Phosphotyrosine-containing proteins during spore germination of Dictyostelium discoideum.

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PHOSPHOTYROSINE-CONTAINING PROTEINS DURING SPORE GERMINATION OF DICTYOSTELIUM DISCOIDEUM.

by

Mona Louise Gauthier

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

Windsor, Ontario, Canada
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ABSTRACT

This present study contributes to the growing hypothesis of signal transduction processes to be the underlining mechanisms which induce *Dictyostelium discoideum* spores to autoactivate and release nascent amoebae. This work focuses on the role of tyrosine phosphorylation during germination and the extracellular signals which govern the phosphorylation/dephosphorylation of specific protein(s) on their tyrosyl residues.

Probing for phosphorytrosine-containing proteins during the germination program of this organism by Western blot analysis using anti-phosphotyrosine antibodies revealed at least ten proteins with relative mobilities between 207kDa and 32kDa. The pattern observed was shared by all strains examined, V12, NC4, SG2 and SG1, independent of the mode of activation. One protein in particular with a molecular weight of approximately 43kDa was intensely labeled on its tyrosine residues during dormancy and was gradually dephosphorylated throughout germination. This protein was identified to be actin. The data presented here demonstrate *in vivo* tyrosine phosphorylation of actin in response to standard growth medium and to material present in the extracellular matrix of the sorocarp. An interesting observation was the direct inverse labeling relationship, where actin labeling corresponded to weak tyrosine phosphorylation. This relationship suggests that phosphotyrosine residues may lie within the monoclonal anti-actin recognition site. The molecular significance of actin tyrosine phosphorylation still remains unknown.

However, it is established that extracellular signals govern this protein's phosphorylation pattern. Incubation with the autoinhibitor not only inhibits spore swelling and emergence, but results in greater actin tyrosine phosphorylation than freshly harvested spores. Upon the addition of the autoactivator, actin tyrosine dephosphorylation begins and spores quickly initiate germination. Recently work has demonstrated thick intranuclear and cytoplasmic actin rods to be present during dormancy. Activation induces their disappearance and swelling causes violent protoplasmic streaming. These immunocytochemical observations coupled with the work presented here point to a cardinal role for actin during dormancy, activation, swelling and emergence. As the link between extracellular signalling and actin reorganization strengthens, it will become clear which events regulate actin polymerization/depolymerization, and thus this will explain the role of actin tyrosine phosphorylation/dephosphorylation.
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INTRODUCTION

Signal Transduction Processes

The ability of cells to adapt and respond to environmental cues such as light, temperature, osmotic pressure, nutrients and hormones as well as the inherent interactions between cells, is of great interest. It has become apparent that cellular responses to extracellular stimuli involve signalling mechanisms that have been highly conserved throughout evolution (Swanson & Simon, 1994; Sprague, 1992). External signals usually exert their function via cell surface receptors to initiate signaling pathways on the cytoplasmic side of the plasma membrane. Activation of cytosolic enzymes through receptor binding yield second messengers which relay information to target proteins or genes through a network of interactions (Kazlauskas, 1994; Berridge, 1993; VanHaastert et al., 1991; Ullrich & Schlessinger, 1990).

G-Protein Coupled Sensory Transduction Pathways

Members of this large receptor family are distinguished from other receptor families by their seven membrane-spanning domains which form docking sites for ligand binding on the cell surface (Savarese & Fraser, 1992). Agonist binding induces conformational change responsible for activating one or more intracellularly localized G-proteins. G-proteins are heterotrimeric, αβγ, and a single cell can express more than six types of α subunit, two types of β subunit and several types of γ subunit (Kleuss et al., 1992). It is hypothesized that the smaller β (35-36kDa) and γ (7-10kDa) subunits form a permanent complex and regulate the larger α (38-52kDa) subunit activity (Neer & Clapham, 1988; Lochrie & Simon, 1988). Ligand-binding transforms the inactive $\alpha_{GDP}\beta\gamma$ complex to an active species $\alpha_{GTP} + \beta\gamma$ (Fig. 1). The duration of the activated species is the time taken to hydrolyze GTP to GDP by the intrinsic GTPase activity of the α subunit.
Figure 1. The regulation of heterotrimeric G-proteins. The open circles represent the inactive forms of the subunits, while the stippled circles represent the active states. Borrowed from Neer (1995).
The rate of GTP hydrolysis varies among the α subunit isoforms (Carty et al., 1990). The reassociation of α and βγ subunits negatively regulates each subunit activity and allows the system to respond again to agonist binding.

In vertebrate systems, G-proteins were traditionally classified by their sensitivity to cholera and pertussis toxins, both toxins, or neither (Katada & Ui, 1982). Cholera toxin catalyzes the transfer of ADP ribose from NAD⁺ to Gα₄ (stimulatory G-protein) rendering the subunit unable to hydrolyze GTP, thus continuously activating target proteins (Kaziro et al., 1991). Alternatively, pertussis toxin catalyzes ADP ribosylation of Gα₁ (inhibitory G-protein), inhibiting its interaction with receptors, and thus blocking GTP binding (Kaziro et al., 1991). The Gα₈ subunit is known to stimulate effector proteins such as adenyl cyclase and regulate Ca²⁺ channels, where Gα₁ has been shown to inhibit adenyl cyclase activity, regulate K⁺ and Ca²⁺ channels, as well as activate cGMP phosphodiesterase (Simon et al., 1991; Gilman, 1987). Gβγ dimers were initially thought to only block adenyl cyclase activation; since then the βγ subunit has been shown to activate ion channels, such as K⁺ channels, as well as positively regulate a number of effector proteins such as phospholipase Cβ (PLCβ), phospholipase A₂ (PLA₂), phosphoinositide 3-kinase (PI3-kinase) and β-adrenergic receptor kinase (Clapham & Neer, 1993). Moreover, it has been proposed that these dimers may act via Ras to stimulate the mitogen-activated protein (MAP) kinase pathway (Crespo et al., 1994). To further complicate matters, effector proteins such as adenyl cyclase and phospholipase C are themselves complex and diverse families comprised of many subtypes which individually interact with G-protein subunits to yield a response. It has now become apparent that many target proteins are coordinated by both βγ and α subunits and their interaction with respective effectors is specialized.

Recently, much attention has focused on transmembrane signalling that regulates intracellular Ca²⁺ and protein phosphorylation. The hydrolysis of phospholipids yields two second messengers responsible for subsequent signalling events. Phosphatidylinositol
bisphosphate (PtdInsP$_2$), stored in the plasma membrane, is hydrolyzed by phospholipase C (PLC) to yield inositol 1,4,5-trisphosphate (InsP$_3$) and diacylglycerol (DAG). InsP$_3$ releases Ca$^{2+}$ from intracellular stores and DAG serves to activate protein kinase C (PKC). Many PLC isoforms have been characterized which respond independently from one another to the signals transmitted by extracellular stimuli. This bifurcating messenger system serves important functions during gametogenesis, fertilization, early development, cell proliferation and motility (see Divecha & Irvine, 1995; Berridge, 1993; Cockcroft & Thomas, 1992, Majerus et al., 1990). Many receptors use this signalling pathway and from what is known to date, PLC is regulated by at least two distinct receptor classes; heterotrimeric G-proteins and tyrosine kinase-linked receptors (Marshall, 1995; Kazlauskas, 1994; Berridge, 1993; Ullrich & Schlessinger, 1990).

Three families of PLC have been identified, β (125-154kDa), γ (145-146kDa) and δ (84-85kDa), and each family has numerous subtypes, such as PLC-β1 and PLC-β2 (Cockcroft & Thomas, 1992). All PLC isoforms share two regions of homology which constitute the catalytic domain. Both PLC-β and PLC-δ are activated via ligand binding to the seven transmembrane spanning domains linked to G-proteins. Evidence for their direct interaction lies in the ability of non-hydrolysable analogues of guanine nucleotides, GTPγS or aluminum fluoride (AlF$_4^-$), to increase hydrolysis of PtdInsP$_2$ (Kahn, 1991; Sternweis & Gilman, 1982), and the ability of pertussis toxoid to inhibit PLC activation in some cell types (Cockcroft, 1987). Alternatively, PLC-γ is stimulated via agonist binding to receptor tyrosine kinases. Receptor autophosphorylation provides docking sites for downstream signal transduction molecules containing src homology domains (Ullrich & Schlessinger, 1990). PLC-γ isoforms contain the src homology domains, SH2 and SH3, where the SH2 domains bind to phosphotyrosyl residues on the cytoplasmic tail of activated receptor tyrosine kinases (eg. PDGF, EGF) and the SH3 domain has been shown to target cytoskeletal microfilaments (Cohen et al., 1995). Evidence that tyrosine phosphorylation could regulate PLC-γ activity was first demonstrated from complementary DNA cloning,
which revealed regions homologous (mentioned above) to tyrosine-kinase related oncogenes (yes, src, fgr, abl, fps, fes, tck and erk, Mayer et al., 1988, Margolis et al., 1989). PLC-γ phosphorylation (hence activation) ensues via EGF and PDGF receptors, where other receptors with tyrosine kinase activity such as insulin receptor do not activate PLC-γ (Margolis et al., 1989; Nishibe et al., 1990).

PLC activation results in PtdInsP₂ hydrolysis to InsP₃ (releasing Ca²⁺) and DAG (activating PKC). These second messengers (Ca²⁺ and PKC) can both regulate PLC activation (Cockcroft & Thomas, 1992). Moreover, protein kinase A (PKA), stimulated through cAMP via adenylyl cyclase activation, can regulate PLC activity (Rhee et al., 1993). It appears that PLC activation either by G-protein coupled receptors or tyrosine-kinase-linked receptors, is similarly affected by these effector proteins (Rhee et al., 1993; Cockcroft & Thomas, 1992; Rhee 1991). Cytosolic Ca²⁺ appears to stimulate PLC activity by either direct interaction or by modulating receptor-mediated responses. However, it still remains unknown if Ca²⁺ activates specific PLC isoforms. In contrast, PKC and PKA inhibit PLC-γ activity by phosphorylating a single serine residue (Ser-1248), thus preventing tyrosine phosphorylation by RTKs (Rhee, 1993). This explains why phorbol esters such as PMA (which increases PKC activity) and cAMP (which increases PKA activity) result in decreased InsP₃ concentration and cytosolic Ca²⁺. Moreover, agents which bind to adenylyl cyclase-coupled receptors such as prostaglandin E2, adenosine, isoproterenol and histamine, and those which directly activate adenylyl cyclase such as forskolin and cAMP analogs, result in the supression of various cellular processes which depend on PtdInsP₂ hydrolysis and Ca²⁺ mobilization (Rhee, 1993). It has been shown that the PLC-β isoform is phosphorylated by PKC but not PKA on serine residues (Ser-887) (Ryu et al., 1990). However, the catalytic activity of this protein in vitro was unaltered. Further experimentation is needed to ensure in vivo serine phosphorylation does not affect receptor-coupled PtdInsP₂ hydrolysis. The activation mechanism of PLC-β has not yet been fully elucidated.
InsP₃ generated from PtdInsP₃ hydrolysis, binds to specialized and ubiquitous tetrameric InsP₃ receptors which span the endoplasmic reticulum (ER). Each receptor subunit binds a single InsP₃ molecule and is also desensitized by high concentrations of this molecule (Hajnoczky & Thomas, 1994). The binding of InsP₃ to its intracellular receptor results in conformational change and initiates the release of Ca²⁺ from the ER (see Berridge, 1993). These Ca²⁺ stores possess three constituents, a pump to sequester Ca²⁺, Ca²⁺ binding proteins (such as calsequestrin and calreticulin) and individualized IP₃R or RYR (ryanodine receptor) channels to release immobilized Ca²⁺ (Fig. 2). IP₃R and RYR channels portray the two main intracellular calcium channels responsible for releasing Ca²⁺ back into the cytoplasm from the endoplasmic reticulum. The distribution of these two receptors varies from cell to cell, where some cell types will house only RYR (skeletal muscle) or only IP₃R (Xenopus oocytes), while others will harbour both (atrial cells, vascular smooth muscle, neurons, chromaffin cells and sea urchin eggs; Berridge, 1993; Parys et al., 1992). It is not yet certain if all four putative binding sites need to be sequentially bound by InsP₃ to induce channel opening or if each individual binding can incite partial channel opening, where the latter could explain variable conducting states during patch recordings (Ehrlich & Watras, 1988). The interaction between InsP₃ receptors and Ca²⁺ oscillations have been shown to be crucial and directly correlated to cell-cycle events such as pronuclear migration, nuclear envelope breakdown, metaphase-anaphase transition of mitosis and cytokinesis (Ciapa et al., 1994). Ca²⁺ itself can induce calcium release from non-mitochondrial pools and is regulated by positive and negative feedback mechanisms.

In non-excitable cells, calcium exerts its function through association with calcium binding proteins. Central to them is calmodulin, the Ca²⁺-calmodulin (CaM) complex which binds to numerous enzymes and modulates their activity. These effector enzymes include CaM-dependent protein kinase, protein phosphatases and adenylyl cyclase (see Ghosh & Greenberg, 1995). It has been shown that Ca²⁺-sensitive cytosolic enzymes,
Figure 2. Intracellular calcium signaling in nonexcitable cells. The abbreviations are defined as follows: R, G-protein-linked receptor; G, G-protein; PLC, phospholipase C or PtdInsP$_2$ phosphodiesterase; InsP$_3$, inositol (1,4,5)trisphosphate; GTP, guanosine triphosphate; GDP, guanosine diphosphate; DG, diacylglycerol; PMCA, plasma membrane Ca$^{2+}$-ATPase pump; ICRAC, Ca$^{2+}$ release-activated current; K+, potassium-selection channel; InsP$_3$R, InsP$_3$ receptor; SERCA, smooth endoplasmic reticulum Ca$^{2+}$-ATPase pump; +, stimulatory regulation; -, inhibitory regulation. Borrowed from Clapham (1995).
such as CaM kinase, can phosphorylate specific transcription factors which regulate the expression of Ca\(^{2+}\)-responsive genes. Moreover, Ca\(^{2+}\) has been reported to be linked to the Ras signalling pathway (Bading & Greenberg, 1991). Increases in cytosolic Ca\(^{2+}\) activate Ras which then associate with serine/threonine kinase Raf, proceeded by a phosphorylation cascade ensuing in the activation of kinase MEK-1, MAP kinases and ribosomal S6 kinases. It will be important to elucidate the mechanism by which Ca\(^{2+}\) promotes Ras activation, since Ras-dependent MAP kinase signaling is integral in the activation of specific transcription factors (Clark & Brugge, 1995).

**Signal Transduction via Tyrosine Kinase-Linked Receptors**

Extracellular information can be transduced across the plasma membrane through receptor tyrosine kinases (RTKs). Ligand binding to the extracellular domain of a RTK leads to the stimulation of the kinase's activity. These events induce tyrosine phosphorylation of numerous downstream effector proteins, as well as the RTK itself. Growth factor receptors with tyrosine kinase activity or receptor tyrosine kinases are both topologically similar. These single transmembrane receptors contain large glycosylated, extracellular agonist binding domains and cytoplasmic domains that have tyrosine kinase catalytic activity (Ullrich & Schlessinger, 1990; Hanks et al., 1988). Upon ligand binding, the receptor undergoes conformational change which induces oligomerization (or dimerization), thus stabilizing the interaction between adjacent cytoplasmic catalytic domains (see Heldin, 1995; Marshall, 1995; Kazlauskas, 1994; Ullrich & Schlessinger, 1990; Sibley et al., 1987). These events do not require repositioning of any amino acid residues within the transmembrane domain. Some ligands are themselves dimeric molecules such as platelet derived growth factor (PDGF) and colony-stimulating factor 1(CSF-1), which allow them to simultaneously form stable interactions with two receptors. Others have monomeric configurations such as epidermal growth factor (EGF), which has also been shown to simultaneously bind two receptors (Heldin, 1995).
variation of these themes, are insulin and insulin-like growth factor 1 (IGF-1), where their receptors exist as homo- or heterodimers. Instead of inducing receptor dimerization, the agonist causes structural change in the preconfigured dimeric receptor, which impels activation.

Oligomerization of RTK leads to autophosphorylation, where one receptor molecule phosphorylates the other (Ullrich & Schlessinger, 1990). Receptors which undergo autophosphorylation can be divided into two classes dependent upon the site of tyrosine phosphorylation (Heldin, 1995). The first class and the most commonly observed, is conserved phosphorylation on tyrosine residues within the kinase domain leading to increased enzyme activity. It is however, not yet known what initiates cross-domain autophosphorylation. The second class of tyrosine phosphorylation sites are found outside the kinase domain, on the carboxy-terminal tail. It is this region that is the most divergent among the RTKs. These phosphorylation sites offer docking posts for downstream signalling molecules which contain Src-homology 2 (SH2) domains. There are several different docking sites outside the kinase region which allow for binding specificity for different signaling molecules. For example, activated PDGF-β receptor contains at least eight autophosphorylated tyrosine residues aside from that which is responsible for kinase activity; this permits specific interaction with eight different effectors (Claesson-Welch, 1994).

Receptor activity, like most enzymic regulation, is under the control of negative feedback mechanisms. Phosphorylation on serine and threonine residues located in the juxtamembrane domain (between the transmembrane and protein tyrosine kinase domains) by PKC results in the inhibition of RTK activity (Ullrich & Schlessinger, 1990). There are at least three known effects of PKC: first, as mentioned above, suppressing receptor kinase activity; second, reducing the binding affinity of the receptor; and third, initiating receptor internalization (Sibley et al., 1987).
Proteins that have Src homology contain three domains, SH2, SH3 and PI1, which are involved in protein-protein interactions (see Cohen et al., 1995; Pawson & Gish, 1992). These domains have been found in all eukaryotes (not prokaryotes), while only PI1 and SH3 are found in yeast. SH2 domains recognize phosphorylated tyrosine residues on the receptor tyrosine kinase cytosolic polypeptide chain, as well as specific binding sites surrounding the phosphotyrosine. It is these secondary binding sequences which dictate SH2 specificity. For example, phosphatidylinositol (PI) 3'-kinase, Ras GTPase-activating proteins and PLC-γ, all contain SH2 domains, but they bind to different autophosphorylated sites on the PDGF-β receptor (Pawson & Gish, 1992). Therefore, the sequences adjacent to the P-Tyr on the carboxy tail of the receptor dictates the binding specificity of SH2-containing signalling molecules. The insulin receptors employ a variation of this theme, where instead of incorporating its SH2 binding sites as a part of the receptor, it uses a SH2-docking protein, IRS-1 (insulin receptor substrate; Pawson & Schlessinger, 1993). Following receptor activation, IRS-1 is phosphorylated on multiple tyrosine residues that lie within its SH2-binding elements.

The SH3 domains (50-75 residues) are usually found in tandem with SH2 domains within a single protein, with the exception of yeast. However, much less is known about the physiological role of SH3 domains and even less is known about its binding specificity (see Cohen et al., 1995). It is thought that binding follows a similar theme of that of SH2, where specificity is determined by variable sequences which lie adjacent to invariant residues. It has also been suggested that it depends on tertiary structure as opposed to a linear sequence. Or even yet, SH3 domains may only rely on crude specificities, while other modular domains have more refined functions. Even though these questions have not been fully elucidated, recent work proposes SH3 domains to play a critical role in some protein-protein interactions. SH3 domains have been found in proteins that localize to the plasma membrane or to the cytoskeleton, such as α-spectrin (an actin-binding protein), non-muscle myosin and ABP-1 protein in S.cerevisiae (Cohen et al., 1995). To
date we can surmise that SH3 domains may be involved in localizing signalling proteins and targetting them to their site of action, the plasma membrane or other intracellular compartments, as well as regulating the movement of proteins within the cytoplasm. The very recently discovered PH domains has been observed in many signalling and cytoskeletal proteins (Cohen et al., 1995). These regions generally have low sequence homology but do share peptide folding structure. Several PH domains have been found to bind the βγ subunits of heterotrimeric G-proteins but their function still remains an enigma. Recently, it was demonstrated that many PH domains were found to be particularly affiliated with lipid vesicles saturated with PtdInsP₂ (Harlan et al., 1994). These results suggest a possible role for PH domains as mediators of protein-lipid interactions, while SH2 and SH3 domains may be mediators of protein-protein interactions.

RTKs relay their signal to several downstream effector proteins, which themselves regulate a network of proteins. The most commonly investigated are Ras-related proteins (small GTP-binding proteins), Src family kinases, p120-GAP, Shc, PI3-kinase, and PLC-γ. The superfamily of small GTP-binding proteins can be divided into four subgroups on the basis of structural and functional homology; Ras-like, Rho-like, Rab-like and ADP ribosylation factor (ADF)-like (see Hall, 1993). Like all GTPases, the above proteins go through cycles of active (bound GTP) and inactive (bound GDP) conformations. The transition between these two states, nucleotide exchange (inactive to active) and GTP hydrolysis (active to inactive), is regulated by guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs), respectively (Bourne et al., 1991). Ras- and Rho-like proteins have been found to be associated with plasma membrane receptors through their association with GEFs and GAPs. The activation of these two GTPase-binding proteins stimulate downstream effector proteins which regulate cellular processes such as growth, development and actin polymerization (McCormick, 1994; Downward, 1992; Williams, 1992). Rab- and ARF-like proteins appear to control the formation and
trafficking of intracellular vesicles (Novick & Brennwald, 1993). These latter proteins have been extensively reviewed, but are not detailed here.

In mammals, ligand-binding to RTK leads to the recruitment of a 23kDa protein with SH3-SH2-SH3 domains (Grb2) to the tyrosine phosphorylated sites on the receptor (see Kazlauskas, 1994). This adaptor protein has also been found in *C. elegans* (Sem-5) and *Drosophila* (Drk) (Pawson & Schlessinger, 1993). Though their SH2-SH3 domains, these proteins act to link activated RTK to the guanine-nucleotide releasing factor, Sos. This latter protein is translocated to the plasma membrane and promotes the release of GDP (bound to inactive Ras), thus enabling GTP binding (bound to active Ras). A variation on this theme occurs for non-receptor tyrosine kinases, such as the T-cell receptor (Kazlauskas, 1994). Receptor activation leads to tyrosine phosphorylation of the ζ receptor chain and subsequent Shc binding via SH2 homology. It appears that the ζ chain-Shc complex acts as a bridge between the activated receptor and binding of the Grb2/Sos complex. Moreover, some receptors recruit p120-GAP through SH2 association. This protein seems to negatively regulate Sos by stimulating Ras-GTPase activity, thus converting active ras to its inactive form (McCormick, 1994). It is unknown whether p120-GAP is in an active state when complexed with the receptor, or if it participates in any other signalling events.

Active ras transfers the signal downstream through association with Raf, which only binds Ras-GTP and not Ras-GDP. Raf is a serine/threonine protein kinase that has been demonstrated to be one of the activators of the MAP kinase pathway (Kazlauskas, 1994; Marshall, 1993; Woodgett, 1992). Raf is transferred to its active state after binding to Ras-GTP as well as additional factors. These additional factors have not been fully elucidated but some studies suggest particular members of the PKC family can phosphorylate Raf, which stimulates Raf autophosphorylation (Kazlauskas, 1994). However, autophosphorylation alone does not enhance MEK phosphorylation. Raf is responsible for the serine/threonine phosphorylation of MAP kinase kinase (MAPKK or
MEK), which in turn stimulates other serine/threonine kinases, MAP kinases. The downstream effectors of MAP kinase are numerous transcription factors, such as c-Jun, c-Myc and p62TcF/E1k-1, which are regulated by phosphorylation and in turn regulate the transcription of growth-dependent genes (Davis, 1993).

Rho-like proteins such as Rho, Rac and CDC42 have only recently begun to be understood. By definition they are GTP-binding proteins and are active when bound to GTP and inactive when in the GDP-bound state. This reaction is enhanced by the GTPase activating protein, rhoGAP, which is also active on rac (see Ridley & Hall, 1993; Downward, 1992). Two similar GAP proteins, Bcr and n-chimerin, stimulate the transition from active rac to the inactive form but have no effect on rho. Additional proteins have been reported to interact in vitro with rho and rac, rhoGDI, smgGDS and rhoGDS. Where rhoGDI keeps both rho and rac in the inactive state by inhibiting GDP dissociation, smgGDS stimulates GTP binding of rho, rac, Ki-ras and rap1 proteins, while rhoGDS enhances guanine nucleotide exchange of rho but not rap (Ridley & Hall, 1992).

The biological function of rho proteins have been linked to cytoskeletal reorganization. More specifically, when rho is ADP-ribosylated by C3 transferase (Clostridium botulinum), cells lose their actin stress fibers and round up (Aktories et al., 1989). A number of growth factors (EGF, PDGF, insulin, thrombin) have been found to elicit a similar response. Recently, Ridley & Hall (1992) demonstrated that lysophosphatidic acid (LPA), present in serum, induced the formation of focal adhesions and actin stress fibers in fibroblasts, much more rapidly than growth factors. The authors hypothesized that LPA and growth factors stimulate rho-dependent responses by elevating the level of active rho, by either activating GEFs or inhibiting GAP proteins. The differential rate of actin stress fibre formation suggests that LPA and growth factors may exert their function via two different signalling mechanisms. Additionally, arachidonic acid metabolites, such as prostaglandins and leukotrienes, have been shown to induce cortical actin polymerization and stress fiber breakdown, respectively (Peppelenbosch et
The authors suggest these metabolites may induce the activity of GEFs or suppress GAP function. The specific downstream targets of rho-GTP remain unknown, but sufficient information points to a role in initiating the signals required for both focal adhesion and stress fiber formation. Rac proteins have also been shown to stimulate actin reorganization but in the form of membrane ruffles (Ridley et al., 1992). Membrane ruffling mainly occurs during spreading and locomotion, as well as in the formation of pinocytotic vesicles. When rac activity is inhibited, fibroblasts accumulate large vesicles which increase in number and in size until the entire cytoplasm is filled (Ridley et al., 1992). These observations suggest either an increase in the pinocytotic mechanism or irregular accretion of trafficking compartments. Alternatively, rac inhibition in confluent cells leads to the accumulation of polymerized actin at the plasma membrane (Ridley et al., 1992). The authors further observed the inability of rac inhibited cells to form new stress fibers, which has lead to their hypothesis that activated rac not only stimulates membrane ruffling, but also induces rho activation and ensuing stress fiber formation.

Phosphatidylinositol 3-kinase (PI3-K) is a membrane enzyme that also interacts with phosphotyrosine residues of activated RTK via its SH2 domain (see Divecha & Irvine, 1995; Kazlauskas, 1994; Foster, 1993). It has also been shown that PI3-kinase can be stimulated by the βγ subunit of G-proteins (Divecha & Irvine, 1995). In mammalian cells, this enzyme phosphorylates the D-3 position on phosphatidylinositol (PtdIns), phosphatidylinositol-4-phosphate (PtdInsP) or phosphatidylinositol-4,5-phosphate (PtdInsP2) to yield PtdInsP, PtdInsP2 and PtdInsP3, respectively. This enzyme consists of two subunits, an 85kDa protein and a 110kDa catalytic subunit (Kazlauskas, 1994). The 85kDa moiety harbours a single SH3 domain, two SH2 domains and a zone of rhoGAP homology. The SH3 domain is located on the amino terminus of the molecule and serves as an as yet unknown function. However, one can hypothesize it binds to proteins which negatively regulate PI3-kinase activity as seen in other PTK (c-Abl and c-Src) or perhaps recruits cytosolic proteins to the membrane as seen in PLC-γ and Grb2 (Kazlauskas,
The SH2 domains, located in the carboxyl-terminal region of the molecule bind to phosphotyrosine residues of activated RTK, which leads to the activation of PI3-kinase activity. The function of the rhoGAP region of p85 has not yet been elucidated. However, the association between PI3-kinase and small GTP-binding proteins has recently been reported (see Kodaki et al., 1994). Activated Ras (via Grb2/Sos binding) directly activates the p85/p110 complex of PI3-kinase. This finding suggests a critical role for Ras as a bifurcating messenger system, as opposed to a singular messenger molecule in the activation of the MEK signalling pathway. The 110kDa subunit has serine/threonine kinase activity, which is capable of phosphorylating the p85 moiety (Divecha & Irvine, 1995). The phosphorylation of the latter regulatory subunit decreases PI3-kinase activity, hence the enzyme controls its own activity.

The products of PI3-kinase activity, PtdInsP, PtdInsP₂ and PtdInsP₃ have themselves individual physiological functions. PtdInsP plays a role in vacuolar protein processing, while PtdInsP₃ has been shown to activate the Ca²⁺- and phorbol ester-independent PKC (see Divecha & Irvine, 1995; Foster, 1993). The role of this PKC isoform has yet to be determined. Alternatively, PtdInsP₃ has been found to stimulate the rate of GTPase activity of Rac, hence increasing the level of GTP binding to yield active Rac (Parker, 1995). As mentioned above, the physiological role of Rac has been linked to change in actin polymerization, whereby membrane ruffling ensues. Aside from the traditional role of PtdInsP₂ hydrolysis to generate InsP₃ and DAG (as discussed previously), PtdInsP₂ also has (like PtdInsP and PtdInsP₃) been linked to cytoskeletal regulation. The PtdInsP₂ profilin interaction plays an important role in the phosphoinositide (PtdIns) cycle and in the regulation of actin filaments. During resting states, PtdInsP₂ can not be hydrolyzed by phospholipase C (PLC) because of its direct association with profilin (see Divecha & Irvine, 1995). However, following receptor activation and subsequent PLC phosphorylation, the PtdInsP₂ profilin complex can be hydrolyzed, allowing profilin to interact with actin filaments, leading to filament
breakdown and the production of profilin-actin complexes. Much is to be learned of the physiological role of signalling phospholipids, their binding specificities and interaction with other signalling pathways. Moreover, as the inositol kinase family grows, it is anticipated that many enzymes will be specifically associated with the cytoskeleton.

Tyrosine phosphorylation of intracellular effector proteins can be also initiated by the receptor family of integrins (see Clark & Brugge, 1995; Juliano & Haskill, 1993). This family of cell surface receptors commonly mediate attachment to the extracellular matrix (ECM), via components therein such as fibronectin, collagen and laminin. Specific classes of integrins also orchestrate cell-cell adhesive interactions. The integrins are commonly composed of α and β subunits, that heterodimerize to yield more than 20 different receptor subtypes. It is ligand binding that initiates receptor clustering and subsequent activation of intracellular integrin-mediated responses. The sites of integrin clustering are known as focal adhesions (focal contacts or focal plaques), which provide a link between matrix components and bundles of actin filaments (stress fibers; Burridge et al., 1992). Several actin-binding proteins aggregate with integrins in focal adhesions, they include α-actinin, talin, vinculin, and tensin (see Clark & Brugge, 1995). Where α-actinin and talin bind specifically to the carboxy-terminus of integrins, the other actin-binding proteins, as well as the two mentioned here, associate through a combination of interactions. These assemblies are thought to significantly contribute in stabilizing cell adhesion and regulating cell shape, morphology and mobility. Not only are focal adhesions important in the above mentioned link between the ECM and intracellular cytoskeletal complexes, but are also sites for the association of signal transduction proteins that regulate cell behavior. Upon receptor engagement, phosphorylation of effector protein tyrosyl residues has been shown to be a common integrin-dependent response, since treatment with tyrosine kinase inhibitors (such as herbimycin A) obstruct the formation of focal adhesions (Burridge et al., 1992). Many of the signalling molecules affected by transmembrane G-protein receptors and receptor tyrosine kinases are also involved in integrin transduction.
responses. These include activation of some members of the serine/threonine kinase family (such as PKC and MAP kinases) and phosphatidylinositol signalling, regulation of integrin-dependent gene expression, elevation of intracellular calcium concentrations, and pH (Clark & Brugge, 1995; Juliano & Haskill, 1993; Burridge et al., 1992; Zachary & Rozengurt, 1992). Central to integrin-mediated signal transduction are focal adhesion kinases (FAK), which are phosphorylated on tyrosine residues (hence activation) following receptor engagement. RTKs (such as PDGF) and G-protein linked receptors (such as those activated through neuropeptides or LPA binding) also induce tyrosine phosphorylation of FAK (Schaller & Parsons, 1994). It has yet to be determined whether there is a common objective to FAK activation or if the stimulus dictates a distinct function. These questions arise because this kinase is capable of affecting (directly or indirectly) many signalling proteins. The cytoskeletal protein, paxillin, associates with FAK, where paxillin is a vinculin-binding protein concentrated in focal adhesions. Subsequent phosphorylation of paxillin (on tyrosine residues) allows for the formation of cytoskeleton-membrane attachments (Burridge et al., 1992). Furthermore, the phosphotyrosine sites on both FAK and paxillin serve as docking sites for proteins containing src homology 2 (such as the family of Src kinases, Csk kinases (which suppress Src activity), Grb2/Sos complex (involved in Ras and ras-related signaling pathways), and PI3-kinase (phospholipid signalling pathway)). It is clear that integrin mediated responses synergize with other receptor pathways, either by increasing or inhibiting their respective signals.

**Spore Germination in Dictyostelium discoideum**

Spore formation in *Dictyostelium discoideum* has been shown to be provoked by intracellular cAMP (Kay, 1989; Riley et al., 1989; Wang et al., 1988; Kay, 1982) and
repressed by the addition of adenosine or differentiation inducing factor (DIF. Early & Williams. 1988). Moreover. Riley & Barclay (1990) propose that ammonia also promotes spore formation by increasing intracellular cAMP concentration. Both cAMP (or its analogues) or calcium ions have been shown to increase the transcription of four spore coat proteins; SP60. SP70, SP87 and SP96 (Yoder et al., 1994; Fosnaugh & Loomis. 1993). Interestingly, cAMP analogues (Br-cAMP) induce the state of SP96 phosphorylation, while an inhibitor of protein kinase activity (K252a) blocks it (Maeda, 1992). The spore coat proteins, SP70 and SP75, are also heavily phosphorylated on their serine residues. To date eight different proteins have been identified that associate with the spore coat (SP96. SP87. SP86. SP85. SP80. SP70. SP60; see Yoder et al., 1994. West & Erdos, 1990). Synthesis of these spore specific proteins begins between the tipped aggregate stage (14 hrs) and the slug stage (16 hrs), and are subsequently compartmentalized in prespore vesicles (West & Erdos, 1990; Devine et al., 1982). During culmination (22 hrs), the prespore vesicle membrane fuses with the plasma membrane, whereby the proteins therein are released into the extracellular matrix of the sorocarp. Once externalization of these coat proteins has ensued, cellulose is synthesized and localized in the matrix between the electron-dense outer layer and plasma membrane proximal inner layer.

The spore mass, surrounded by a viscous matrix is supported by a column of nonviable stalk cells (see Raper, 1984). The individual spores suspended within the sorocarp matrix are kept in a dormant state by the activity of endogenous autoinhibitors and by the maintenance of high osmotic pressure (Cotter, 1975). Discadenine, present in the extracellular matrix has been proposed to be the autoinhibitor, since it prevents spore germination (Abe et al., 1976). It remains however, unclear to what capacity (directly or indirectly) this compound is involved in spore formation, maintenance of dormancy or reacquisition of dormancy.
When the sorocarp collapses onto the substratum, the spores and the extracellular matrix proteins contained within, are dispersed. The spores are therefore, not held within a high osmotic environment, nor is the concentration of the autoinhibitor high enough to maintain dormancy. Under favourable environmental conditions, the spores will autoactivate and synchronously germinate to yield nascent myxamoebae. The germination program consists of four stages, spore activation, lag period, spore swelling, and the emergence of nascent amoebae (Cotter, 1981). The spontaneous activation, or autoactivation, is characterized by the production and secretion of a low molecular weight, phosphorylated adenine derivative, the autoactivator, from spores during early swelling (Cotter & Glaves, 1989; Glaves & Cotter, 1989). Spores presumably bind and respond to this germination-specific factor, which results in synchronous spore swelling and amoebal emergence throughout the entire population (Cotter & Glaves, 1989). Among the environmental conditions which regulate activation are spore density, oxygen tension, pH, temperature, and osmotic pressure (Cotter & Glaves, 1989; Cotter, 1981; 1975). The concentration of spores must be at least $10^7$ spores/ml to induce synchronous germination within the population. This density ensures the spores survival for another round of asexual development if food is not plentiful. Oxygen consumption is the primary indication that spores have committed to germination, which infers the critical role for mitochondrial oxidative phosphorylation (Cotter & George, 1975). Moreover, inhibitors of mitochondrial electron transport, such as azide or cyanide, result in deactivation (Cotter et al., 1976). The optimum pH of the medium surrounding dormant spores is in the range of 6.0 and 7.0; values below or above this range reduce the total percentage of emerged amoebae (Cotter & Raper, 1968). Interestingly, Riley & Barclay (1990) recently demonstrated that both high pH and ammonia stimulate the increase of intracellular cAMP, while high internal cAMP has been shown to prolong or block germination (Virdy et al., 1995). Suboptimal (4°C) and supraoptimal (50°C) temperatures result in the maintenance of dormancy and the death of spores, respectively (Cotter & Raper, 1968).
However, heat shocking spores at 45°C for 30 minutes preceding incubation at room temperature ensues synchronous germination (Cotter & Raper, 1968). Moreover, shock treatment by the addition of the protein denaturant, DMSO, also results in synchronous germination (Cotter et al., 1976). This latter activation treatment is presumed to shock mitochondrial function, whereby rapid oxygen uptake ensues following the removal of this agent. Spores can also be induced to germinate with the hydrophobic amino acids tryptophan, phenylalanine and methionine (Cotter & Raper, 1966).

Regardless of the mode of activation, the wild-type strains, NC4 and V12, and the spontaneous germinating daughter mutants, SG1 and SG2, all share the morphological changes which accompany germination (Dahlberg & Cotter, 1978; Cotter & Dahlberg, 1977). The lag period (during autoactivation) of the premature maturation mutants, SG1 and SG2, is much shorter than that of their parental strains and more closely resembles that of heat or DMSO activated spores. Furthermore, wild-type strains must age 10-14 days in the sorocarp before acquiring the ability to spontaneously germinate, presumably due to the presence of late spore maturation genes (Cotter & Glaves, 1989). However, young (2 day old) wild-type spores can be exogenously activated by either heat or DMSO shock. It is not yet known what events are occurring during the aging process to allow for spontaneous germination nor the mutation responsible for the SG phenotype. Moreover, spontaneously germinating spores require protein synthesis to progress to the swelling stage, as shown by the inhibition of swelling by cycloheximide. In contrast heat-induced activation only requires de novo synthesis for emergence (Dahlberg & Cotter, 1978). These authors suggest it is the autoactivator that is synthesized de novo, since heat-activated spores do not produce nor secrete the autoactivator prior to swelling. From these data, it is clear that autoactivation and exogenous activation occur via two different mechanisms.
Autoinhibitor-Autoactivator Interactions

When crude autoinhibitor preparations (extracellular matrix of freshly harvested sorocarps) are added to dormant spores, they remain in this state until they are washed free of the inhibitor (Cotter & Glaives, 1989; Dahlberg & Cotter, 1978). The addition of crude autoactivator (present in the supernatant of freshly germinated spores) advances the onset of swelling by approximately 1 h (Cotter & Glaives, 1989). These authors further examined the possibility that both the autoinhibitor and autoactivator compete for the same binding site on the spore surface. However, their results indicate that these two factors may exert their function independently via two different mechanisms, since the autoactivator can override any effect imposed by the autoinhibitor. As mentioned above, it appears the autoactivator is synthesized de novo in spontaneous germinating spores and secreted into the surrounding medium, which results in a autocatalytic cascade with ensuing synchronous germination of the entire population. The effects of the autoactivator are limited to the spontaneous germination process, since those spores activated by exogenous treatment (heat shock) do not synthesize nor secrete the autoactivator (Cotter & Glaives, 1989; Dahlberg & Cotter, 1978). Moreover, activator preparations are not denatured or destroyed during heat treatment, since they retain their ability to stimulate germination to the same potency as untreated autoactivator (Cotter & Glaives, 1989). The process of autoactivation (response to exogenous autoactivator) and the production of autoactivator (by individual spores) appear to be independent of one another; i.e., spores incubated at slightly higher than optimum temperatures (31°C) still produce internal autoactivator, but fail to release it, hence germination in the population is inhibited (Cotter & Glaives, 1989).

Signal Transduction during Germination

Traditional signalling molecules that regulated many cellular and developmental processes (detailed above) such as those mediated via receptor activation have only
recently been examined during the germination program in *Dictyostelium discoideum*. To no suprise, second messenger molecules (such as cAMP and calcium) and regulatory protein kinases (such as protein kinase A, protein kinase C, and CaM-dependent protein kinases) were the first to be investigated (Virdy *et al.*, 1995; Lydan & Cotter, 1995; 1994; Lydan *et al.*, 1994a; 1994b). These works have established that signal transduction processes are indeed involved in the underlying mechanisms which regulate spore dormancy, spore aging, and spore germination.

Intracellular levels of cAMP have been found to be approximately 10-fold higher in quiescent spores than those levels found in nascent amoebae, suggesting a gradual decline during autoactivation (Virdy *et al.*, 1995). The addition of cAMP analogues and adenylyl cyclase activators both suppress germination of autoactivated but not heat activated spores. The effects of these pharmacological agents on autoactivation versus other activation methods again demonstrates differential germination mechanisms. It appears that high cAMP keeps the spore in a dormant state, possibly via the activation of PKA and subsequent serine/threonine phosphorylation of dormancy-dependent proteins. Moreover, disruption of the *acg* (adenylyl cyclase-germination) gene results in a phenotype with spontaneous germination characteristics (SG) (Pitt *et al.*, 1992; K. Virdy, T. Sands, D. Cotter, G. Pitt, P. Devreotes, unpublished). Moreover, ACG has been shown to transverse the plasma membrane and serve as its own receptor (Pitt *et al.*, 1992). Receptor engagement leads to the activation of its catalytic domain and subsequent cAMP accumulation. The ligand has yet to be determined, however, it has been hypothesized that the ACG protein may be an osmosensor (Schaap & Cotter, unpublished).

Autoactivation appears to occur through InsP₃-dependent signalling and subsequent Ca²⁺ mobilization (Lydan & Cotter, 1995). Pharmacological studies have revealed a positive role for phospholipase C activity, during autoactivation, leading to the hydrolysis of PtdInsP₂ to yield InsP₃ and DAG. However, PKC does not seem to play a role in autoactivation (Lydan *et al.*, 1994b). Ca²⁺ mobilization (probably from the
endoplasmic reticulum) and subsequent efflux from the spore is required for activation to occur, and is dependent on the amount of autoactivator present (Lydan & Cotter, 1995). Furthermore, this calcium flux occurs independent of the mode of activation. The rise of intracellular Ca\(^{2+}\) appears to associate with calmodulin, where this latter protein has been shown to mediate the events leading to spore swelling (Lydan & Cotter, 1994; 1994b). Prior to emergence, regardless of the mode of activation, a net influx of Ca\(^{2+}\) and subsequent association with calmodulin must occur for emergence to ensue. Interestingly, CaM-dependent kinase activity does not appear to play a role in either autoactivated or heat activated spore germination (Lydan et al., 1994a). It will be interesting to elucidate the specific target proteins of the calcium-calmodulin complex, since its inhibition arrests both swelling (of autoactivated spores) and emergence (independent of activation mode). The upstream events leading to the activation of PLC remain unknown. From the literature, there exist two possible receptor families which could stimulate PLC activity, G-protein linked and tyrosine kinase-linked receptors. Recently, Khosla et al. (1994) demonstrated that RasG (Ras for growth) is synthesized during the transition between swelling and emergence, independent of the mode of activation. Furthermore, the addition of a protein tyrosine phosphatase inhibitor, vanadate, totally blocked germination initiated by autoactivation (Cotter et al., 1990). The authors suggest that vanadate inhibited membrane traffic flow necessary for autoactivation to ensue. More specifically, this agent could be affecting the proteins directly associated with the synthesis and secretion of the autoactivator.
Objectives of this investigation

From recent work, it has become clear that several signal transduction processes regulate germination. The scope of this work will focus on the possible role of tyrosine phosphorylation during the germination program in spores of Dictyostelium

1. **Determine whether phosphotyrosine-containing proteins are present during germination of D. discoideum strains SG1, SG2, NC4 and V12.** Each of these commonly used laboratory strains will be incubated under optimal environmental conditions to allow for autoactivation. At each hour during the germination program, spores will be collected and analyzed by Western blotting using commercially available anti-phosphotyrosine antibodies.

2. **Determine if the developmental pattern of phosphotyrosine-containing proteins differs with different activation methods.** Since it well established that autoactivation and heat activation occur via two distinct mechanisms, the pattern of P-Tyr containing proteins from both activation methods will be compared. Moreover, the pattern observed in aged wild-type spores (NC4 and V12) will be compared to young immature spores of the same strains. This may establish a possible role of tyrosine phosphorylation during spore aging leading to autoactivation-competency.

3. **Determine the biological significance of these phosphotyrosine-containing proteins.** Spores will be treated with a potent and specific tyrosine kinase inhibitor, herbimycin A, to determine a possible developmental role of intracellular protein tyrosine kinases (Gauthier et al., 1995). Furthermore, the receptor tyrosine kinase inhibitor, tyrphostin A46 (Lyall et al., 1989), will be used to determine whether the autoactivator or autoinhibitor exert their function via this receptor family.
4. **Determine whether signal transducing G-proteins are specifically involved during the germination program of strain SG2.** An activator of signal transducing G-proteins, aluminum fluoride (Sternweis & Gilman, 1982), will be used to determine whether the autoactivator or autoinhibitor exert their function via the transmembrane spanning domains leading to G-protein activation.

5. **Determine whether the autoinhibitor or autoactivator affect any specific protein(s) that are phosphorylated on tyrosine residues.** Phosphotyrosine containing proteins will be analyzed after treatment with cycloheximide, prolonged incubation with autoinhibitor preparations and the addition of exogenous activator preparations. Furthermore, commercially available anti-actin antibodies will determine whether actin is among the phosphotyrosine containing proteins, as has been found during asexual development in this organism.
MATERIALS AND METHODS

I. Culture Conditions

A variety of strains of cellular slime molds were used throughout this study. All cultures were maintained on glucose salts medium (containing: 1.0 g NH₄Cl, 3.0 g KH₂PO₄, 6.0 g NaHPO₄, 0.13 g MgSO₄ and 20.0 g Bacto Agar (Difco) in 1L of ddH₂O. 10 mL of 0.4 g/mL of sterile glucose (Fisher) was added after the above medium was autoclaved). Agar plates each received 15mL of the above agar medium.

Spore germination buffer contained 1.04 g KH₂PO₄ in 1 L of ddH₂O. The pH of the 0.01 M phosphate buffer was adjusted to 6.5 with KOH prior to sterilization. Spores were aseptically loop transferred from stock cultures to a conical tube containing germination buffer along with a loopful of Escherichia coli B/r. The suspension was mixed by vortexing and 1.0 to 1.5 mL of inoculum was transferred to each glucose salts agar plate. All plates were incubated at room temperature. Approximately 24 hrs after initial inoculation, plates were shaken to ensure an even distribution of myxamoebae over the agar surface. Fruiting bodies were formed about 3 to 4 days after inoculating the plates.

II. Conditions for Spore Germination

All spores were harvested from agar plates by holding a microscope slide above the agar surface while gently rotating the petri dish. Sori were rinsed from the microscope slide into 5 to 10 mL of ddH₂O in a 50 mL beaker. The suspension was then poured into a 15 mL plastic conical centrifuge tube and centrifuged at setting 6 in a table top IEC Clinical Centrifuge to pellet the spores. The supernatant containing the sorocarp matrix material was poured off and saved as "crude autoinhibitor preparation", while the pellet was resuspended in germination buffer and recentrifuged. This washing procedure was repeated.
1) Autoactivation

Spore pellets were resuspended in germination buffer (pH 6.5) at a concentration of 2-3 \times 10^7 spores/mL (determined with a hemacytometer) and stirred with 10 mm magnetic bars. Volumes were usually kept below 4 mL in 1 X 10 cm glass tubes. Wild-type strains, NC4 and V12 were aged 10-14 days prior to harvesting. Whereas, the mutant strains SG1 and SG2 (generated from parent strains, NC4 and V12, respectively), were aged only 2 days prior to harvesting.

ii) Heat activation

Spore pellets were resuspended in germination buffer (pH 6.5) and placed in a Braun Thermomix II circulating water bath and heat shocked at 45°C for 30 minutes. All spore strains germinated in response to heat shock, independent of spore density and age (Cotter & Glaves, 1989; Dahlberg & Cotter, 1978).

Percent spore germination was monitored microscopically at 1 hour intervals with a Zeiss phase contrast microscope. A minimum of 200 objects was scored as dormant (phase-bright, oval-shaped spores), swollen (phase-dark, distended and irregularly shaped spore cases), or emerged (appearance of myxamoebae, not enclosed in spore cases).

III. Autoinhibitor and Autoactivator Studies

i) Autoinhibitor

Crude autoinhibitor preparations were collected as per Cotter & Glaves (1989). Following harvesting of SG2 spores, the suspension was poured into 15 mL plastic conical tubes and centrifuged at setting 6 in a table top IEC Clinical Centrifuge. The supernatant containing sori matrix material was then filtered through a millipore filter of 0.2μmeter pore size and frozen at -20°C until use.
ii) Autoactivator

Crude autoactivator preparations were collected as per Cotter & Glaves (1980). Following the onset of germination (swelling) of SG1 spores, the suspension was poured into 50 mL plastic tubes and centrifuged at top speed in an IEC Clinical Centrifuge. The supernatant was then filtered through a millipore filter of 0.2 μm pore size and frozen at -20°C until further use.

To test the ability of treated or untreated spores to respond to the autoinhibitor or autoactivator, SG2 spores at 2-3 × 10⁷/mL were diluted 1:1 with an autoactivator or autoinhibitor preparation and monitored microscopically as described above.

IV. Pharmacological Agents

In order to understand the role of tyrosine phosphorylation during germination, tyrosine kinase inhibitors were added prior to the onset of germination and the spores were scored as dormant, swollen or emerged, as described above. Herbimycin A (BioMol) was dissolved at 1 mM in 100% DMSO. Dormant spores were treated with 10 μM and 20 μM Herbimycin A, while control spores were treated with the respective percentage of DMSO (Gauthier et al., 1995). Tyrphostin A46 (CalBiochem) was dissolved to 27 mM in 100% DMSO. Dormant spores were treated with 40 μM, 80 μM, 100 μM, and 200 μM tyrphostin A46 (Lyall et al., 1989), while control spores were treated with the respective concentration of DMSO. All spores were saponin permeabilized (100 μg/ml, Europe-Finner & Newell, 1987) before inhibitor treatment.

In order to ascertain the role of signal transducing G-proteins during germination, aluminum fluoride was produced by first making independent stock solutions of 0.5 mM AlCl₄⁻ and 200 mM NaF (Sigma) in ddH₂O (Sternweis & Gilman, 1982). It has been shown that in the presence of 5 mM F⁻, Al³⁺ will primarily exist as AlF₄⁻. Therefore, 25 μM AlCl₄⁻ and 10 mM NaF, 12.5 μM AlCl₄⁻ and 5 mM NaF, and 2.5 μM AlCl₄⁻ and 1 mM NaF were mixed and represented as 10 mM, 5 mM and 1 mM AlCl₄⁻, respectively. The noted
AlCl₄⁻ concentrations were added to culture which were made permeable with saponin as detailed above. It should be noted that the only other metallic ion that can substitute for Al³⁺ and yield similar potency is Be²⁺ (Sternweis & Gilman, 1982).

V. Nutrient Shifts

Harvested SG2 spores were washed and resuspended in germination buffer (pH 6.5) at a concentration of 2-3 x 10⁷/mL. Spores underwent autoactivation to yield nascent amoebae after 5 hours of incubation at room temperature. Alternatively, spores at the above concentration were heat shocked at 45°C for 30 minutes, then incubated at room temperature. Nascent amoebae were starved in phosphate buffer (pH 6.5) for 2 hours while shaking on a Gyratory Shaker (Model G2; New Brunswick Scientific Co.) at 180 rpm at room temperature. Cells were then centrifuged at top speed for 15 seconds in an IEC Clinical Centrifuge and resuspended in TM growth medium (containing: 10.0g trypticase peptone, 5.0g yeast extract, 10.0g glucose, 0.35g NaHPO₄, 1.2g K₂PO₄ in 1L of ddH₂O). Cells were collected every 5 minutes for 25 minutes and subsequently boiled in SDS sample buffer (described below). After 25 minutes, the remaining cells were washed free of growth medium, resuspended in phosphate buffer (pH 6.5), and sampled at 2 and 4 hours, lysed (in SDS), boiled and frozen at -20°C until further use.

VI. One Dimensional Electrophoresis

In preparation for electrophoretic protein separation, 2 mL aliquots of spores were removed at times indicated, washed free of germination buffer, and centrifuged at maximum setting in a IEC Clinical Centrifuge for 15-20 sec. Pellets were resuspended at least 1:1 in lysis buffer (containing: 1% TritonX-100, 1 µg/mL leupeptin, and 1 mM phenylmethylsulfonyl fluoride (PMSF)). To mechanically disrupt spore membranes, glass beads of 0.25-0.32 mm were added to the resuspended spores and the mixture was vortexed for approximately 10 minutes. The spore suspension was then centrifuged at
500g for 2 minutes to remove the glass beads. The supernatant was collected and thoroughly mixed with 5X sample buffer (containing: 250 mM Tris-HCl (pH 8.8), 50% glycerol, 10% sodium-dodecylsulfate (SDS), 25% beta-mercaptoethanol (BME) and 0.25% bromophenol blue). The diluted extracts were boiled for 4 minutes, clarified by centrifugation in a Fisher microcentrifuge (Model 235B, Allied Co.) at 12,500g for 10 minutes at 4°C, and the supernatants were stored at -20°C until needed.

Soluble protein was quantified according to the method of Bradford (1976) using the BioRad Protein Assay. Protein standards were made using bovine serum albumin (Sigma). Sample dilutions were prepared at 1:1000 in ddH₂O prior to the reaction with protein-dye binding solution (BioRad). Protein assay volume was reduced to a final volume of 1 mL in order for each sample to be measured spectrophotometrically at 595 nm in a standard microtube using a Beckman DU-65 spectrophotometer equipped with a batch sampler (Beckman Instruments).

In all cases, protein (10-20 µg/lane) was separated by electrophoresis in 12.5% sodium dodecylsulfate (SDS) polyacrylamide gels using the BioRad Mini Protein II Electrophoresis System by the method of Laemmli (1970). The 12.5% separating gel (containing: 3.1ml 40% bis-acrylamide (BioRad), 2.5ml 1.5M Tris-HCl (pH 8.8), 100µl 10% SDS, 4.25ml ddH₂O) was degassed, under house vacuum, for 10 minutes before adding the polymerization agents (5µl N,N,N',N'-tetramethylethylenediamine (TEMED) and 50µl 10% ammonium persulfate (APS)). The solution was poured into gel formers and overlayed with ddH₂O. After polymerization (~30-45 minutes), a 4% stacking gel was formed above the separating gel (containing: 1.3ml 40% bis-acrylamide (BioRad), 2.5ml 0.5M Tris-HCl (pH 6.8), 100µl 10% SDS, 6.1ml ddH₂O). This solution was degassed as per the separating gel but the polymerization agents contained 10µl TEMED and 50µl 10% APS. A 10 well slot former was inserted into the stacking gel and allowed to polymerize for approximately 45-60 minutes. The electrophoretic chamber was immersed in running buffer (pH 8.3) (containing: 9.0g Tris base, 43.2g glycine, 3.0g SDS in 600ml
ddH$_2$O) The voltage was initially set at 80-90V until the bromophenol marker dye had travelled through the stacking gel; a constant voltage of 135V was then applied until the marker dye had reached the bottom of the gel. Prestained rainbow molecular weight markers (BioRad) were run in parallel lanes in order to determine the relative molecular weights of the proteins of interest.

VII. Western Blotting

Protein profiles were transferred from 12.5% polyacrylamide gels to nitrocellulose membranes in a BioRad mini-trans-blot cell using ice cold blotting buffer (pH 8.3) (containing: 25 mM Tris base, 193 mM glycine and 20% methanol). Transfer was made possible by applying a constant voltage of 100V for 60 minutes employing a frozen cooling unit.

Nitrocellulose blots were blocked with 4% BSA in 20 mM Tris base, 150 mM NaCl, 0.05% Tween 20 (Sigma) for 30 minutes at 37°C. When probing for phosphotyrosine containing proteins, the blocked membranes were incubated with recombinant anti-phosphotyrosine antibodies (RC20-E120AP; Transduction Laboratories) at 1:2500 dilution for 30 minutes at 37°C (the RC20 antibodies are offered preconjugated with the secondary antibody, alkaline phosphatase). When probing for actin, the blocked membranes were incubated with monoclonal anti-actin (amoeba) antibodies (Sigma) at 1:75 dilution for 1 hour at 22°C. The secondary antibody, alkaline phosphatase conjugated anti-mouse IgG produced in goat (Sigma), at 1:15,000 was used to detect the anti-actin primary antibody. Between each incubation, blots were washed 3 times for 5 minutes in blocking buffer. Visualization of both phosphotyrosine containing proteins and actin was with the chromogenic substrates for alkaline phosphatase (50mM MgCl2, 1.65mg/mL 5-bromo-3-chloro-indolyphosphate, 12.7mg/mL nitro blue tetrazolium (GibcoBRL) in 0.1 M Tris-HCl (pH 9.5) and 0.1 M NaCl).
RESULTS

I. The Presence of Phosphotyrosine Containing Proteins during Germination of
*Dictyostelium discoideum* Spores.

i) Autoactivated Spores

Recently, phosphotyrosine signalling has been shown to be important in the regulation of *Dictyostelium discoideum* multicellular development (Howard et al., 1994; Howard et al., 1992; Schweiger et al., 1990). It was therefore of interest to investigate the pattern of phosphotyrosine containing proteins during the germination program of this organism. The premature maturation mutants, SG1 and SG2 of *Dictyostelium discoideum*, were used primarily to elucidate proteins containing phosphotyrosine residues. The germination requirements of these two strains have been well characterized (Cotter & Dahlberg, 1977; Dahlberg & Cotter, 1978; Cotter & Glaves, 1989), particularly, their autoactivation competency which bypasses the prolonged aging and maturation time required by wild-type strains.

These mutants, SG2 and SG1, were incubated under optimal conditions (see material and methods) and allowed to autoactivate. Germination kinetics were scored as quiescent, swollen or emerged, every hour for 5 hours using phase-contrast microscopy (Fig. 3A and 4A, respectively). Both strains demonstrated the same pattern of tyrosine phosphorylated proteins. The RC20-E120AP antibody recognized at least 10 bands, all with relative mobilities between 207kDa and 32kDa. The most noticeable and consistent pattern of bands migrated between 93kDa and 32kDa, as determined by molecular weight standards run in parallel lanes. Figure 3B and 4B illustrate an ~93kDa protein present throughout germination of these strains, possibly the same protein previously observed in vegetative cells which becomes more prominent at the tipped aggregate stage (12 hours in multicellular development) (see Appendix 1; Howard et al., 1992). Both 72kDa and 55kDa proteins were faintly phosphorylated, whereas the 65kDa was strongly labelled thoughout
Figure 3. Phosphotyrosine containing proteins during autoactivation of premature maturation mutant, SG2, spores. Spores of the mutant strain SG2, were incubated under optimal conditions and allowed to autoactivate. Germination kinetics are shown in panel A, where (●) is the % of swollen spores in the population and (▲) is the % of emerged nascent amoebae. At time 0 and each hour succeeding the initiation of germination, 2 mL aliquots were collected, ground and 25µg/µl of protein was loaded in each lane and separated by 12.5 % SDS-PAGE. Panel B illustrates Western blot analysis using anti-P-Tyr antibodies revealing those proteins containing phosphotyrosyl residues. Molecular weight standards are in Daltons X 1000.
Figure 4. Phosphotyrosine containing proteins during autoactivation of premature maturation mutant, SG1, spores. Spores of the mutant strain SG1, were incubated under optimal conditions and allowed to autoactivate. Germination kinetics are shown in panel A, where (●) is the % of swollen spores in the population and (▲) is the % of emerged nascent amoebae. At time 0 and each hour succeeding the initiation of germination, 2 mL aliquots were collected, ground and 20μg/μl of protein was loaded in each lane and separated by 12.5 % SDS-PAGE. Panel B illustrates Western blot analysis using anti-P-Tyr antibodies revealing those proteins containing phosphotyrosyl residues. Molecular weight standards are in Daltons X 1000.
germination. From Fig. 3B and 4B, a 43kDa species is very strongly labelled during quiescence, it thereafter undergoes substantial dephosphorylation. A doublet of ~39kDa and a 32kDa species were observed only when spores were dormant, while a 36kDa species increases in intensity during swelling (2-3 hours).

In order for wild-type spores to autonomously synthesize the autoactivator or respond to exogenous autoactivator (Dahlberg & Cotter, 1978), they must age in the sorocarp for 10-14 days, presumably, due to the existence of late spore maturation genes (Cotter & Glaves, 1989). The pattern of phosphotyrosine containing proteins during the germination program of the wild-type strains, V12 (Fig. 5B) and NC4 (Fig. 6B), were similar to that of the mutant daughter strains SG2 and SG1, respectively. In contrast to aged wild-type strains, young wild-type spores (1-3 days old) do not respond to or produce the autoactivator (Cotter & Dahlberg, 1977). The protein phosphorylation on tyrosine residues of these young spores was slightly different than that of the aged wild-type spores (see Fig 5B and Fig. 6B, lane A), in that the 55kDa doublet is barely visible and the labelling of the 43kDa species is of low intensity compared to that of the aged quiescent spores. Overall, autoactivation-competent spores (SG2, SG1, aged V12 and NC4) house the same pattern of phosphotyrosine containing proteins during germination. The process of wild-type aging, to acquire autoactivation-competency, appears to require tyrosine phosphorylation of the 55kDa and 43kDa species.

ii) Heat Activation

Supraoptimal heating (45°C for 30 minutes) initiates germination, independent of density and age, by yet an unknown pathway. In order to determine if the pattern of phosphotyrosine containing proteins is inherent to the germination program and not specific to autoactivation, heat activated spores were probed with P-Tyr antibodies. The heat activated mutant strain, SG2 (Fig. 7B), and aged wild-type strains, V12 (Fig. 8B) and NC4 (Fig.9B), revealed similar phosphotyrosine labelling patterns to that of autoactivated
Figure 5. Phospho-tyrosine containing proteins during autoactivation of aged wild-type strain, V12, spores. Spores of the wild-type strain V12, were incubated under optimal conditions and allowed to autoactivate. Germination kinetics are shown in panel A, where (●) is the % of swollen spores in the population and (▲) is the % of emerged nascent amoebae. At time 0 and each hour succeeding the initiation of germination, 2 mL aliquots were collected and ground, while lane A represents young immature V12 spores. 25μg/μl of protein was loaded in each lane and separated by 12.5% SDS-PAGE. Panel B illustrates Western blot analysis using anti-P-Tyr antibodies revealing those proteins containing phospho-tyrosyl residues. Molecular weight standards are in Daltons X 1000.
Figure 6. Phosphotyrosine containing proteins during autoactivation of aged wild-type strain, NC4, spores. Spores of the wild-type strain NC4, were incubated under optimal conditions and allowed to autoactivate. Germination kinetics are shown in panel A, where (●) is the % of swollen spores in the population and (▲) is the % of emerged nascent amoebae. At time 0 and each hour succeeding the initiation of germination, 2 mL aliquots were collected and ground, while lane A represents young immature V12 spores. 25μg/μl of protein was loaded in each lane and separated by 12.5 % SDS-PAGE. Panel B illustrates Western blot analysis using anti-P-Tyr antibodies revealing those proteins containing phosphotyrosyl residues. Molecular weight standards are in Daltons X 1000.
A

% Emergence

% Swollen

0 1 2 3 4 5

0 20 40 60 80 100

0 20 40 60 80 100

B

A 0 1 2 3 4 5

207

139

84

41.7

32
Figure 7. Phosphotyrosine containing protein during heat activation of mutant strain, SG2, spores. Spores of the mutant strain SG2, were heat activated and allowed to germinate. Germination kinetics are shown in panel A, where (●) is the % of swollen spores in the population and (▲) is the % of emerged nascent amoebae. At time 0 (lane A), after 30 min heat shock (lane B) and each hour succeeding the initiation of germination, 2 mL aliquots were collected and ground. 25μg/μl of protein was loaded in each lane and separated by 12.5 % SDS-PAGE. Panel B illustrates Western blot analysis using anti-P-Tyr antibodies revealing those proteins containing phosphotyrosyl residues. Molecular weight standards are in Daltons x 1000.
Figure 8. Phosphotyrosine containing proteins during heat activation of aged wild-type strain, V12, spores. Spores of the wild-type strain V12, were heat activated and allowed to germinate. Germination kinetics are shown in panel A, where (●) is the % of swollen spores in the population and (▲) is the % of emerged nascent amoebae. At time 0 (lane A), after 30 min heat shock (lane B) and each hour succeeding the initiation of germination, 2 mL aliquots were collected and ground. 25μg/μl of protein was loaded in each lane and separated by 12.5% SDS-PAGE. Panel B illustrates Western blot analysis using anti-P-Tyr antibodies revealing those proteins containing phosphotyrosyl residues. Molecular weight standards are in Daltons X 1000.
Figure 9. Phosphotyrosine containing proteins during heat activation of aged wild-type strain, NC4, spores. Spores of the wild-type strain NC4, were heat activated and allowed to germinate. Germination kinetics are shown in panel A, where (●) is the % of swollen spores in the population and (▲) is the % of emerged nascent amoebae. At time 0 (lane A), after 30 min heat shock (lane B) and each hour succeeding the initiation of germination, 2 mL aliquots were collected and ground. 25μg/μl of protein was loaded in each lane and separated by 12.5 % SDS-PAGE. Panel B illustrates Western blot analysis using anti-P-Tyr antibodies revealing those proteins containing phosphotyrosyl residues. Molecular weight standards are in Daltons X 1000.
spores, respectively. However, it appears that the 30kDa species in the wild-type strains (V12 and NC4) is more intensely phosphorylated upon heat shock and thereafter undergoes dephosphorylation. The most intensely labelled protein, the 43kDa species, follows the same pattern of dephosphorylation as the spores progress through germination, independent of the mode of activation.

Although young (1-3 days) wild-type spores do not autoactivate, they will initiate germination via external activating treatments such as heat or DMSO shock (Cotter & Dahlberg, 1977; Dahlberg & Cotter, 1978). Therefore, young V12 (Fig. 10B) and NC4 (Fig. 11B) spores were heat shocked and probed for phosphotyrosine containing proteins. The phosphotyrosine patterns during heat activation of these young wild-type spores were similar to that their aged counterparts.

It can therefore be concluded that the changes in phosphorylation on tyrosine residues of the proteins observed during both autoactivation and heat activation are inherent to spore germination in this organism.

II. The Effects of Tyrosine Kinase Inhibitors on Germination Kinetics of *Dictyostelium discoideum*

In order to understand the physiological significance of tyrosine phosphorylation, the effect of two known inhibitors of tyrosine kinase activity (herbimycin A and tyrphostin A46) were examined.

i) Herbimycin A

Herbimycin A is a benzoquinonoid ansamycin antibiotic that has been found to reverse oncogenic transformation impelled by pp60v-src and inhibits tyrosine phosphorylation induced by the src kinase (Uehara *et al.*, 1989). More recently, this agent has been shown to inhibit T-cell protein-tyrosine kinases, lck and fyn, and to impair T-cell
Figure 10. Phosphotyrosine containing proteins during heat activation of young wild-type strain, V12, spores. Young spores (1-2 days old) of the wild-type strain V12, were heat activated and allowed to germinate. Germination kinetics are shown in panel A, where (●) is the % of swollen spores in the population and (▲) is the % of emerged nascent amoebae. At time 0 (lane A), after 30 min heat shock (lane B) and each hour succeeding the initiation of germination, 2 mL aliquots were collected and ground. 25μg/μl of protein was loaded in each lane and separated by 12.5 % SDS-PAGE. Panel B illustrates Western blot analysis using anti-P-Tyr antibodies revealing those proteins containing phosphotyrosyl residues. Molecular weight standards are in Daltons X 1000.
Figure 11. Phosphotyrosine containing proteins during heat activation of young wild-type strain, NC4, spores. Young spores (1-2 days old) of the wild-type strain NC4, were heat activated and allowed to germinate. Germination kinetics are shown in panel A, where (●) is the % of swollen spores in the population and (▲) is the % of emerged nascent amoebae. At time 0 (lane A), after 30 min heat shock (lane B) and each hour succeeding the initiation of germination, 2 mL aliquots were collected and ground. 25µg/µl of protein was loaded in each lane and separated by 12.5 % SDS-PAGE. Panel B illustrates Western blot analysis using anti-P-Tyr antibodies revealing those proteins containing phosphotyrosyl residues. Molecular weight standards are in Daltons X 1000.
receptor-mediated activation, tyrosine substrate phosphorylation, and PLC activity (June et al., 1990) In Dictyostelium, autoactivation ensues via Ins(1,4,5)P₃-dependent Ca²⁺ release (Lydan & Cotter, 1995), hence PLC plays a central role in this signalling cascade.

Herbimycin A was added at 10µM and 20µM concentrations (Gauthier et al., 1995) to saponin permeabilized spores prior to the onset of germination. The spores were then scored as dormant, swollen or emerged myxamoebae. Treatment with this agent at 10µM and 20µM did not affect the time of maximum spore swelling (Fig. 12A) or the rate of amoebal emergence (Fig. 12B) compared to untreated controls. The overall post-activation lag was prolonged due to the presence of the solubilizing agent. DMSO at 2%, in both the treated and control spores. Only concentrations above 5% have been shown to inhibit mitochondrial function and concurrently obstructed spore swelling until the spores are freed of this agent (Cotter et al., 1976).

The results shown in Fig 12A and 12B can be explained two-fold; first, the src family of PTKs are not present in Dictyostelium or this family of PTK is not involved in the intracellular signalling mechanisms required for germination. The former is highly unlikely since the src kinase family is expressed in almost all cell types (Jove & Hanafusa, 1987), where as the latter is possible. However more experimentation is needed to elucidate the function of this family of PTKs in Dictyostelium. Second, the experimental design did not include prolonged (24 hrs) pretreatment with herbimycin A as was shown to be necessary to inhibit early substrate tyrosine phosphorylation induced by T-cell receptor activation (June et al., 1990).

**ii) Tyrphostin**

The tyrphostins are a series of low molecular weight compounds designed to bind to the protein-tyrosine kinase substrate domain of membrane receptors, hence inhibiting their kinase activity (Calbiochem). These compounds resemble tyrosine and erbstatin moieties and are slightly hydrophobic which allow them to traverse the plasma
Figure 12. Effects of the PTK inhibitor herbimycin A on SG2 autoactivated spore germination. Dormant SG2 spores were incubated with 2% DMSO as the control (●), 10μM (■) and 20μM (▲) herbimycin A at time 0 and allowed to progress through autoactivation. Spore germination kinetics are shown in panels A (% swollen spores) and panel B (% emerged nascent amoebae).
membrane. Recently, tyrphostins have been shown to block EGF-receptor autophosphorylation and subsequent endogenous substrate phosphorylation (Lyall \textit{et al.}, 1989).

Tyrphostin at concentrations of 20\,\mu M, 40\,\mu M, 80\,\mu M and 100\,\mu M was added to saponin permeabilized spores prior to the onset of germination. There were no significant changes in maximum spore swelling (Fig. 13A) or the rate of amoebal emergence (Fig 13B) between untreated control spores and those treated with 20\,\mu M, 40\,\mu M and 80\,\mu M tyrphostin. Those spores treated with 100\,\mu M tyrphostin yield a significantly higher percentage of swollen spores and emerged amoebae than control spores. However, western blotting revealed no significant changes in the pattern of phosphotyrosine labelling when spores were treated with 100\,\mu M tyrphostin (Fig. 14A) compared to untreated control spores (Fig. 14B). The observed decrease in the overall label intensity of all proteins from panel B (Fig. 14) was due to reduced incubation time with the chromogenic substrates.

Since it has been shown that the endogenous autoinhibitor is present in the spore matrix, it is hypothesized here, that the autoinhibitor may be exerting its signalling mechanism (keeping the spore dormant) via a receptor tyrosine kinase, since quiescent spores harbour highly tyrosine phosphorylated proteins. However, tyrphostin at 100\,\mu M and 200\,\mu M, did not reverse the effects of exogenously added autoinhibitor (Fig. 15), suggesting the autoinhibitor may exert its function other than via a RTK. Alternatively, it is possible that prolonged pretreatment (16-20 hours) of the spores with this agent is necessary to inhibit endogenous substrate phosphorylation and subsequent cellular trafficking as has been shown in NIH3T3 cells (Lyall \textit{et al.}, 1989). Further experimentation under these latter conditions is necessary to elucidate the possible link between the autoinhibitor and RTK activation.
Figure 13. Effects of the RTK inhibitor tyrphostin A46 on SG2 autoactivated spore germination. Dormant SG2 spores were incubated with 3.0% DMSO as the control (▲), 20μM (■), 40μM (○), 80μM (●), 100μM (●) tyrphostin A46 and allowed to progress through autoactivation. Panel A illustrates the % of swollen spores, while panel B represents the % of emerged amoebae in the population.
Figure 14. Effects of the RTK inhibitor tyrphostin A46 on phosphotyrosine containing proteins during autoactivation of SG2 spores. Spores of the mutant strain SG2, were incubated under optimal conditions and allowed to autoactivate. At time 0 and each hour succeeding the initiation of germination 2mL aliquots were collected and ground. 25µg/µl of protein was loaded in each lane and separated by 12.5% SDS-PAGE. Subsequent Western blot analysis using anti-P-Tyr antibodies revealed those proteins containing phosphotyrosyl residues. Panel A represents control germination pattern, while panel B represents spores treated with 100µM tyrphostin at time 0. Molecular weight standards are in Daltons X 1000.
Figure 15. Effects of the RTK inhibitor tyrphostin A46 on SG2 spores preincubated with autoinhibitor. SG2 spores were incubated with a crude autoinhibitor preparation prior to the addition of 0.4% DMSO as the control (○), 100μM (□) and 200μM (△) tyrphostin. Symbols ● & ▲ represent the % of swollen and emerged spores, respectively, not incubated with the autoinhibitor, nor the agent.
III. The Effects of Aluminum Fluoride on Germination in *Dictyostelium*

Aluminum fluoride (AlF₄⁻) mimics the γ-phosphate of GTP, thereby activating G-proteins independent of receptor activation (Sternweis & Gilman, 1982; Bomsel & Mostov, 1992). Furthermore, Kahn (1991) demonstrated that aluminum fluoride is not an activator of the monomeric (smaller) GTPases, hence the effect of this agent is targeted toward larger Go-proteins. Browning *et al.* (1995) have shown that concentrations greater than 10mM AlF₄⁻ are toxic to *D.discoideum* amoebae, therefore only 1mM, 5mM and 10mM concentrations were used to determine the ability of this agent to reduced or accelerate the rate of germination in autoactivated spores. When added at time 0, aluminum fluoride dose dependently prolonged the time of maximum spore swelling and reduced amoebal emergence. At 5mM—10mM, AlF₄⁻ prolonged the peak time of spore swelling by 1 hour, reduced the total number of swollen spores by 5% and 25% (Fig. 16A), and inhibited 17% and 45% of the spores to release nascent amoebae, respectively (Fig. 16B). Spores treated with 1mM AlF₄⁻ were unaffected (Fig. 16A & 16B). When 10mM AlF₄⁻ was added at 90 minutes, spores displayed similar kinetics to those treated at time 0, where the peak time of spore swelling was delayed by 1 hour, the maximum number of swollen spores was reduced by 35% (Fig. 17A) and the release of nascent amoebae was inhibited by 65% (Fig. 17B).

IV. The Role of Actin Tyrosine Phosphorylation

i) Nutrient Shifts

³¹P-NMR analysis has shown dormant spores to be nutrient-rich, while nascent amoebae harbour much lower endogenous reserves, thus relying on the surrounding medium for growth (Cotter *et al.*, 1992; Klein *et al.*, 1990). Since there appears to be a dramatic change in the phosphotyrosine labelling of the p43kDa species from a nutrient-
Figure 16. Effects of the signal transduction G-protein activator, aluminum fluoride, on autoactivated SG2 spore germination. Spore germination kinetics were scored when SG2 spores were incubated with 1mM (▲), 5mM (■) and 10mM (●) aluminum fluoride, while control spores are represented by (●). Panel A illustrates the % of swollen spores in the population and panel B, the % of emerged amoebae.
Figure 17. The effects of aluminum fluoride added 90 minutes after the onset of SG2 spore germination. SG2 spores were incubated under optimal conditions to allow for autoactivation. At 90 minutes, 10mM aluminum fluoride (■) was added, while control spores are represented by (●). Spore germination kinetics were scored as the % of swollen spores (panel A) in the population and % of emerged amoebae (panel B).
rich environment (dormancy) to a starvation environment (emergence), nutrient manipulations were conducted to further observe these changes. Spontaneously germinated SG2 amoebae were starved for two hours in non-nutrient buffer to initiate early developmental gene expression and then transferred to standard growth medium (TM). At 5 minute intervals for 25 minutes, cells were collected and analyzed by protein immunoblotting with anti-P-Tyr antibodies. Phosphorylation of the 43kDa protein increased substantially when transferred to growth medium from non-nutrient medium (Fig. 18A). The intensity of p43 phosphorylation was detected within 5 minutes and remained strongly labelled while amoebae were in growth medium. The signal was faintly detectable when cells were washed and returned to non-nutrient buffer for 2h and 4h (Fig. 18A). The pattern of p43 tyrosine phosphorylation was similar to that of amoebae generated from heat activation (Fig. 18B). It was necessary to examine the response of heat activated amoebae to nutrient shifts since it has been shown they exhibit a longer lag period before acquiring aggregation-competency (Cotter et al., 1992; Cotter et al., 1990).

The p43 protein which undergoes dynamic changes in tyrosine phosphorylation in response to nutrition was found to be actin (Fig. 18C). When the above samples were probed with anti-actin, the observed signal was much stronger in cells that were starved than those in nutrient medium. There appears to be a direct inverse relationship between anti-P-Tyr and anti-actin labelling patterns (Fig. 18 A, B & C).

ii) Germination via Autoactivation

Similar to previous experiments which used RC20-E120AP serum as a probe (Fig. 3 & 19A), cell extracts from autoactivated spores were analyzed by western blotting using anti-actin serum (Fig. 19B). The band previously identified as p43, which underwent gradual tyrosine dephosphorylation throughout germination, exhibited the reverse pattern when probed with anti-actin. As seen in Figures 18, anti-actin antibodies did not recognize
Figure 18. Changes in protein tyrosine phosphorylation and actin in response to nutrient shifts of SG2 amoebae. SG2 nascent amoebae generated from autoactivation (panel A) and heat activation (panel B) were starved in non-nutrient medium for 2 hours (designated time 0) and transferred to standard growth medium. At 5 minute interval for 25 minutes in growth medium, cells were collected and lysed. After 25 minutes, the remaining cells were washed and transferred back to non-nutrient medium and sampled at 2 and 4 hours. 15μg/μl protein was loaded in each lane and separated by 12.5% SDS-PAGE. Subsequent Western blot analysis using anti-P-Tyr antibodies revealed those proteins containing phosphotyrosyl residues. Panel C represents the same samples used in panel B but probed using commercially available monoclonal anti-actin antibodies.
Figure 19. Changes in protein tyrosine phosphorylation and actin during autoactivated SG2 spore germination. Spores of the mutant strain SG2 were incubated under optimal conditions and allowed to autoactivate. At time 0 and every hour thereafter, 2mL aliquots were collected and ground. 25μg/μl of protein was loaded in each lane and separated by 12.5% SDS-PAGE. Panel A illustrates Western blot analysis using anti-P-Tyr antibodies, while panel B represents the same samples used in panel A but probed using commercially available monoclonal anti-actin antibodies.
the intensely labelled tyrosine phosphorylation of p43 of dormant spores but began to faintly recognize the p43 protein during maximum swelling (3 hours), thereafter the signal gradually increased upon emergence. These results again suggest a reverse signal intensity correlation between tyrosine phosphorylation and actin, where intense actin labelling corresponds to weak tyrosine phosphorylation.

**iii) Autoactivator and Autoinhibitor**

In order to gain some insight into the relationship between actin tyrosine phosphorylation with autoinhibition, autoactivation and protein synthesis, SG2 spores were incubated under various conditions and probed with anti-P-Tyr antibodies. Protein synthesis has been shown to be required for both swelling and amoebal emergence in autoactivated spores (Dalhberg & Cotter, 1978). When spores were treated with cycloheximide at 200µg/mL and probed with anti-P-Tyr antibodies at 5h (Fig. 20B, Lane 3), the actin P-Tyr signal was intermediate when compared to that of dormant spores (Fig. 20B, Lane 1) and emerged amoebae (Fig. 20B, Lane 2). Since actin tyrosine dephosphorylation continues in the presence of cycloheximide and cycloheximide has been shown to inhibit the production and secretion of the autoactivator (Dalhberg & Cotter, 1978), it can be concluded that the autoactivator may not directly stimulate the dephosphorylation of actin. This however, does not rule out an indirect role between the autoactivator and protein tyrosine phosphatases. Purified autoactivator would elucidate these unknowns. When SG2 spores were incubated with a crude autoinhibitor preparation, obtained from freshly harvested mutant spores, autoactivation is completely inhibited (Fig. 20A). After a 5h incubation period with the autoinhibitor preparation, actin tyrosine phosphorylation was substantially greater (Fig. 20B, Lane 4) in comparison to that of dormant spores (Fig. 20B, Lane 1). It appears that incubation with the autoinhibitor preparation may exhibit its function (keeping the spore in a dormant state) via phosphorylation of actin on its tyrosine residues.
Figure 20. Changes in actin phosphotyrosine in response to protein synthesis inhibition and the autoinhibitor. SG2 spores were incubated under various conditions and germination kinetics were scored as previously described. At the times indicated, 2mL aliquots were collected and ground. 25µg/µl of protein was loaded in each lane and separated by 12.5% SDS-PAGE. Subsequent Western blot analysis revealed those proteins containing phosphotyrosyl residues. Panel A illustrates the % cf of emerged nascent amoebae from control spores (○), spores incubated with 200µg/ml cycloheximide (△), spores incubated with a crude autoinhibitor preparation (□). Panel B illustrates the phosphotyrosine containing proteins of control dormant spores (lane 1), control emerged amoebae (lane 2), spores incubated with 200µg/ml cycloheximide for 5h (lane 3) and spores incubated with a crude autoinhibitor preparation for 5h (lane 4).
It has been previously shown that the autoactivator overrides all inhibitory effects of the autoinhibitor (Cotter & Glaves, 1989). This was once again demonstrated (Fig 21A), where spores incubated with the autoinhibitor for 2h quickly began the initiation of gemination when a 1:1 dilution of autoactivator was added. As demonstrated in Fig. 20B, the presence of the autoinhibitor induces further tyrosine phosphorylation of actin, however, when the autoactivator is added, actin undergoes rapid tyrosine dephosphorylation until it is faintly observable upon amoebal emergence (Fig. 21B). These results strongly suggest a link between extracellular matrix material (surrounding spores in the sorocarp) and actin tyrosine phosphorylation, while the autoactivator promotes tyrosine dephosphorylation of actin.
Figure 21. Changes in actin tyrosine phosphorylation in response to the autoinhibitor and autoactivator. SG2 spores were incubated with a crude autoinhibitor preparation for 2h, at which time half of the population underwent a 1:1 dilution with an autoactivator preparation, while the other half remained in the presence of the autoinhibitor. Panel A represents spore germination kinetics where (●) and (▲) represent the % of swollen and % emerged amoebae in the population following the addition of the autoactivator, while (■) represents prolonged incubation with the autoinhibitor. At the times indicated, 2mL aliquots were collected, ground and 25μg/μl of protein was loaded in each lane and separated by 12.5% SDS-PAGE. Subsequent Western blot analysis revealed those proteins containing phosphotyrosyl residues. Panel B shows the phosphotyrosine containing proteins of samples collected every hour from spores incubated with the autoinhibitor then the autoactivator.
DISCUSSION

The data presented here is novel information elucidating the pattern of tyrosine phosphorylation during germination. One protein in particular was the central locus due to its dramatic pTyr changes in response to nutrient shifts, the autoinhibitor and the autoactivator. This protein was identified to be actin, and appears to play a cardinal role during the spore germination program in *Dictyostelium discoideum*.

I. Phosphotyrosine-containing proteins during the germination program of *D. discoideum*.

Recently, several proteins have been reported to be phosphorylated on tyrosine residues during the multicellular development of this organism (Howard et al., 1992; Schweiger et al., 1990). Additionally, two protein-tyrosine kinase genes, DPYK1 and DPYK2, have been identified and found to be developmentally regulated (Tan & Spudich, 1990). It was therefore anticipated that some or all of the tyrosine phosphorylated proteins would be present during spore germination of *Dictyostelium*. Western blotting using recombinant anti-phosphotyrosine antibodies revealed at least 10 proteins with relative mobilities between 207kDa and 32kDa. The most consistent pattern of bands migrated between 93kDa and 39kDa, as determined by molecular weight standards run in parallel lanes. The most pronounced phosphotyrosine labelled proteins, 65kDa, 43kDa, 39kDa, 36kDa and 32kDa, were observed in dormant spores. The 65kDa protein was heavily phosphorylated throughout the germination program. While the 43kDa species was intensely labelled during quiescence and was gradually dephosphorylated upon amoebal emergence. The 39kDa and 32kDa proteins were only observed during dormancy, and presumably rapidly dephosphorylated upon spore activation. Whereas, the 36kDa protein was found to increase in labeling intensity during swelling and emergence. These patterns were observed in all strains examined, V12, NC4, SG2 and SG1, independent of activation method or age. These results therefore, suggest that phosphorylation and
dephosphorylation on tyrosine residues of the observed proteins is inherent to spore germination. There were however, inconsistencies with respect to total labelling intensity of the blots presented and the appearance of higher molecular weight proteins. This can only be explained by the differential times the blots were exposed to the chromogenic substrates and the transfer ability (from gel to membrane) of some high molecular weight proteins, respectively. These obstacles can be overcome by immunoprecipitating pTyr-containing proteins with the antibody and subsequent incubation with [$\gamma$-32P]ATP.

II. The physiological significance of these pTyr-containing proteins during germination

The availability of potent pharmacological agents allows for physiological manipulations of signal transduction processes. Two known inhibitors of tyrosine kinase activity (herbimycin A and tyrphostin A46) were used to examine their effects on spore germination kinetics. With the methods used here, herbimycin A did not affect the kinetics of autoactivation. It is possible that PTKs do not play a role in the germination program, since dephosphorylation of pTyr-containing proteins is the main observable event.

Incubation with 100μM tyrphostin A46 appeared to increase the percentage of spore swelling and amoebal emergence. However, other investigators have reported that prolonged pretreatment (16-24h) with this agent, as well as herbimycin A, is necessary to inhibit endogenous substrate phosphorylation. Therefore, these experiments must be conducted before any speculation can be made with respect to tyrosine kinase activity during autoactivation. Furthermore, monitoring germination kinetics with the addition of protein tyrosine phosphatase inhibitors, such as phenylarsine oxide (PAO) or sodium orthovanadate could provide fundamental insights to the importance of tyrosine dephosphorylation of the proteins observed here. Subsequent western blot analysis of the latter treatments using P-Tyr antibodies may reveal additional pTyr-containing proteins, since pTyr events are under the stringent regulation of phosphatases. It should be noted, that sodium orthovanadate does inhibit germination of autoactivated spores, but has no
effect on heat activation (Cotter et al., 1990). However, these treated spores have not yet been analyzed by western blot analysis.

III. The role of signal transducing G-proteins

Aluminum fluoride mimics the γ-phosphate of GTP, thereby activating G-proteins independent of receptor activation (Sternweis & Gilman, 1982; Bomsel & Mostov, 1992). This agent has been shown to dose-dependently inhibit chemotaxis toward cAMP and folate in Dictyostelium amoebae (Browning, 1995). Additionally, AlF$_4^-$ along with GTPγS has demonstrated an important role for heterotrimeric G-proteins in phagocytosis and cell fusion during the sexual life-cycle of this organism. The work here demonstrates aluminum fluoride dose-dependently inhibited germination of autoactivated spores. At the outset these results seemed to support the work of Virdy et al. (1995), whereby activation of adenylyl cyclase and subsequent cAMP accumulation keeps the spore in a dormant state. However, it has been revealed that spores harbour an adenylyl cyclase protein (ACG) specifically active during germination (Pitt et al., 1992). It was shown that ACG has a single transmembrane span which serves to connect an extracellular domain (for ligand-binding) to an intracellular catalytic domain (for enzyme activity). ACG was also shown to be insensitive to guanine nucleotides which distinguishes it from ACA (adenylyl cyclase for aggregation) since the latter is regulated by a G protein-linked receptor, cAR1. The results presented here are therefore not straight forward. However, if ACA was still present in the dormant spore, even though it is not transcribed, AlF$_4^-$ could activate the guanine nucleotide-binding regulatory component of the enzyme. Subsequent c$^+$ accumulation could than mimic the osmotic response elicited by ACG, thus rendering the spore dormant.
IV. Identification of a p43 species which undergoes phosphotyrosine changes

Reversible and highly regulated tyrosine phosphorylation of the p43 species was identified to be actin. Several studies have reported actin to be phosphorylated on both serine/threonine and tyrosine residues. A cell membrane kinase from human placenta has been shown to phosphorylate actin on both serine and tyrosine residues in response to insulin receptor engagement (Machicao & Wieland, 1985; Machicao et al., 1983). Additionally, F-actin polymerization has been reported to be governed by phosphorylation in B lymphocytes and in Amoeba proteus (Melamed et al., 1991; Sonobe et al., 1986).

The data presented here demonstrated in vivo tyrosine phosphorylation of actin in response to standard growth medium and material present in the sorocarp extracellular matrix (possibly the autoinhibitor). Elegant work by Schweiger et al. (1992) and Howard et al. (1993) have demonstrated tyrosine phosphorylation of Dictyostelium actin to be associated with cell-shape changes. Whereby inhibiting protein tyrosine phosphatases (with phenylarsine oxide) or by transferring starved cells to a nutrient medium caused cells to firstly lose their tipped pseudopods and later become rounded and lose mobility. These effects could be reversed by incubating cells with 2,3-dimercaptopropanol or transferring cells back into a starvation medium. Intracellular particle movement could still be observed during these cell shape alterations, therefore oxygen depletion or inhibition of ATP synthesis was ruled out. The data presented here support the finding of rapid actin tyrosine phosphorylation in response to nutrient shifts. A novel observation was the direct inverse labelling relationship, where intense actin labelling corresponded to weak tyrosine phosphorylation. This relationship suggests that phosphotyrosine residues may lie within the monoclonal anti-actin recognition site. However, the molecular significance of actin tyrosine phosphorylation still remains unknown. The above inverse labelling correlation was also observed during germination of autoactivated spores. Incubation with the autoinhibitor not only inhibits spore swelling and emergence, but results in greater actin tyrosine phosphorylation than freshly harvested dormant spores. Upon the addition of the
autoactivator, actin tyrosine dephosphorylation begins and spores quickly initiate germination. It appears that the autoinhibitor directly induces tyrosine phosphorylation of actin, while the autoactivator indirectly promotes its dephosphorylation. This latter observation comes from experiments where dephosphorylation still occurs in the presence of a protein synthesis inhibitor, whereby spore swelling does not take place.

From these data it can be hypothesized that dormant spores are nutrient-rich (as demonstrated by NMR analysis, Klein et al., 1990) and encapsulated in an environment which promotes tyrosine phosphorylation of actin, thus keeping spore actin in bundles (Sameshima et al., 1994) resulting in the round shape of the spore. Upon activation (by the autoactivator), metabolism ensues rendering the spore limited in its nutrient reserves. Subsequent dephosphorylation of actin could be linked to actin depolymerization, followed by intracellular filamentous/stress fiber formation resulting in the extension of pseudopods (hence spore coat swelling). Interestingly, Sameshima et al. (1994) and Kishi et al. (1994) have shown that dormant spores harbour thick elongated nuclear rods which render the nucleus lemon-like in shape. Immunelectron micrographs using anti-actin antibodies also revealed these structures in the cytoplasm. During swelling, these groups observed protoplasmic streaming and subsequent disappearance of intranuclear and cytoplasmic actin rods. These authors further report that the actin rods found in mature dormant spores morphologically differ from those found from spores exposed to stress conditions such as DMSO. This could explain why the signalling pathway necessary to induce autoactivation is not present during exogenous activation (heat or DMSO). This information together with the data presented here, strongly suggest an important role for actin reorganization during germination and those events to be governed by extracellular signalling and exogenous stress conditions.
V. A model for the regulation of germination by the autoinhibitor and autoactivator

The information presented in this study provide the foundation of a model which links the signal transduction processes observed in other work. This type of model is only speculative and should put into context the information to date and provide possible avenues for future experimentation.

The viscous matrix surrounding each spore within the sorocarp keeps the spores in a dormant state. High osmotic pressure, inherent to dormancy within the sorocarp, leads to adenylyl cyclase activation and subsequent cAMP accumulation (Virdy et al., 1995, Pitt et al., 1992; Schaap & Cotter, unpublished). In turn, cAMP stimulates protein kinase A activity, whereby this latter enzyme phosphorylates (activates) dormancy-dependent proteins. Moreover, matrix protein(s) directly stimulates tyrosine phosphorylation of actin, thus keeping the spore round in shape. The signalling pathway leading to tyrosine kinase activity remains unknown. There are many possibilities; PKA or PKA-dependent proteins could activate a specific tyrosine kinase responsible for the pTyr of actin. More likely, a receptor tyrosine kinase may bind a ligand present in the extracellular matrix (possibly the autoinhibitor) that specifically stimulates tyrosine kinase activity (see Fig. 22). The receptor type may also be G protein-linked since it has been shown that tyrosine kinase activity can be stimulated by a pertussis toxin-sensitive G-protein (Melamed et al., 1992).

When the sorocarp collapses onto the substratum, the spores and the extracellular matrix proteins are dispersed. The concentration of the autoinhibitor is no longer the strength to maintain dormancy. During the lag period (between activation and spore swelling), the autoactivator is synthesized and secreted to the extracellular environment. Spores respond to this factor when bound to the cell surface via as yet an unidentified receptor. It is presumed that the ligand binds to G-protein linked receptors. It will therefore be important to distinguish between this Go-isoform and that which renders the
Figure 22. Diagramatic representation of the signalling events occurring during autoactivation. The abbreviations are as follows: AC, adenylyl cyclase; cAMP, cyclic AMP; PKA, protein kinase A; RTK, tyrosine kinase-linked receptor; TK, tyrosine kinase; pTyr actin, tyrosine phosphorylation of actin; PLC, phospholipase C; PIP₂, phosphatidylinositol 4,5-bisphosphate; IP₃, inositol 1,4,5-trisphosphate; Ca²⁺ᵢ, intracellular calcium; Ca²⁺ₑ, extracellular calcium; CaM, calmodulin; PP, protein phosphatase 2B (calcineurin); ----, possible indirect links.
spores dormant, since it has been shown that the autoinhibitor and the autoactivator do not compete for the same binding site (Cotter & Glaves, 1985). Autoactivator binding leads to phospholipase C (PLC) activation and subsequent phosphatidylinositol 4,5-bisphosphate (PtdInsP₂) hydrolysis to yield inositol 1,4,5-triphosphate (InsP₃, Lydan & Cotter, 1995). InsP₃ then triggers the mobilization of Ca²⁺ from intracellular stores, presumably the endoplasmic reticulum. Ca²⁺ complexes with calmodulin to alter the activity of effector proteins (Lydan et al., 1994a; 1994b, Lydan & Cotter, 1994). The Ca²⁺/CaM complex could activate protein phosphatase 2B (calcineurin), belonging to the family of serine/threonine-specific phosphatases but which also has phosphotyrosyl phosphatase activity (Donella et al., 1994). Interestingly, Tisa & Cotter (1979) reported substantially high levels of acid phosphatase activity throughout germination, independent of activation mode. These authors also observed alkaline phosphatase specific activity to increase significantly during swelling and level off post-emergence. It is possible that the activity observed was that of calcineurin, however, the use of PP2B specific inhibitors, such as cyclosporin A or FK506, should elucidate the specific physiological role of this protein. Since the two recently uncovered protein tyrosine phosphatase gene products in Dictyostelium are not capable of dephosphorylating actin on tyrosine residues (Howard et al., 1994), calcineurin could possibly fulfill this role, thus allowing cytoskeletal reorganization to ensue. This reorganization would allow for spore swelling, where a net efflux of Ca²⁺ out of the spore occurs (Lydan & Cotter, 1994) and a net influx of this ion just prior to emergence. This influx is essential for the activation of Ca²⁺/CaM-dependent proteins necessary during amoebal emergence (see Fig. 22; Lydan et al., 1994a; 1994b). It should be noted that the autoactivator overrides any effect imposed by the autoinhibitor, presumably as shown in other systems (Rhee, 1993), by countering the inhibitory effect of cAMP and PKA on PtdInsP₂ hydrolysis. Exogenous activation (heat or DMSO) appears to bypass receptor activation but directly releases Ca²⁺ from intracellular stores (Lydan & Cotter, 1995). Interestingly, methods used for exogenous activation have been shown to
cause reorganization of filamentous actin (Sameshima et al., 1994, Kishi et al., 1994, McRobbie & Newell, 1985), which could explain the progression through germination without receptor engagement. As the link between extracellular signalling and actin reorganization strengthens, it will become clear which events regulate actin polymerization/depolymerization, and thus this will explain the role of actin tyrosine phosphorylation/dephosphorylation.
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APPENDIX I

Effects of tyrosine kinase inhibitors during asexual development in *D. discoideum.*

In order to put into context the role of tyrosine phosphorylation leading to spore germination, phosphotyrosine containing proteins during early developmental stages, as well as terminal cell differentiation and sporulation were examined. Elucidating this pattern of phosphorylation during the cyclical developmental program may provide fundamental insights into the role of tyrosine phosphorylation in *Dictyostelium discoideum.*

INTRODUCTION

*Dictyostelium discoideum* harbours two tyrosine kinase genes, DPYK1 and DPYK2, which have been shown to be developmentally regulated (Tan & Spudich, 1990). These genes appear to be evolutionary hybrids, displaying both tyrosine and serine/threonine kinase motifs. However, the specific physiological importance of these gene products has yet to be investigated. It has also been demonstrated that this organism contains two protein tyrosine phosphatase genes, PTP1 and PTP2 (Howard et al., 1994; 1992). Both genes have been shown to be preferentially expressed in prestalk and anterior-like cells (involved in the formation of the upper and lower cups and basal discs of the fruiting body) during the multicellular stages of development. In order to understand the physiological significance of tyrosine phosphorylation, the effects of two known inhibitors of tyrosine kinase activity (genestein and herbimycin A) were examined. Additionally, the effects of these inhibitors on phosphotyrosine-containing proteins were examined by probing western blots with recombinant anti-pTyr antibodies.
RESULTS

The developmental program of the axenic strain AX3D was carried out on 1.5% agar plates as per the method of O'Day (1979). Genistein at 10μM, 100μM and 1mM was added prior to pouring plates, while 0.1% DMSO was used as the control. Vegetative cells were pretreated for 30 minutes with the corresponding concentration of genistein prior to the onset of development. Developmental stage progression was recorded every 4 hours for 24 hours. It was found that treatment of cells with genistein had no effect on developmental timing or phenotype morphology compared to controls (data not shown). Moreover, there were no observable changes in the phosphotyrosine labeling pattern from genistein treatment (Fig. 23). These results support the findings of Browning et al. (1995), that demonstrated genistein to inhibit chemotaxis of wild-type cells to folic acid in a dose-dependent manner, but had no effect on the chemotactic ability of aggregate-competent cells to cAMP.

Since genistein inhibits tyrosine kinase activity via competitive inhibition with respect to ATP, a more specific inhibitor (herbimycin A) was used to elucidate the physiological significance of tyrosine phosphorylation. This latter agent inhibits the tyrosine phosphorylation mediated by src kinase as well as targets this protein for degradation. As above, 1.5% agar plates contained 5μM or 10μM herbimycin A, while control plates contained 0.5% DMSO. Vegetative cells were pretreated with the corresponding concentration of herbimycin A for 30 minutes prior to the onset of development. Developmental stages were photographed using an inverted microscope (Zeiss, W.Germany) at 4000X, every 4 hours for 24 hours. Figure 24 illustrates that treatment with herbimycin A at 5μM and 10μM resulted in progression through early development 1-2 hrs and 2-4 hrs faster than control cells, respectively. Aggregation territories of cells treated with 10μM herbimycin A are well established at 4h, while control cells have only begun this stage. At 16h the treated cells (herbimycin A) have
Figure 23. The effects of genistein on AX3D fruiting bodies (24h) phosphotyrosine containing proteins. The developmental program was carried out on phosphate buffer (pH 6.5) agar (Lane 1); on phosphate buffer (pH 6.5) and 0.1% DMSO agar (Lane 2); phosphate buffer (pH 6.5) and 100µM genestein agar (Lane 3); phosphate buffer (pH 6.5) and 1mM genestein agar (Lane 4). Cells were pretreated for 30 minutes with the corresponding genestein concentrations, as well, control cells were pretreated with 0.1% DMSO prior to the onset of development. Upon fruiting body formation (24h) spores were collected, ground and separated by 12.5% SDS-PAGE. 25µg/µl of protein was loaded in each lane and subsequent Western blot analysis using anti-P-Tyr antibodies revealed those proteins containing phosphotyrosyl residues. Molecular weight standards are in Daltons X 1000.
Figure 24. The developmental time course of strain AX3D, untreated and treated with 5μM and 10μM herbimycin A. AX3D amoebae were pretreated for 30 minutes with 5μM and 10μM herbimycin A, while control cells were treated with 0.5% DMSO prior to the onset of development. The developmental program was carried out on phosphate buffer (pH 6.5) agar containing the corresponding concentration of herbimycin A. Developmental stages were photographed using an inverted microscope at 4000X every 4h for 24h.
clearly developed slugs, while control cells are in late tipped aggregate stage. Furthermore, fruiting bodies of treated cells are well formed at 22h, while terminal differentiation of control cells did not occur until 24h. Whole cell lysates from control and treated (10μM herbimycin A) cells were analyzed by western blotting using anti-pTyr antibodies (Fig. 25). There were no observable changes in the phosphotyrosine labelling pattern from treatment with this agent compared to control cells. To further understand the method by which chemotaxis was advanced via herbimycin A treatment, a radial bioaassay for comparative analysis of chemotactic ability was performed (method of O'Day, 1979). Unlike the reliability and accuracy of this method when using wild-type cells, the axenic strain, AX3D, did not behave as expected (data not shown). The latter strain retained its ability for chemotaxis toward both folate and cAMP, even after prolonged starvation (24 hrs). Additional work is needed to elucidate the effects of herbimycin A on chemotaxis.

The rapid progression through early development via the inhibition of protein-tyrosine phosphorylation mimics the effects of DdPK2 overexpression and subsequent high levels of PKA (cAMP-dependent protein kinase) activity; however, herbimycin A treatment does not mimic the sporogenous phenotype observed in these mutants (Anjard et al., 1992). Interestingly, Wilson & Kaczmarek (1993) demonstrated that endogenous protein-tyrosine phosphatases may be activated by PKA, which offers a possible link between these results and those previously observed.

The phosphotyrosine pattern observed throughout development is similar to the results obtained by Howard et al. (1994). More specifically, actin is increasingly phosphorylated on its tyrosyl residues as development progresses. Since the samples analyzed here are whole cell lysates, comprised of both prestalk and prespore cell types, it would be interesting to reveal the developmental pTyr pattern of each cell type. This information may elucidate the molecular and behavioural differences of prestalk and prespore actin.
Figure 25. The effects of herbimycin A on phosphotyrosine containing proteins during the developmental time course of strain AX3D. AX3D amoebae were pretreated for 30 minutes with 10μM herbimycin A, while control cells were pretreated with 0.5% DMSO prior to the onset of development. The developmental program was carried out on phosphate buffer (pH 6.5) agar containing the corresponding concentration of herbimycin A. Cells were collected every 4h, ground and 25μg/μl of protein was loaded in each lane and separated by 12.5% SDS-PAGE. Subsequent Western blot analysis using anti-P-Tyr antibodies revealed those proteins containing phosphotyrosyl residues. Time represents hours into development: 0, vegetative; 4, pre-aggregation; 8, aggregation; 12, tipped aggregate; 16, slugs; 20, culmination; 20-24, fruiting body maturation. C and T represent control (0.5% DMSO) and treated cells (10μM herbimycin A), respectively. Molecular weight standards are in Daltons X 1000.
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