers (upper left-hand corner) of the spore to the top (lower right-hand corner). **Panel A** is a photograph of an Ax2 spore that was subjected to a z-series to illustrate that cellulose is present in the spore coat layers of Ax2. **Panel B** represents Ax3 and was treated the same as Panel A and shows the same cellulose patterns.
Figure 47.
Figure 48. Confocal analysis with the aid of Congo Red for cellulose presence in dormant spore coat layers of *Dictyostelium discoideum*, strains V12, JH10, and CN'.

The performance of a z-series illustrates several submicron sections through spores from the bottom layers (upper left-hand corner) of the spore to the top (lower right-hand corner). **Panel A** is a photograph of a V12 spore that was subjected to a z-series to illustrate that cellulose is present in the spore coat layers of V12. **Panel B** represents JH10 and **Panel C** represents CN', both were treated the same as Panel A and show the same cellulose patterns.
Figure 49. Confocal analysis with the aid of Congo Red for cellulose presence in dormant spore coat layers of *Dictyostelium discoideum*, SpiA− and SplA−. The performance of a z-series illustrates several submicron sections through spores from the bottom layers (upper left-hand corner) of the spore to the top (lower right-hand corner). Panel A is a photograph of a SpiA− spore that was subjected to a z-series to illustrate that cellulose is present in the spore coat layers of SpiA−. Panel B represents SplA− and was treated the same as Panel A, however Panel B shows a different cellulose pattern. SplA− does not just show cellulose in the spore coat layers, it also shows small amounts throughout the spore.
Figure 50. Confocal analysis with the aid of Congo Red for cellulose presence in dormant spore coat layers of *Dictyostelium discoideum*, strains RegA⁻, Aeg⁻, and the aca⁻ [PKA-C] overexpressor. The performance of a z-series illustrates several submicron sections through spores from the bottom layers (upper left-hand corner) of the spore to the top (lower right-hand corner). **Panel A** is a photograph of a RegA⁻ spore that was subjected to a z-series to illustrate that cellulose is present in the spore coat layers of RegA⁻. **Panel B** represents Aeg⁻ and **Panel C** represents aca⁻ [PKA-C] overexpressor, both were treated the same as Panel A. Panel A and B show similar cellulose spore coat layer patterns, however the aca⁻ [PKA-C] overexpressor (Panel C) appears to have similar characteristics resembling SplA⁻, where cellulose is present in both throughout the spore and in the spore coat layers.
DISCUSSION

Upon exhaustion of food sources, vegetative cells of *D. discoideum* aggregate to become multicellular and differentiate into dormant spores and stalk cells in a fruiting body. In the spores spherical vesicles and crenate mitochondria are both immobile (Cotter *et al.*, 1969; Kishi *et al.*, 1994), indicating that the actin cytoskeleton is generally inactive. In spores of *D. discoideum* three actin filaments are bundled to form a novel tubular structure and the tubules are then organized into rods. The stretched bundles of actin filaments are referred to as actin rods (Fig 9). The bundling of actin filaments is required for several functions in many types of cells (i.e.) contractile rings in dividing cells. They are also induced in the cytoplasm or the nucleus by exposing cells to stress conditions such as dimethyl sulfoxide (DMSO) or heat shock (Fukui, 1978; Iida *et al.*, 1986; Welch and Suhan, 1985).

Past studies demonstrated that a 43 kDa protein (actin) in the dormant spores of *D. discoideum* is tyrosine phosphorylated and subsequently dephosphorylated during germination (Gauthier *et al.*, 1997). Recently, it has been shown that 50% of the total actin in dormant spores of *D. discoideum* is tyrosine phosphorylated, and that maintaining high levels of actin phosphorylation may correlate with the inactive state of the actin cytoskeleton (Kishi *et al.*, 1998). These data indicate that tyrosine phosphorylation of actin is required for the maintenance of a dormant state and the viability of *Dictyostelium* spores. Using the same methods, the question arises, is actin tyrosine phosphorylation required to maintain the viability and dormant state for all variants of *D. discoideum*?

The experiments performed in this study have collected novel information regarding actin tyrosine phosphorylation in various *D. discoideum* strains. Actin tyrosine phosphorylation was observed in all but the late culmination stage (22 hours) of those *D. discoideum* strains (wild type NC4, V12, JH10, and mutant strains SG1, SG2, Ax2, Ax3, CN-, RegA-, and SpiA-) analyzed (Fig 17, Panel A – 26, Panel A, respectively) during the sporulation process and the dormancy acquisition. On average the actin tyrosine phosphorylation levels increased up to the fourth to sixth day and there after slowly
decreased as the spores reached the age of 14 days. This was expected because past experiments have shown that the phosphorylation levels increase at the end of the culmination stage when spores have matured morphologically and physiologically (Kishi et al., 1998). Perhaps it would have been beneficial to collect samples during the 23, 26, and 30 hours of development in addition to those collected to gain a better understanding of the actin tyrosine phosphorylation patterns demonstrated at the earlier stages of development.

Comparison studies show that the wild type NC4 (Fig 17), parent strain of the spontaneous germinator, SG1 (Fig 20) showed similar actin tyrosine phosphorylation patterns over the 14 days of development observed. The same holds true for V12, the parent strain (Fig 18) of the spontaneous germinator, SG2 (Fig 21). Due to minute amounts of protein collected for the SplA- strain, Western blot analysis could not be performed on this strain thus a comparison between this strain and its parent, JH10 (Fig 19) can not be commented on at this time. Lastly, the axenic Ax3 (Fig 23), parent strain of the phosphodiesterase deletion mutant, RegA- (Fig 25) showed slightly different patterns. RegA- exhibited stronger actin tyrosine phosphorylation levels throughout its development, whereas Ax3 exhibited weaker actin tyrosine phosphorylation levels throughout the 14 days of development.

PKA and RegA are considered to be main characters in the regulation of spore dormancy and germination. RegA is an intracellular cAMP phosphodiesterase specific for the regulatory subunit of PKA (Shaulsky et al., 1996). RegA plays an important regulatory role upon PKA which is required to maintain spore dormancy (van Es et al., 1996). RegA activity has been shown to be affected by a transmembrane, two component, histidine kinase transduction cascade DHKB and is activated by phosphorylation (Thomason et al., 1999; Zinda and Singleton, 1998). This cascade in dormant spores 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 mutant RegA\(^-\) is considered to be a negative regulator of PKA. 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. A model (Fig 51) proposed by Clements in 1998, 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 \textit{et al.}, 1996) depending on the activity of RegA. The response regulator that dephosphorylates RegA to inactivate it is likely a type of phosphatase. Also, SplA, whose mutant spores do not possess actin tyrosine phosphorylation (data not shown), may act downstream of PKA playing a role in actin phosphorylation (Kishi \textit{et al.}, 1998). The Western blot analysis result for RegA\(^-\) (Fig 25) shows that germination is strongly retarded, which directly correlates with the above ideas presented.

A protein load of 1.0 \(\mu\)g was sufficient to reveal a dense banding pattern at the 43 kDa region, as revealed through Western blot analysis for those \textit{D. discoideum} strains (wild type NC4, V12, JH10, and mutant strains SG1, SG2, Ax2, Ax3, CN\(^-\), RegA\(^-\), and SpiA\(^-\)) analyzed (Fig 17, Panel B – 26, Panel B respectively). Western blot results for the wild type NC4 revealed an interesting band at 36 hours of development (Fig 17, Panel B, lane 3, indicated by arrow), that was determined to be 31 kDa. In comparison, the phosphodiesterase deletion mutant, RegA\(-\) that lacks the phosphodiesterase believed to be responsible for the cleavage of cAMP with the cells of \textit{Dictyostelium} (Shaulsky \textit{et al.}, 1996) also demonstrated an interesting band at 22 hours of development (Fig 25, Panel B, lane2, indicated by arrow), that was determined to be 64 kDa. To date these findings have not been reported by others within the field, thus at this time it is too early to make any concrete conclusions, for more experiments need to be pursued.
Figure 51. A model for the regulation of *D. discoideum* spore dormancy. From Clements, 1998.
Figure S1.
As originally described by Raper (1935) the spores in the sorus of strain, North Carolina 4 (NC4) were capsule shaped. The opposite mating type, Virginia 12 (V12) also forms capsule shaped spores which are somewhat larger than NC4 ( Cotter and Raper, 1968c; Raper, 1984). Many strains of D. discoideum in use throughout the world have been derived from the NC4 strain by genetic drift or by selection. For example, a strain obtained from Maurice Sussman (strain B) produces oval to round spores which have altered germination characteristics when compared to strains NC4 and V12 ( Cotter and Raper, 1968c). The axenic strains Ax2 and Ax3 have been derived from strain B and therefore form oval to round spores with altered germination characteristics when compared to strain NC4 (Virdy et al., 1999).

It can be seen that Dictyostelium research conducted by different laboratories around the world will depend on the origin and the type of strain in use. Thus, as a preliminary investigation it is important to study the spore morphology of individual D. discoideum strains to grasp a better understanding of the organism at study. All present studies performed were based on those strains that originated from Dr. Cotter's laboratory (subject to genetic drift and/or selection). Figure 27, Panel A through 27 Panel M illustrates the following D. discoideum strains respectively: wild type NC4, V12, JH10, and mutant strains SG1, SG2, Ax2, Ax3, CN⁻, RegA⁻, SpiA⁺, SplA⁺, Acg⁻, and the aca⁻ [PKA-C] overexpressor. The wild type NC4, V12, SG1, and SG2 were found to be oval or elliptical in spore shape and long in spore length (average of 8–13 µm, depending on strain). Where as the majority of the other strains analyzed, JH10, Ax2, Ax3, CN⁻, RegA⁻, SpiA⁺, and Acg⁻ were found to be oval to round in spore shape and short in spore length (average of 5-7 µm, depending on strain). The two strains of interest are SplA⁻ (Fig 27, Panel K) and the aca⁻ [PKA-C] overexpressor (Fig 27, Panel M) because they do not resemble dormant spores, they are round and short, and appear to look more like amoebae, than dormant spores.

D. discoideum spores survive harsh environmental conditions such as dehydration, heat, osmotic pressure, as well as non-nutrient conditions (Raper, 1935). Actin plays a very important role in regulating spores in the dormant state. Actin rods aid
in giving the dormant spore rigidity, shape, and the ability to remain inert until favorable conditions allow it to germinate. Past studies conducted show formation of fruiting bodies of wild type NC4 at temperatures less than 19 °C results in oval to round spores, with normal germination characteristics (Cotter, 1973). Room temperature (21 °C) is the optimal temperature for growth of *Dictyostelium* spp. (Raper, 1984).

To better understand how temperature effects the shape of the spore, its cytoskeleton structure, cold resistant properties, and actin tyrosine phosphorylation levels, experiments were performed at room temperature (RT) (21-23 °C) and 15 °C over a period of 10 days to determine the effects of decreasing temperatures on the *D. discoideum* strains NC4, SG1, SG2, Ax3, and RegA⁻. Figure 28 illustrates Panels A-F: where NC4 is Panel A (RT) & B (15 °C); SG1 is Panel C (RT) & D (15 °C); and SG2 is Panel E (RT) & F (15 °C). Figure 30 illustrates Panels A-F: where NC4 is Panel A (RT) & B (15 °C); Ax3 is Panel C (RT) & D (15 °C); and RegA⁻ is Panel E (RT) & F (15 °C). Cell morphology was observed at 1, 5, and 10 days of development for both RT and 15 °C grown cultures for all strains mentioned above. Overall there was a decrease in spore length and width (diameter) for all strains observed, thus it can be concluded that there is a possible change in the cytoskeleton structure by growing cultures at low temperatures.

In addition to observing the cell morphology, Western blot analysis was also performed to determine if tyrosine phosphorylation levels of actin are effected by temperature change. Figure 29 outlines the Western blot analysis for the *D. discoideum* strains NC4, SG1, and SG2 at RT and 15 °C, where Fig 29A shows the densitometric analysis and Fig 29B shows the blots for the levels of actin tyrosine phosphorylation. Figure 31 is a mirror image of Figure 29 (Western blot analysis) except it examines the actin tyrosine phosphorylation levels for the *D. discoideum* strains NC4, SG1, SG2, Ax3, and RegA⁻. Those experiments at 15 °C exhibit a similar pattern to the control at RT, with the difference being an overall decrease in actin tyrosine phosphorylation for all days observed (1, 5, and 10 days).
These findings could be the result of two possible explanations: (1) dormant spores at 15 °C may not be able to form the same amount of actin in comparison to RT, thus possibly decreasing their rate of survival; (2) growth at 15 °C may slow the actin formation process, thus producing lower amounts of actin (lower phosphorylated actin levels) as compared to the control at RT. These results show both spore shape and size, as well as the levels of actin tyrosine phosphorylation are decreased when they are exposed to low temperatures in comparison to the RT results. It would be beneficial to repeat these experiments at higher temperatures (30 °C) and compare all three temperature (RT, 15 °C, and 30 °C) to get a better understanding of how temperature effects the shape of the spore, its cytoskeleton structure, cold/heat resistant properties, and actin tyrosine phosphorylation levels of the various mutants. With the aid of confocal microscopy it would also be advantageous to analyze the amount of actin present in the spore for each temperature and establish if there are any significant differences between the temperatures.

The cell wall of the spore consists of four morphological distinct layers (outer, middle, and inner layer surrounded by plasma membrane) (Fig 11). Cellulose is only found in the middle spore coat layer (Roseness and Wright, 1974). Cellulose plays an important role in conferring structural strength to slugs, sheaths, stalks, and spore walls in *D. discoideum*, therefore making it a notable subject to study. The confocal microscope was used to view Congo Red stained, 4 day-old *D. discoideum* strains for presence of cellulose in the middle spore coat cell wall (Table 1). All spore strains analyzed were found to contain cellulose in the spore coat cell wall layer.

Figures 32 through 36 are photographs demonstrating the presence of cellulose in the middle spore coat cell wall layer of the *D. discoideum* strains, wild type NC4, V12, JH10, and mutant strains SG1, SG2, Ax2, Ax3, CN', RegA', SpiA', SplA', Acg', and the *aca'* [PKA-C] overexpressor. It is important to note that some strains may appear to have more cellulose content then others based on the intensity of the Congo Red stain. This
experiment can only detect if there is cellulose present, it can not measure the amount of cellulose present in the spore.

The next experiment tested whether cellulose was present or absent in the spore coat cell wall during the germination process. Past experiments have proved that amoebae do not make cellulose while they are growing, developing cells accumulate cellulose after the mound stage. During culmination, cellulose is deposited in the stalk tube and in the spore coats and stalk cell walls (Fig 14c and 14h) (Blanton, 1993; Blanton, 1997; and Blanton, 2000). As mentioned previously, cellulose is also found in the spore coat. During spore germination, degradation of the cellulose in the spore's cell wall by cellulase is required to allow the amoeba to emerge (Ramalingam et al., 1992). The presence and location of cellulose in different stages of the life cycle of Dictyostelium can be demonstrated by the use of the fluorescent cellulose dye, Congo Red.

Cellulose was found to be present in dormant spores, partially in swollen spores, and in empty spore capsules, and was absent in all amoebae analyzed (Table 2). From the information provided previously, these results were expected. It was noticed that the empty capsules stained more intensely than the dormant spores, this is most likely due to the fact that the empty capsules are hollow, whereas the dormant spores contain amoebae, thus the argon-krypton laser passes more light through the empty capsules, making them look like they have more cellulose, when in fact they should have the same amount if not less than a dormant spore.

Due to space constraints only three stages of germination (dormant spores, swelling, emergence) are shown in Figures 37 – 41. The fourth stage (nascent amoebae) is shown in the appendix (Figure 52). The majority of images show swollen spores as seen in Fig 37, Panel D for SG1, or emerged amoebae as seen in Fig 39, Panel B for V12, and some of empty capsules as seen in Fig 41, Panel H for aca’ [PKA-C], and of course nascent amoebae are undetectable under an argon-krypton laser when stained with Congo Red.
It is thought that there are weak associations between the plasma membrane and the spore coat, in which these may be sufficient to provide polarity to the coat during its morphogenesis. Past studies (Cotter et al., 1969) suggest the two outer layers split and can retract during the swelling phase of germination, exposing the outer surface of the inner layer, which remains continuous over the surface of the cell. Completion of germination disrupts the inner layer resulting in its shedding, thus causing lysis of the spore cell wall and allowing the emergence of the viable amoebae.

This next experiment was a comparative study to determine whether there is a difference in where the spore cell wall splits, for example the natural germination process (spore swelling) versus the mechanical disruption of the cell wall with the aid of small glass beads (spore splitting). The results outlined in Table 3 show that all the strains analyzed were found to contain cellulose in the middle spore coat cell wall layer during both spore coat splitting processes; however mechanical (Fig 42, Panel A) spore coat splitting method (glass beads) produced stronger Congo Red staining spores with no established spore coat splitting patterns, whereas natural (Fig 44, Panel C) spore coat splitting method (germination) produced weaker Congo Red staining spores and demonstrated the same pattern of spore coat splitting.

In conclusion, these results suggest that there is a certain location within the spore coat cell wall that is a common tearing or splitting point during the natural germination process (Fig 45, Panel C). Whereas the mechanical method appears to chew apart the spores and shows no common point in the spore coat cell wall where the spore would split (Fig 43, Panel A). The staining intensity could be due to the same reason as described earlier. The mechanically treated spores may lose their inner contents during the grinding process, whereas the naturally treated spores may still contain the amoebae, thus producing lower levels of Congo Red staining. The z-series done through confocal imaging exhibits the above findings (Fig 43, and Fig 44).

The last experiment was performed to determine if cellulose is present throughout the spore or if it is only present in the cell wall compartment, by performing z-series with
the use of confocal microscopy. A z-series produces several submicron sections through an individual spore from the bottom layers of the spore to the top. It was shown that all of the *D. discoideum* strains examined have cellulose present in their spore coat layers (Table 4). Figure 46, Panel A best illustrates where the cellulose is located within the dormant spore. It was also noted that SplA' (Fig. 49, Panel B) and the aca' [PKA-C] overexpressor (Fig. 50, Panel C) show small amount of cellulose throughout the spore. The best explanation for this finding may be due to the individual mutations and how they effect the biosynthesis of cellulose during the development of these two strains. Unfortunately there is not enough known about these mutants, thus further analysis is needed to understand the meaning behind these results.

From past studies, it was clear that further investigation of the role of actin tyrosine phosphorylation in mutants of *D. discoideum* was required. The spore morphology and actin tyrosine phosphorylation patterns exhibited at both RT and 15 °C for all ages of development examined demonstrated that actin, whether it be actin rods, filaments or tubules, play a crucial role in maintaining spore dormancy and the viability of the spore. Cellulose also plays an important role in the development of *D. discoideum* by adding structural strength to slugs, sheaths, stalks, and spore walls. Several studies have been conducted in the past in the study of cellulose, however little has concentrated on the mutants of *D. discoideum*, therefore making it an important subject to study. Experiments revealed that cellulose: was present in the middle layer of the spore coat cell wall; was present in dormant, swelling, and emerging spores, but absent in nascent amoebae; demonstrated that there is a certain location within the spore coat cell wall that is a common tearing or splitting point during the natural process (germination), but not for the mechanical process (glass beads); and was present throughout the spore. It is fair to say that even though there are no remarkable actin or cellulose findings in any of the aforementioned experiments, it is not to say that they are not important characters in the life of the cellular slime mold, *D. discoideum*.
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APPENDIX

Figure 52. Spores of *Dictyostelium discoideum*, SG1 viewed under fluorescence microscopy with the aid of the fluorescent dye, calcofluor used to detect cellulose in spores. **Panel A** is a photograph of a dormant spore (bottom left) and amoebae (top right), whereas **Panel B** represents the same photograph showing the dormant spores stained with calcifluor (bright blue) whereas the amoebae do not stain with calcifluor (colourless). **Panel C** is a photograph of a swollen spore (bottom right) and amoebae (top left), whereas **Panel D** represents the same photograph showing the dormant spores stained with calcifluor (bright blue) whereas the amoebae do not stain with calcifluor (colourless).
Figure 52.
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Phosphorylation during Sporulation, Spore Dormancy, Spore 
Aging, and Senescence in *Dictyostelium discoideum*. American 
Society for Microbiology, 100th General Meeting. Los Angeles, 
California.

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Meeting. Atlanta, Georgia.