Cdc2 links the cell division cycle to differentiation in *Dictyostelium discoideum*

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ABSTRACT

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Dictyostelium discoideum

Kimchi Doquang

In *Dictyostelium discoideum,* a multicellular organism stems from individual amoebae in response to nutrient depletion. Despite the vastly different strategy used to generate multicellular structures, processes associated with animal development including cell sorting, pattern formation and cell-type choice are mirrored to a remarkable degree in the development of *Dictyostelium.* Morphogenesis ultimately leads to the formation of a fruiting body composed of dormant spores mounted on a rigid stalk of dead cells. The simplicity of this dichotomous choice makes *Dictyostelium* a model system for the study of pattern formation in developing organisms. Several lines of evidence suggest that cell-type biases are directly linked to the cell cycle. Cells starved early in the cell cycle preferentially become the stalk cells while those starved in later phases of the cell cycle favour the spore pathway [Weijer, C., Duschl, G., David, C.N. (1984) *J. Cell Sci.,* **70**, 133-145; MacDonald, S.A., Durston, A.J. (1984) *J. Cell Sci.,* **66**, 195-204; Gomer, R.H., Firtel, R.A. (1987) *Science,* **237**, 758-762; Ohmori, T., Maeda, Y. (1987) *Cell Differentiation,* **25**, 11-18; Zimmerman, W., Weijer, C. (1993) *Developmental Biology,* **160**, 178-185; Araki, T., Hajime, N., Takeuchi, I., Maeda, Y. (1994) *Developmental Biology,* **162**, 221-228]. The underlying mechanism linking the

cell cycle to cellular differentiation is unclear. To gain a better understanding of the relationship we have undertaken the analysis of proteins controlling the cell division cycle. In this work we describe a mutation in Cdc2 kinase, a pivotal cell cycle control element, and demonstrate that the cell-cycle control mechanism contributes directly to cell-type biases.

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CONTRIBUTIONS OF AUTHORS

The data presented in this thesis represents the work of the author with the exception of the following:

-RNA extractions from cold-synchronized cells were done by Dr. Harry MacWilliams.

-Gfp/Bfp images were taken and formatted by Emanuel Clausing.

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LIST OF ABBREVIATIONS

A: Alanine

*act6*P: Actin 6 promoter

*act15*P: Actin 15 promoter

ATP: Adenosine 5′-triphosphate

AX: Axenic

Bfp: Blue fluorescent protein

β-gal: β-galactosidase

Bp: Base pairs

BCIP : Bromochloroindolyl phosphate

BrdU: Bromodeoxyuridine

BSA: Bovine serum albumin

BSR: Blasticidin resistance

C: Celsius

Ca_i: Free internal calcium

cAMP: Cyclic adenosine 3′, 5′-monophosphate

Car: cAMP receptor

Cdc: Cell division cycle

CDK: Cyclin dependent kinase

CRS: Cytoplasmic retention signal

Dd: *Dictyostelium discoideum*

DIF: Differentiation inducing factor

DNA: Deoxyribonucleic acid

dNTP: Deoxyribonucleotide triphosphate

Dox: Doxycycline

DTT: Dithiothreitol

ecmA: Extracellular matrix A

EDTA: Ethylenediaminetetraacetic acid

EGTA: Ethylene glycol-bis(β-aminoethyl ether)

EP: Electroporation

F: Phenylalanine

FACS: Fluorescence activated cell sorter

FSC: Forward light scatter

G1: Growth phase 1

G2: Growth phase 2

G418: Geneticin

GDP: Guanosine 5′-diphosphate

Gfp: Green fluorescent protein

GTP: Guanosine 5′-triphosphate

KO: Knockout

M: Mitosis

MMS: Methyl methanesulfonate

NES: Nuclear export signal

nm: Nanometer

PCR: Polymerase chain reaction

pNBT: Nitroblue tetrazolium

PK: Protein kinase

PpiA: Peptidyl prolyl cis trans isomerase

Rb: Retinoblastoma

RNA: Ribonucleic acid

RNR: Ribonucleotide reductase

S: Serine

SDS: Sodium dodecyl sulfate

S. pombe: *Schizosaccharomyces pombe*

S. cerevisiae: *Saccharomyces cerevisiae*

T: Threonine

TA: Transactivator

TBS: Tris buffer saline

TCA: Trichloroacetic acid

Tet: Tetracycline

TRE: Tetracycline response element

UV: Ultraviolet

X-gal: 5-bromo-4-chloro-3-indolyl-β-D-galactoside

Y: Tyrosine

1. INTRODUCTION

The human body consists of over 200 different cell types that harmoniously arise from a single fertilized egg (Murray and Hunt, 1993). Elucidating the mechanisms involved in generating cell diversity remains a primary objective for researchers studying developmental biology and teratology. Numerous processes associated with embryonic development including cell sorting, pattern formation and cell-type regulation are eloquently displayed on a minimal scale in the social amoeba *Dictyostelium discoideum*. *Dictyostelium* is naturally found in forest topsoil where it feeds on bacteria, grows and multiplies by mitotic division. Once food supplies diminish the cells initiate an interesting survival mechanism. Growth ceases and the cells enter a twenty-four hour developmental pathway ultimately leading to the formation of a multicellular organism. Starvation in *Dictyostelium* therefore induces multicellular development in which an amoeba becomes one of essentially two possible cell types: stalk or spore. The relative simplicity of this system makes *Dictyostelium* an excellent model for the study of fundamental processes that take place during development.

1.1. Development

1.1.1. Aggregation

Four to five hours after starvation, independent *Dictyostelium* cells interact with each other through the periodic production and secretion of cyclic adenosine 3′, 5′-monophosphate (cAMP). During aggregation, cAMP is recognized by the major cAMP receptor, Car1, embedded in the plasma membrane of *Dictyostelium* cells. Car1 is a typical serpentine receptor that interacts with trimeric G-proteins. When cAMP binds to

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Car1, GDP is exchanged for GTP in the G-protein complex. The $\beta\gamma$ complex dissociates from the α subunit of the G protein leading to the activation of adenylyl cyclase. The activated enzyme subsequently converts ATP into cAMP, causing the rapid release of cAMP into the environment. Moreover, the dissociated α subunit modulates downstream events leading to the activation of genes whose protein products are involved in cell motility. Thus neighbouring cells respond to the pulse of cAMP in two ways: they begin to move towards the source of cAMP and they produce bursts of the chemoattractant cAMP themselves. As a consequence, the signal is propagated outwards causing the mass movement of up to 100 000 amoebae towards a central point (reviewed by Loomis in 1996).

1.1.2. Postaggregation

Movement towards an aggregation centre is highly orchestrated. Guided by waves of cAMP the amoebae unite forming streams of cells. The cells eventually converge at a central point and mount on top of each other forming a mound. At this stage the cells adhere to each other giving rise to a cohesive organism. A nipple-shaped tip subsequently emerges from the mound. With the capacity to respond to light as well as various temperature and moisture gradients the apex becomes the organizing part of the structure. The tip extends upwards and the entire structure eventually falls over to form a migrating slug of about 1 mm in length. The slug migrates with its anterior tip slightly elevated in search of grounds suitable for further development (reviewed by Mutzel *et al.*, 1995).
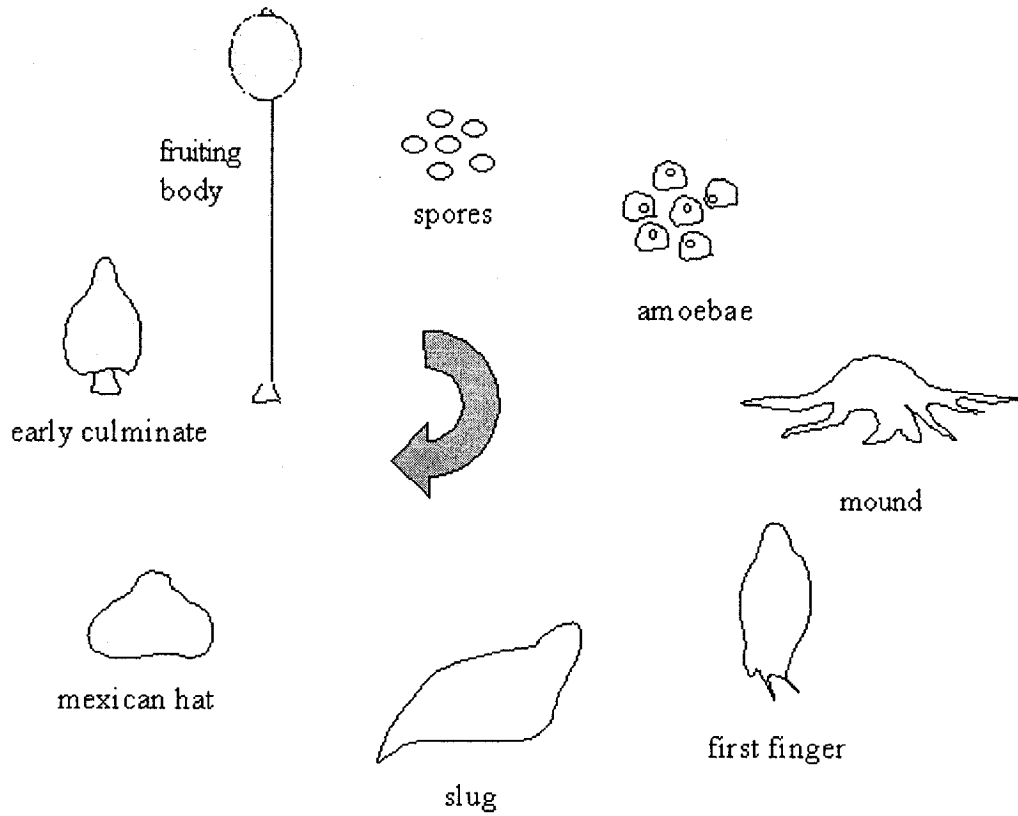
Figure 1. *Dictyostelium discoideum* **life cycle.**

Starving *Dictyostelium* cells enter a twenty-four hour developmental pathway leading to

the formation of a fruiting body.

1.1.3. Late development

About 18 hour after starvation, the slug ceases migration and starts to culminate. Cells originally at the front of the slug force their way through the cell mass thereby forming a tube. Cells from the top subsequently dive into the tube creating a "reverse-fountain" effect (Bonner, 1967). As more cells from the apical region plunge into the tube, cells that were originally at the back of the slug are hoisted upwards resulting in the formation of a fruiting body. A mature fruiting body consists of a ball of spores resting on a tapered stalk. The stalk cells are dead but the spores are now ready to be dispersed to richer environments. Dormant spore cells are encapsulated in a layer of carbohydrates and as such are resistant to adverse environmental conditions including high temperatures and arid conditions (Cotter *et al.*, 1992).

1.2. Cell-type proportions

Differentiation (the choice of becoming stalk or spore) begins shortly after aggregation. Precursors of the stalk and the spore cells are identifiable biochemically and cytologically, and are partially segregated during the slug stage. Cells occupying the posterior 80% of the slug are predisposed to become spore cells while cells located in the remaining anterior region are of a stalk nature (MacWilliams and Bonner, 1979). They are given the names "prespore" and "prestalk" because of their eventual fates. Approximately 10% of the cells in the posterior zone are very similar to the prestalk cells. They are called anterior-like cells and are found distributed at random throughout the rear of the slug (Sternfeld and David, 1982).

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"reverse-fountain"
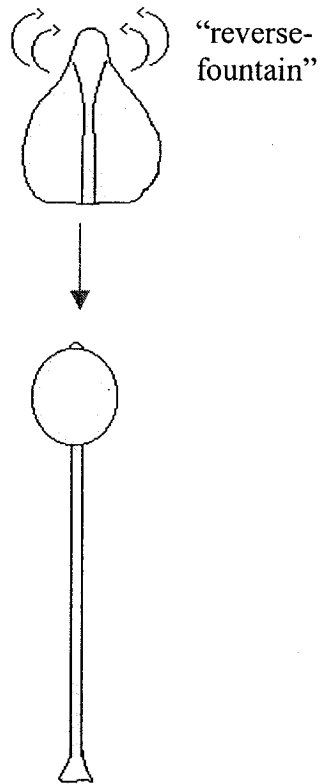
Figure 2. Culmination

Cells from the anterior region of the slug dive into the cell mass and give rise to a mature fruiting body.

1.3. Factors regulating cell fate

Proper cell proportioning is a critical factor for the multicellularity of *Dictyostelium*. If the spore mass is too large then the stalk may collapse under the excessive weight. If the stalk is too short then spore dispersal may be compromised (Brown and Firtel, 2000). Accordingly a *Dictyostelium* slug is capable of regenerating both prestalk and prespore cells when portions of the structure are micro-surgically removed. For instance when the prestalk region is excised, the anterior-like cells move forward and redifferentiate into prestalk cells to regenerate the missing segment. Concomitantly some of the prespore cells re-differentiate into anterior-like cells (Sakai, 1973; Sternfeld and David, 1982). This mechanism ensures the adequate presence of different cell-types so as to produce a proportioned fruiting body. The ability to restore the missing cell-type suggests that cell fate is dependent upon cell-cell interactions within the aggregate. Based on these observations it was postulated that cell-type choice is controlled by morphogens in the immediate vicinity of the developing cells (MacWilliams and Bonner, 1979; Meinhardt, 1983). Diffusible molecules capable of influencing cell fate have in fact been isolated (reviewed by Weeks and Gross, 1991). One such factor is DIF-1 that promotes stalk cell differentiation while inhibiting spore formation (Town *et al.*, 1976; Kopachik, 1983; Kay and Jeremy, 1983). DIF-1 promotes calcium uptake, and elevated levels of intracellular calcium have been linked to prestalk differentiation (Azhar *et al.*, 1997). Thus some substances act synergistically to incite stalk formation. The action of DIF appears to be mediated by intracellular pH since treating cells with a weak base favours spore formation even in the presence of the
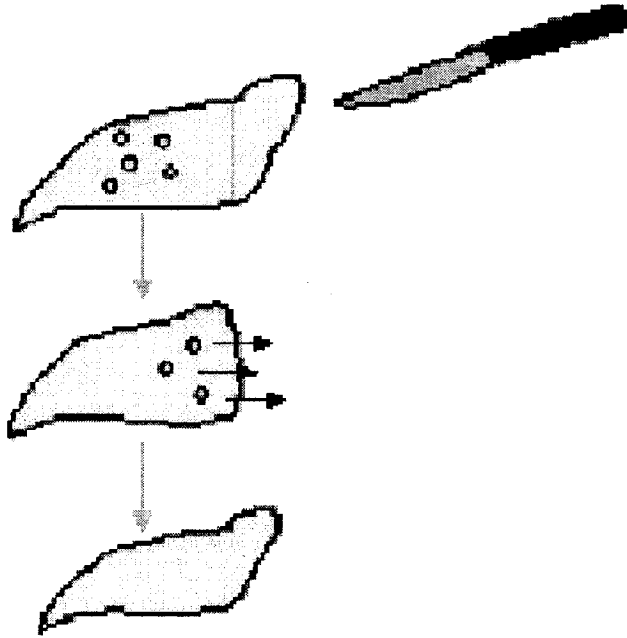
Figure 3. *Dictyostelium* **redifferentiation.**

Dictyostelium slug regenerates the missing anterior, prestalk region.

morphogen (Gross *et al.*, 1983; Wang *et al.*, 1990). Accordingly ammonia, a by-product

of protein degradation, acts by stimulating spore cell differentiation (Gross *et al.*, 1983).

The observation that DIF-1 is present at higher concentrations in the prespore region as

opposed to the prestalk area of the slug contradicts the morphogen gradient theory

(Brookman *et al.*, 1987). Also if the spatial distribution of morphogenic substances alone

controlled cell fate then one might expect cells exposed to similar concentrations of a

particular morphogen to produce identical cell types. Based on this conjecture Gomer

and his colleagues studied the developmental fate of cells starved under conditions that

prevented cell-cell contact. Briefly the cells were washed free of nutrients and plated at a

low density thereby preventing intercellular communication. The cells were treated with

purified DIF-1. Although each cell was exposed to equivalent amounts of the morphogen

only 20 percent differentiated into prestalk cells (a value similar to the percentage

observed in slugs) (Gomer *et al.*, 1986; Gomer and Firtel, 1987; Clay *et al.*, 1995). These

results suggest that cell-type specification is not solely based on external morphogenic

factors. So how does a group of apparently identical cells give rise to different cell types?

1.4. Correlating cell differentiation to the cell cycle

Experiments performed over thirty years ago revealed a pre-existing

heterogeneity in a starved *Dictyostelium* population. *Dictyostelium* cells fractionated on

density gradients manifested cell-fate biases (Takeuchi, 1969). It was later shown that

different cell fractions were in alternate phases of the cell cycle (Weijer *et al.*, 1984a).

Hence it has been suggested that the position of an individual cell in its cell cycle at the

onset of starvation influences cell fate (Weijer *et al.*, 1984b; MacDonald and Durston,

1984; Gomer and Firtel, 1987; Ohmori and Maeda, 1987; Zimmerman and Weijer, 1993; Araki *et al.*, 1994).

A typical somatic cell cycle is divided into two distinct parts known as interphase and mitosis. Interphase consists of two growth phases known as G1 and G2 that bracket a single period of DNA replication termed S (synthesis). Mitosis, on the other hand, is restricted to a brief period of the cell cycle and entails nuclear division. Mitosis is marked by condensation of the replicated DNA into microscopically visible chromosomes. The chromosomes bind to microtubules emanating from the centrioles allowing the former to align at the equator of the cell. The chromosomes consequently separate from each other and migrate to opposite ends of the cell. As the chromosomes near the poles a contractile ring pinches the cell into two through a process know as cytokinesis. Cleavage produces two identical cells each containing a full set of chromosomes (Pines and Rider, 2001).

When employing *Dictyostelium* as a model to study cell division, potential complications may arise due to cell cycle anomalies. In contrast to other well studied eukaryotic systems *Dictyostelium discoideum* has a very short or absent G1 phase. After mitosis the daughter cells appear to enter S immediately resulting in a cell cycle that consists primarily of a G2 phase (Weijer *et al.* 1984a; Zimmerman and Weijer, 1993; Saran, 1999).

The idea that population heterogeneity in *Dictyostelium*, ascribed by the cell cycle position at the onset of starvation, provides the grounds for initial cell-type choice is nonetheless appealing. Experimental evidence corroborates this claim. Prespore and

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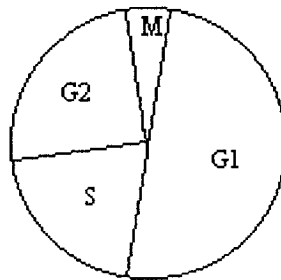
A.   B.
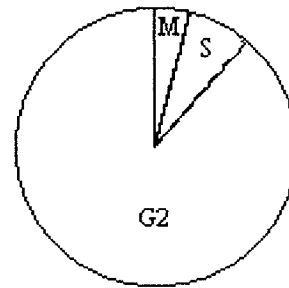
Figure 4. (A) Typical somatic cell cycle and (B) *Dictyostelium discoideum* **cell cycle.**

In contrast to other eukaryotic cell cycles, the cell cycle of *Dictyostelium discoideum*

lacks a G1 phase.

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prestalk cells can be identified by the gene markers they express. As an example, prestalk differentiation is most often measured by the expression of reporter genes from the *ecmA* and *ecmB* promoters (Jermyn and Williams, 1991). Precursors of stalk and spore cells also can be identified by the expression of specific cell-surface antigens. For instance cathepsin protease 2 is expressed in prestalk cells while SP70 is expressed exclusively on prespore cells. Either marker can be recognized by specific antibodies (Gomer *et al.*, 1986). Using these distinctive characteristics, synchronous cultures of cells in S or early G2 were found to form slugs with fewer prespore cells than normal when starved. In parallel experiments, slugs derived from cells synchronized in late G2 formed developmental structures containing a high percentage of prespore cells (Gomer and Firtel, 1986; Weijer *et al.*, 1984b). Using a low cell-density differentation assay to eliminate cellular communication, it was shown that cells in the early phases of the cell-cycle showed a prestalk bias while those occupying later positions in the cycle preferentially developed into spores (Gomer and Firtel, 1987). When S phase inhibitors were used to lengthen the duration of S, the percentage of prestalk cells increased (Gomer and Amman, 1996). These results indicate that initial cell-type selection is somehow linked to the cell cycle. Indeed prestalk cells arise from cells that are in S, M or the early G2 phase at the onset of starvation. Prespore cells on the other hand arise from cells that are in late G2 phase of the cell cycle at the time of nutrient depletion (Weijer *et al.*, 1984b; MacDonald and Durston, 1984; Gomer and Firtel, 1987; Ohmori and Maeda, 1987; Zimmerman and Weijer, 1993, Araki *et al.*, 1994). Based on mounting evidence it is therefore likely that the cell cycle provides the grounds for population heterogeneity necessary for inital cell-type choice. Conceiveably, morphogenic gradients then reinforce

proper spatial distribution and proportioning (MacWilliams and Bonner, 1979; Krefft *et al.*, 1984).

1.5. Regulation of the cell division cycle

Although relatively little is known about the *Dictyostelium* cell cycle comprehensive studies have been done on other eukaryotic organisms including yeast. Conveniently, many of the key proteins involved in cell cycle regulation have counterparts in other systems, including humans, thus implying that the mechanisms controlling the cell division cycle have been preserved throughout the evolution of eukaryotes.

1.5.1. Cell cycle checkpoints

Coordination of complex processes involved in the cell cycle are crucial to preserve genome integrity. For instance, DNA must be fully and faithfully replicated before the cell attempts to divide. Failure to do so may lead to genomic instabilities thus increasing the likelihood of diseases such as cancer (Hartwell and Kastan, 1994). Checkpoints that operate throughout the cell cycle arrest or delay progression until conditions are suitable for the resumption of growth. Several checkpoints are operative during the cell cycle. A major checkpoint occurs in late G2 and controls entry into mitosis. A second checkpoint known as START or the restriction point in unicellular and multicellular eukaryotes respectively, functions in late G1. To progress through this block cells need not only attain a threshold size but also must possess the functional machinery required for DNA replication. Cells that have passed the G1/S checkpoint are committed

to divide. Cells failing to pass G1/S on the other hand enter an indeterminate resting state known as Go (Murray and Hunt, 1993).

1.5.2. Ions involved in cell cycle regulation

A transient increase in free intracellular calcium is correlated to progression through G1/S. Treatment with the calcium ionophore, A23187, releases cells from Go arrest. Whether induced by natural or artificial means a surge in free calcium influences the cell cycle by allowing cells to resume growth. At fertilization the rapid rise in internal calcium levels generates a sustained increase in intracellular pH that in turn stimulates DNA and protein synthesis (Whitaker and Patel, 1990). A momentary rise in free calcium therefore enables cells to proceed through the G1/S checkpoint and initiate DNA replication.

A role for calcium in cell cycle regulation also can be inferred from calcineurin, a calcium-calmodulium dependent phosphatase. Calcineurin is a critical component of the immune system (Lin *et al.*, 1991). It catalyzes dephoshorylation events required for cytokine gene expression (Clipstone and Crabtree, 1992). The physiological role of calcineurin has been elucidated primarily through the use of immunosuppressive compounds such as FK506. This drug physically associates with its intracellular receptor, FKBP12 (FK506 binding protein), and the complex, in turn, targets calcineurin (Lui *et al.*, 1991). Inhibitors of calcineurin cause cell cycle arrest at the G1/S transition (reviewed by Schreiber in 1992).

FKBPs have been identified in a wide array of systems including bacteria, yeast and vertebrates suggesting that these enzymes may be involved in cellular functions

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beyond those of immune response (Heitman *et al.*, 1992). Indeed, FKBPs catalyse the interconversion of the cis-trans isomers of prolyl residues and may thus play a role in basic processes including protein folding and trafficking. In the absence of FK506, FKBP12 interacts with the IP3 and ryanodine receptors (Cameron *et al.*, 1995; Jayaraman *et al.*, 1992). It is thought that FKBP12 controls gating of these Ca^{2+} channels by recruiting calcineurin and faciliating dephosphorylation events necessary for Ca^{2+} flux and G1/S progression.

1.5.3. Cyclin-dependent kinases

Cyclin-dependent kinases are key regulators of the cell cycle. CDKs are defined by their ability to phosphorylate S or T residues in S/T-P motifs and are also recognized by the presence of a conserved domain known as PSTAIRE (Murray and Hunt, 1993). Most eukaryotic systems possess multiple CDKs each with a defined role. In landmark studies done on Xenopus, CDK was discovered as the catalytic subunit of mitosis promoting factor (Newport and Kirschner, 1984). Traditionally the CDK involved in initiating mitosis is known as Cdc2 (Cdc28 in *S. cerevisiae*) (reviewed by Mendenhall and Hodge, 1998). Hitherto all systems contain a *cdc2* gene and these share a high degree of sequence similarity (Liu and Kipreos, 2000). Homologous Cdc2 proteins from systems as diverse as yeast and human are in effect functionally interchangeable (Lee and Nurse, 1987; Krek *et al.*, 1992). The ability to exchange Cdc2 between species enforces the notion that eukaryotic cell cycles are governed by similar principles.

Levels of CDKs remain relatively constant throughout the cell cycle. Virtually all controls are accomplished by post-translational modifications. In the case of the mitotic

Cdc2, activation requires explicit events- cyclin binding, nuclear import of the Cdc2/cyclin complex, stimulatory/inhibitory phosphorylations and dephosphorylations on critical sites (reviewed by Nurse, 1990).

1.5.4. The cyclins

As inferred by their name, the activity of cyclin-dependent kinases relies critically upon the binding of cyclins for proper function. In view of this, monomeric CDK subunits exhibit low protein kinase activity (Booher et al., 1989). Initially identified in sea urchin eggs, cyclins are proteins whose levels oscillate during the cell cycle (Evans et al., 1983). All cyclins possess a conserved domain known as the "cyclin box" necessary for CDK binding. The crystal structure of human Cdc2 bound to cyclin A has been resolved (Debont et al., 1993). It serves as a model for other CDK-cyclin interactions. In the complex the cyclin box contacts the PSTAIRE domain of the cyclin-dependent kinase subunit. Although binding has very little influence on the structure of cyclin, the interaction has an immense impact on the CDK. The catalytic site undergoes major conformational changes leading to a dramatic increase in protein kinase activity.

Most organisms express multiple cyclins. In mammalian systems, cyclins D1, D2, D3 and D4 (cyclins 1, 2 and 3 in yeast) are expressed during the G1 phase of the cell cycle. They are collectively known as the G1-cyclins and regulate events associated with DNA replication. Cyclins A and B (equivalently known as B-type cyclins in yeast) are predominately expressed during G2 and M and are therefore referred to as the mitotic cyclins (Murray and Hunt, 1993). The binding of cyclin B to Cdc2 is required for progression through mitosis (Draetta et al., 1989; Pines and Hunter, 1989).

Cyclins are under tight post-translational control. Proteolytic degradation is mediated by an ubiquitin-dependent mechanism. Cyclins contain a conserved RTALGDIGN sequence known as the destruction box located approximately 40 residues from the start of the protein. The addition of ubiquitin to L residues downstream of the destruction box targets cyclin for degradation (Glotzer *et al.*, 1991). Conjugates are subsequently captured by cytosolic proteosomes and degraded into free amino acids (Ciechanover and Schwartz, 1994). Temporal control of cyclins is crucial for proper mitotic timing. In fact, proteolytic degradation of the cyclin B subunit inactivates Cdc2 and is necessary for cells to exit mitosis (Murray *et al.*, 1989).

1.5.5. Nuclear localization of *cdc2*

Subcellular translocation of the Cdc2-cyclin B complex also is necessary for initiating mitotic entry. Cyclins possess a nuclear localization signal (Murray and Hunt., 1993). When Cdc2 is bound to cyclin B it becomes a target for importin β (Moore *et al.*, 1999). The carrier protein shuttles the Cdc2-cyclin B complex from the cytoplasm to the nucleus. During the G2 phase of the cell cycle nuclear import is negated by nuclear export (Hagting *et al.*, 1998). Phosphorylation of the nuclear export sequence, NES, on the cyclin B subunit prevents the nuclear export protein, Crm1, from binding, leading to a nuclear accumulation of Cdc2 (Hagting *et al.*, 1998; Yang *et al.* 1998).

1.5.6. Control of Cdc2 by phosphorylation

Successive phosphorylation and dephosphorylation events on Cdc2 also are necessary for proper mitotic timing. Three residues (T161, T14 and Y15) act as

phosphoacceptors. Phosphorylation of T161 by the Cdc2-activating kinase is essential for Cdc2 function (Ducommun *et al.*, 1991; Gould *et al.*, 1991). Conversely, phosphorylation of the T14 and Y15 residues impedes Cdc2 activity. Both residues are located within the ATP binding region of the kinase (Debondt *et al.*, 1993). Prior to mitosis, Cdc2 is held in an inactive state via phosphorylation at these two critical sites. Inhibitory phosphorylation of Cdc2 is controlled by the membrane bound Myt1 kinase that preferentially targets T14 (Mueller *et al.*, 1995; Lui *et al.*, 1997). Phosphorylation at the Y15 site is accomplished exclusively by the nuclear Wee1 kinase (McGowan and Russel, 1993). When conditions are appropriate for cellular division, Cdc2 is sequentially dephosphorylated at T14 and Y15 resulting in mitotic entry (Lundgren *et al.*, 1991; Borgne and Meijer, 1996). Dephosphorylation is achieved by the inactivation of Myt1 and Wee1 kinases and the simultaneous activation of Cdc25 phosphatase (Russel and Nurse, 1986; Millar and Russel, 1992; Lammer *et al.*, 1998). It is of interest to note that the G2/M checkpoint in lower eukaryotes operates by means of Y15 phosphorylation alone (Rhind and Russel, 1988; Gould and Nurse, 1989).

The inhibitory role of Y phosphorylation on Cdc2 was initially addressed in yeast cells by replacing the Y15 amino acid with the structurally similar, non-phosphorylatable F. Cells expressing the mutant protein showed marked advancement into mitosis (Gould and Nurse, 1991). Similar experiments were performed in mammalian cells using Cdc2AF, a mutated version of Cdc2 that cannot be phosphorylated on either T14 or Y15. Overexpression of Cdc2AF or transient expression using a tetracycline repressible promoter resulted in the premature occurrence of events associated with mitosis,

including chromatin condensation, suggesting that these sites are crucial to maintain proper G2 delay (Krek *et al.*, 1992; Jin *et al.*, 1996).

1.6. Cell cycle response to DNA damage

Exposure to DNA damaging agents that cause genomic alterations induces cell cycle arrest (Hartwell and Kastan, 1994). The delay is presumably intended to provide the cells an opportunity to repair damaged chromosomes. Ample evidence suggests that DNA mutagenesis stabilizes the phosphorylated state of Y15 in *Aspergillus nidulans*, fission yeast (*Schizosaccharomyces pombe*) and mammalian cells (Rhind *et al.*, 1997; Poon *et al.*, 1996; Ye *et al.*, 1997). The ability to maintain a phosphate group on Cdc2 at this critical site is most likely due to the inactivation of the Cdc25 phosphatase. Damaged DNA activates Chk protein kinases that, in turn, phosphorylate Cdc25 on S216. This phosphate group creates a docking site for protein 14-3-3, allowing the inhibitor to bind to Cdc25 by this way sequestering it in the cytoplasm (Ford *et al.*, 1994). Reports indicate that cyclin B also is retained in the cytoplasm in response to DNA damage. Expression of a nuclear cyclin B abrogates the G2 checkpoint suggesting that nuclear trafficking of cyclin B also controls mitotic entry after DNA damage (Jing *et al.*, 1998; Toyoshima *et al.*, 1998).

1.7. Cell cycle control elements in *Dictyostelium discoideum*

Although the cell cycle of *Dictyostelium* is skewed, pivitol cell cycle control proteins common to those of yeast and mammalian systems have nonetheless been identified. The gene coding for the putative *Dictyostelium* Cdc2 has been isolated.

Homology of *Dd.cdc2* to that of other systems is revealed by sequence comparison. The protein contains a PSTAIRE peptide motif common to all CDKs and necessary for cyclin recognition and binding. The protein shares on average 60% sequence identity with the Cdc2 proteins of other systems. Furthermore the *Dd.cdc2* gene can complement a temperature sensitive *cdc28⁻* strain of *Saccharomyces cerevisiae* just like the human homologue rescues a conditional yeast *cdc2* mutant (Michaelis and Weeks, 1992; Lee and Nurse, 1987). Thus there is a notable conservation of the *cdc2* gene both structurally and functionally.

The gene coding for cyclin B also has been isolated. Like the mammalian and yeast counterparts mRNA levels of cyclin B fluctuate throughout the cell cycle reaching a peak at the G2/M boundary (Luo *et al.*, 1994).

1.8. Existing models linking the cell cycle to differentiation

1.8.1. The "putative-shift point"

Current *Dictyostelium* literature provides conjectural models that explain the nature of cell sorting. Maeda and his colleagues contend that cells exit the growth phase and enter development at a particular moment in the cell cycle coined the "putative-shift" or "PS point" (Maeda *et al.*, 1989). The switch occurs in late G2 approximately 2.5 hours before mitosis (Figure 5). Cells starved just prior to the PS-point initiate development immediately whereas cells located after this critical transition progress through M, S and most of G2 before entering development. The model implicates differential chemotaxis in cell sorting. It has been shown that cells entering development early are most efficient

"PS-point"
Exit growth; enter
development
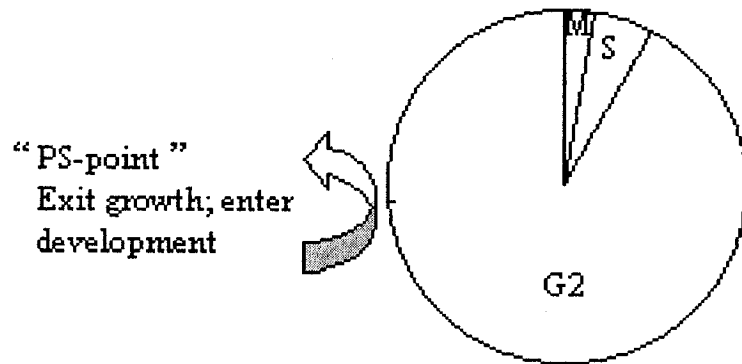
Figure 5. Location of the "putative-shift point" in the *Dicytostelium* cell cycle.

Starving cells exit the cell cycle at the "putative-shift" point and initiate development (Maeda *et al.*, 1989). Cells reaching the "PS-point" first pre-empt the spore pathway coercing latecomers to become stalk cells.

at producing and responding to cAMP pulses and have a greater ability to initiate aggregation centres (Wang et al., 1988; McDonald, 1986). Hence cells that initiate development precociously assume positions within the cell mass that expose them to levels of morphogens promoting spore differentiation (Ohmori and Maeda, 1986).

Although these interpretations are valid there are inconsistencies. Assuming cells initiate development by crossing the putative-shift point one would expect a delay in the developmental time course of cells located in the early phases of the cell cycle (after the PS point). For cells positioned immediately after the PS-point the time lag should theoretically be the duration of one cell cycle. In detail, if a cell cycle lasts seven hours then cells located just after the PS- point should enter development approximately seven hours after the onset of starvation while cells positioned just prior to the PS-point should initiate development immediately. Using synchronized cell isolates, however, it was shown that the time discrepany between the earliest and the latest developers was at most 4 hours (Ohmori and Maeda, 1987). Thus although substantial evidence correlates the cell cycle to cell differentiation the unifying mechanism remains unclear.

1.8.2. Prespore and prestalk biases may be related to the cell cycle

Recently, it has been proposed that the Dictyostelium cell cycle contains a Start-like transition in mid G2, analogous to the G1 checkpoint in mammalian and yeast cells, thus raising the possibility that this may be an important control site in the cell cycle of this organism (MacWilliams et al., 2000). Appropriately, genes whose products are normally associated with entry into S phase accumulate in mid G2. Regulators include the small subunit of riboncleotide reductase involved in manufacturing the building

blocks of DNA. Studies done on *rnrB* demonstrate that the gene is expressed in a cell cycle specific fashion. While the transcript is usually maximal in G1/S in other eukaryotes, *rnr*B message peaks during mid-G2 in *Dictyostelium* (MacWilliams *et al.*, 2000).

Evidence obtained using synchronized cultures indicates that cells in the early phases of the cell cycle preferentially differentiate into stalk cells. The region of the cell cycle specifying prestalk bias varies somewhat depending on the experimental conditions. If one defines S phase as the reference point, prestalk preference spans a region from –3.0 hours to 0 hours (Araki *et al.*, 1994) or 0 hours to +3.0 hours (Weijer *et al.*, 1984) (Figure 6). Taken together these results encompass 6 hours of an 7 hour cell cycle implying a rather casual relationship between the cell cycle and cell differentiation. Current synchronization regimes can produce cell cycle distortions, however, and dissimilar results have been obtained when comparing the various approaches (MacWilliams *et al.*, 2000). Studies performed using asynchronous cultures demonstrate a much tighter correlation between the cell cycle and cell-type choice. Gomer and Firtel (1987) have shown that single cells induced to develop express specific stalk or spore antigens depending on the cell cycle phase at the time of starvation. Cells switched from a prespore to a prestalk bias in late G2 and reverted back to a prespore preference in mid G2 (Figure 7). The first switch may correspond to the G2/M checkpoint while the second switch at mid G2 is consistent with the *Dicty*-START (MacWilliams *et al.* 2000).

A.
B.

● Spore
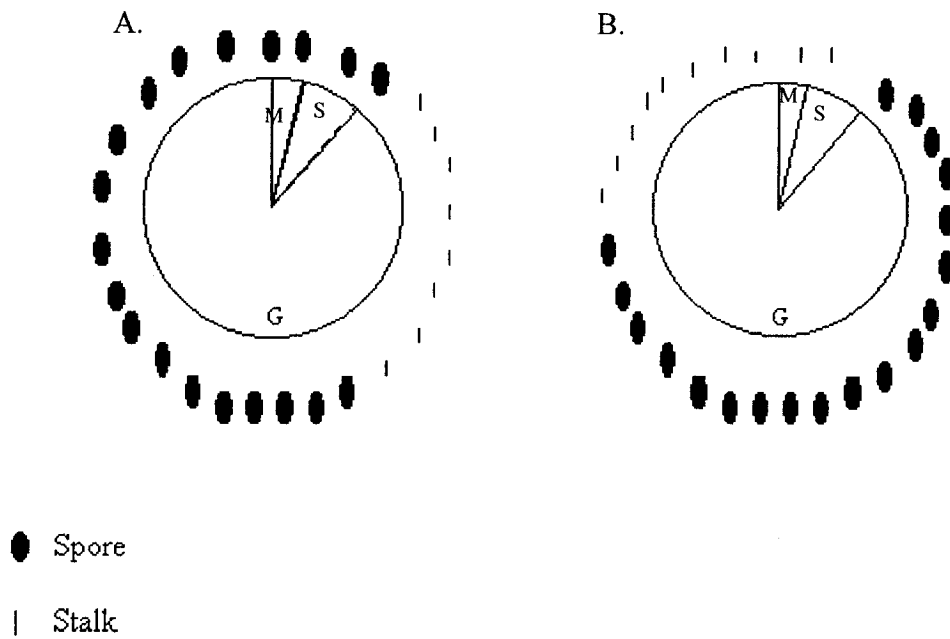
| Stalk

Figure 6. Differentiation preferences observed using (A) high density synchrony (Weijer *et al.*, 1984) and (B) cold-shift synchrony (Araki *et al.*, 1994).

Prestalk preference extends from 0 hours to +3 hours and −3 hours to 0 hours depending on the synchronization method used.
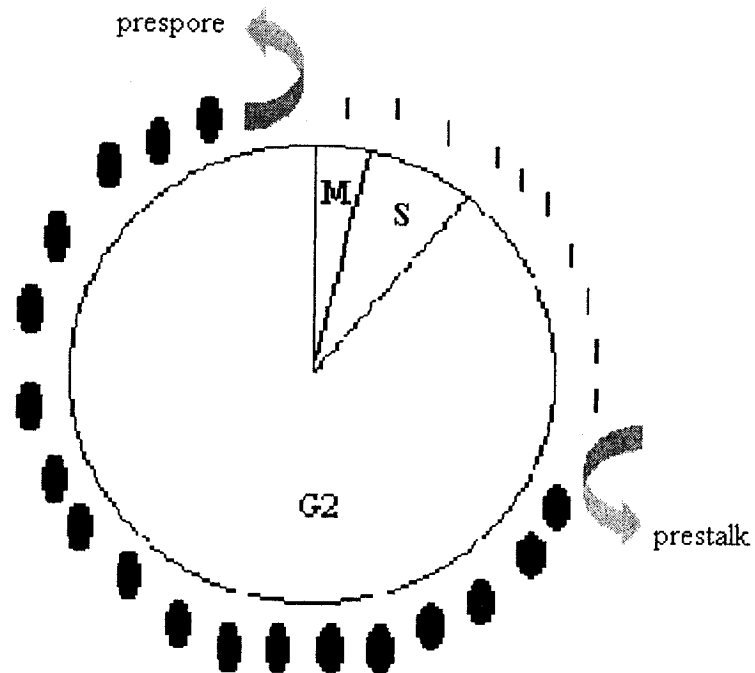
Figure 7. Differentiation preferences observed using asynchronous cultures.

Without synchronization, cells switch from a prespore preference to a prestalk bias seemingly at the G2/M transition but revert back to a prespore preference in mid-G2 (Gomer and Firtel, 1987; Zimmerman and Weijer, 1993).

To gain a better understanding of the relationship between the cell cycle and developmental fate in *Dictyostelium* we have initiated an extensive study of the proteins that regulate the cycle checkpoints. In particular we approached the matter by altering cell cycle regulated proteins and examining the consequential effects on cell fate. In this present work we describe the Y21 mutation of the *Dd.cdc2* gene (equivalent to the *cdc2* Y15 residue in yeast) and its subsidiary role in *Dictyostelium* development. In addition we disrupted the *Dd.ppiA*, a gene that is expressed specifically at the *Dicty*-start transition. Our results show a direct role for Cdc2 in cell-type choice and help define the relationship between the cell-division cycle and differentiation in *Dictyostelium*.

2. MATERIALS AND METHODS

2.1. Generation of mutants

2.1.1. Site-directed mutagenesis

Primers used in this study are listed in Table 1. The *Dictyostelium discoideum* *cdc2* gene (a gift from G. Weeks) was subcloned from pGEX*cdc2* into the *Bam*HI and *Eco*RI sites of the bacterial vector pBluescript KS (+). The strategy used for site-directed mutageneis (Ho *et al.*, 1989) is depicted in Figure 8. The 5′ region of the gene was amplified with the universal and *cdc2*Y21F antisense primers. The 3′ region was amplified with the reverse and *cdc2*Y21F sense primers. The PCR reactions contained 1 X reaction buffer (20 mM Tris-Cl, 10 mM KCl, 10 mM $(NH_4)SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100, 100 μg/mL nuclease free bovine serum albumin, pH 8.8), 200 μM of dNTPs, 100 ng of DNA template, 250 ng of each primer, and 2.5 units of *Pfu*TURBO DNA polymerase (Stratagene, La Jolla, California, USA) in a total volume 100 μL. Following an initial cycle of denaturation at 94°C for 5 minutes, annealing at 40°C for 30 seconds and extension at 72°C for 3 minute, 20 additional cycles were carried out at 94°C for 30 seconds, annealing at 40°C for 30 seconds and extension at 72°C for 3 minute using an Eppendorf thermocycler (Mastercycler gradient, Eppendorf, Hamburg, Germany). The two PCR-amplified gene fragments corresponding to the 5′ and 3′ sequences of *Dd.cdc2* were treated with *Dpn*I to degrade the original, methylated DNA template. PCR products were cleaned using Qiaquick PCR purification columns (QIAGEN Inc., Mississauga, Ontario, Canada) and served as template DNA for a second round of PCR. Fusion and extension polymerase chain reactions were

Table1. Primers designed for Polymerase Chain Reactions

PRIMER	SEQUENCE	USE
reverse primer	5'-AGCGGGATAACAATTTCACACAGG-3'	Site-directed mutagenesis of *cdc2*
cdc2Y21F sense	5'-GGTGAAGGTACCTTGGTAAAG-3'	Site-directed mutagenesis of *cdc2*
cdc2Y21F antisense	5'-CTTTACCAAGGTACCTTCACC-3'	Site-directed mutagenesis of *cdc2*
universal primer	5'-GTTGTAAAACGACGGCCAGT-3'	Site-directed mutagenesis of *cdc2*
*Bgl*II *cdc2*	5'-GAAGATCTATGGAATCAGATGGAGG-3'	Cloning *cdc2* into dox response plasmid
*Sph*I *cdc2*	5'-ACATGCATGCGAAAAAACTAGTATCAAGATCACC-3'	Cloning *cdc2* into dox response plasmid
*Sal*I *ppi* 5'	5'-ACGCGTCGACGGCATTACATGGTGTTGATGAA-3'	*ppiA* disruption
*Eco*RV *ppi* 5'	5'-CCGATATCGGTGGAATATTACCTTTACCTTA-3'	*ppiA* disruption
*Xba*I *ppi* 3'	5'-GCTCTAGACCCTCTAAAACTTTATAGTCAAGTC-3'	*ppiA* disruption
*Spe*I *ppi* 3'	5'-CGACTAGTTTCAAAACCTTGATCTCACC-3'	*ppiA* disruption
pDneoII antisense	5'-CAATTGATGGACGACCCGAGC-3'	Confirm presence of *act6cdc2*

Overhangs are in bold, restriction endonuclease sites are underlined and base substitutions are in red.

27
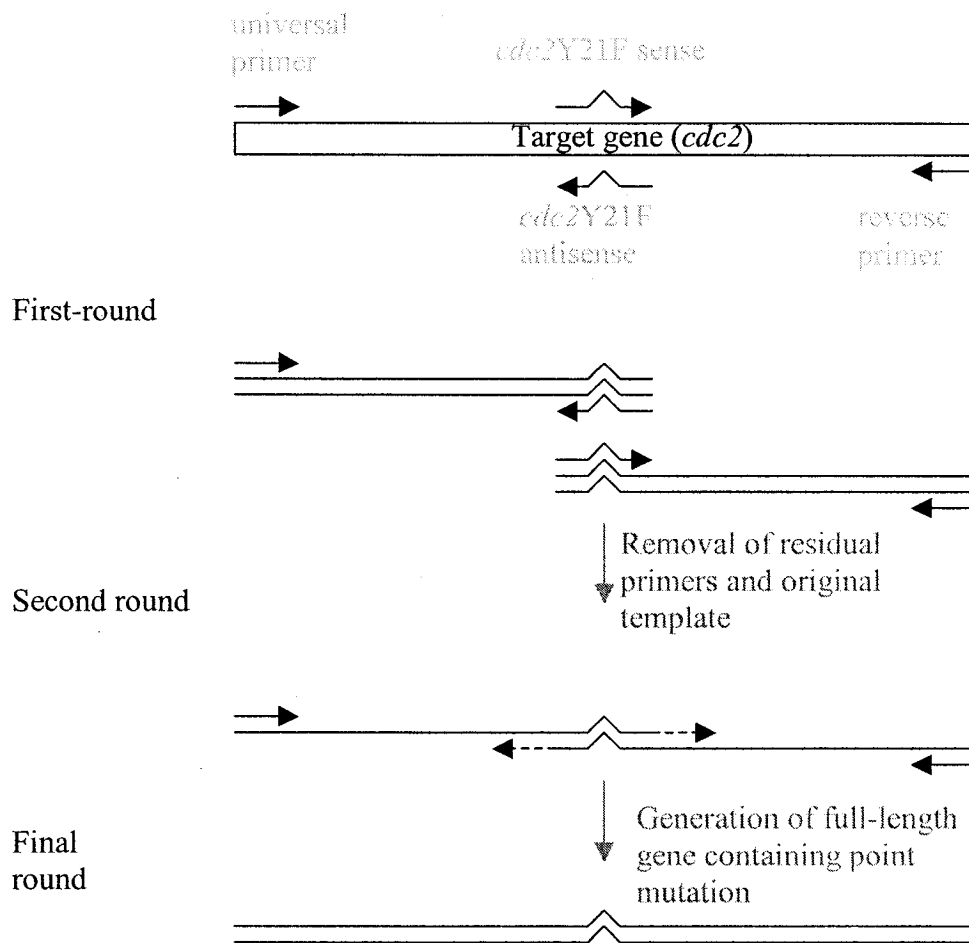
Figure 8. Strategy for site-directed mutagenesis of *Dd.cdc2* gene

The 5′ and 3′ portions of the gene were amplified in two independent reactions. The two

PCR products were combined and serve as template for the amplification of the mutant

gene sequence.

performed with the two external primers (universal and reverse) using the conditions described above.

2.1.2. Plasmid construction

The vector act6*cdc2*Y21F (Figure 9) was made by treating the PCR-mutagenesis products with *Bam*HI and *Eco*RI. The fragment was ligated into the corresponding sites of the *D.discoideum* expression vector pDneoII (Witke, 1987). The cloning step places the gene under control of the constitutive *actin6* promoter. The wild type *cdc2* gene was directly subcloned from pBluescript KS (+) into the *Bam*HI and *Eco*RI sites of pDneoII to make *act6cdc2*.

To clone *cdc2* into the tetracycline response plasmid, the gene (and the mutant counterpart) was amplified from pDneoII using the oligonucleotides *Bgl*II*cdc2* and *Sph*I*cdc2* listed in Table 1. PCR products were treated with the appropriate endonucleases and cloned into the *Bgl*II/*Sph*I sites of MB38 to make TRE-$P_{min}cdc2$Y21F (Figure 10).

2.1.3. Gene disruption of the *Dictyostelium discoideum ppiA*

The strategy for disrupting *Dd.ppiA* gene is depicted in Figure 11. The primers used for this reaction are listed in Table 1. The 5′ and 3′ genomic fragments were amplified separately. The reaction contained 1 μM of each primer, 200 μM dNTPs, 50 ng of genomic DNA, 0.5 units of *Taq* DNA Polymerase in a 100 μl volume of 1 X buffer (20 mM Tris-HCl, 1.5 mM $MgCl_2$, 25 mM KCl, 0.05% Tween-20, 100 μg/ml BSA, pH 9.5). Amplification was carried out using the following thermal cycles; 94°C for 5 minutes,
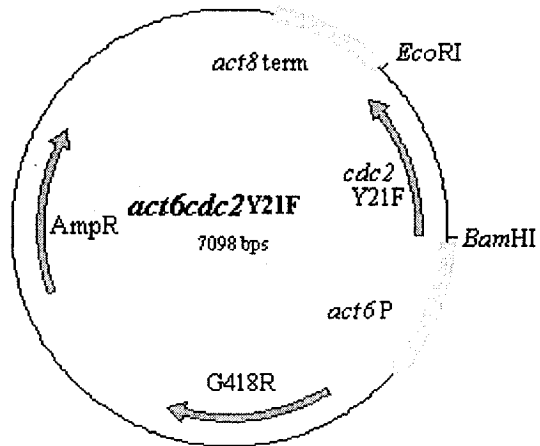
Figure 9. *act6cdc2*Y21F **vector.**

The vector contains a cassette conferring resistance to geneticin. The target gene is placed under the control of the constitutive *Dictyostelium discoideum actin6* promoter.
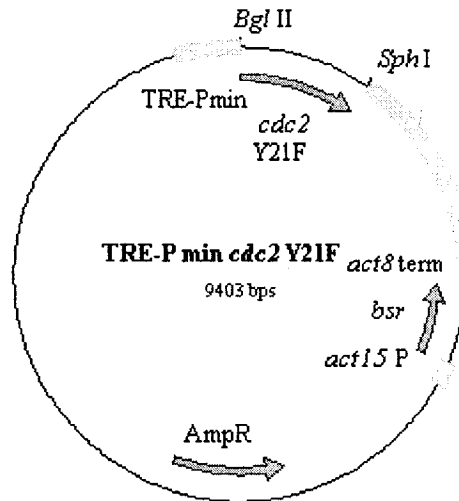
Figure 10. Response plasmid TRE-P$_{min}$cdc2Y21F.

The vector contains the ColEI origin for propagation, a blasticidin resistance cassette composed of the blasticidin deaminase gene under the control of the *Dictyostelium discoideum actin15* promoter and *actin8* terminator, and the doxycycline inducible promoter TRE-P$_{min}$.

50°C for 2 minutes, 68°C for 3 minutes then 30 cycles of 94°C for 1 minute, 50°C for 2 minutes, 68°C for 3 minutes and finally one segment of 94°C for 1 minute, 50°C for 2 minutes, 68°C for 10 minutes. Finally, 2% of the PCR product was analyzed on a 0.8% agarose gel cast in TAE (40mM Tris-acetate, 1mM EDTA, pH 8.0). The 5′ and 3′ genomic fragments were cloned into the *SalI/EcoRV* and *SpeI* sites of the pRHI119 plasmid (a gift from R. H. Insall), respectively, to make *ppi::bsr*.

2.2. Amplification and extraction of plasmid DNA

Individual vectors were introduced into *E. coli* strain XL1Blue (F′::Tn10(Tet^r) proA$^+$B$^+$ lacI^{q}Δ(LacZ)M15/ recA endA1 gyrA96(NaI^r) thi-1 hsdR17 (rk-mk-) glnV44 relA1 lac) by electroporation for amplification. Extraction and purification of the circular DNA was accomplished using the alkaline lysis method (Sambrook *et al.*, 1989).

2.3. Transformation of *Dictyostelium discoideum*

Vectors were introduced into *Dictyostelium discoideum* strain AX2 by the calcium phosphate method (Nellen *et al.*, 1987). Briefly cells were grown to a density of 1 X 10^6 cells/ml and 1 X 10^7 cells were transferred to a 100 mm Petri dish and left undisturbed for 30 minutes to permit adherence to the plastic. The growth medium was replaced with 10 ml of MOPS buffer (5 g/l yeast extract (Oxoid), 10 g/l proteose peptone (Oxoid), 55.5 mM dextrose, 6.2 mM MOPS, pH 7.1). Cells were allowed to recover overnight in axenic HL5 medium (Watts and Ashworth, 1970). Clonal transformants were selected 16 hours after glycerol shock in HL5 medium supplemented with 10 μg/ml

of G418 sulfate (BioShop, Burlington, Ontario, Canada). The medium was changed every two days.

To trace the fate of cells, double transformants were generated. The non cell-type specific reporter construct *act6lacZ* (a gift from H.K. MacWilliams) was introduced into AX2 cells. Additionally the cells were transformed with the *act6cdc2* or *act6cdc2*Y21F plasmids. Transformations were performed using the calcium chloride/ glycerol shock method. Routinely, 18 μg of *cdc2* vector (*act6cdc2* or *act6cdc2*Y21F) and 2 μg of vector carrying the gene coding for β-galactosidase were used in each transformation. To select for *cdc2*Y21F/*lacZ* double transformants a method developed by H.K. MacWilliams was employed (personal communication). Cells from a single transformation plate were harvested by centrifugation and suspended in KK2 (17 mM KH_2PO_4/K_2HPO_4, pH 6.2) at a density of 5 X 10^7 cells/ml. Next, 100 μl of the suspension (5 X 10^6 cells) were spread uniformly on 1.5% agar in KK2 buffer plus 100 μg/ml G418 in combination with *Klebsiella aerogenes*. The bacterial selection plates were incubated at 22°C for 3-4 days until fruiting bodies were macroscopically visible. Clonal isolates were blotted onto 9 cm nitrocellulose filters (Millipore, Bedford, Massachusetts, USA), frozen and subsequently fixed for twenty minutes in 1% glutaraldehyde (SIGMA, St. Louis, Missouri, USA). The filters were washed extensively in Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4 and 1 mM $MgSO_4$, pH 7.0) until the wash solution was free of bacteria. Filters were stained for β-galactosidase activity as described (Dingermann *et al.*, 1989). After incubation in the stain solution for 1 hour at room temperature the filters were incubated in 3% TCA for 10 minutes, rinsed twice with H_2O and allowed to air dry. Transformants with a moderate β-galactosidase expression level were selected to start a new culture.

Figure 11. *ppiA* **disruption by homologous recombination.**

The 5′and 3′ fragments of *ppiA* were PCR amplified separately and cloned into the *SalI/EcoRV* and *SpeI* sites of the pRHI119, respectively. The resulting vector, *ppi::bsr*, was cleaved with *SalI/NotI* and introduced into *Dictyostelium* cells. Homologous recombination between the incoming DNA and the resident *ppiA* sequence results in gene disruption.
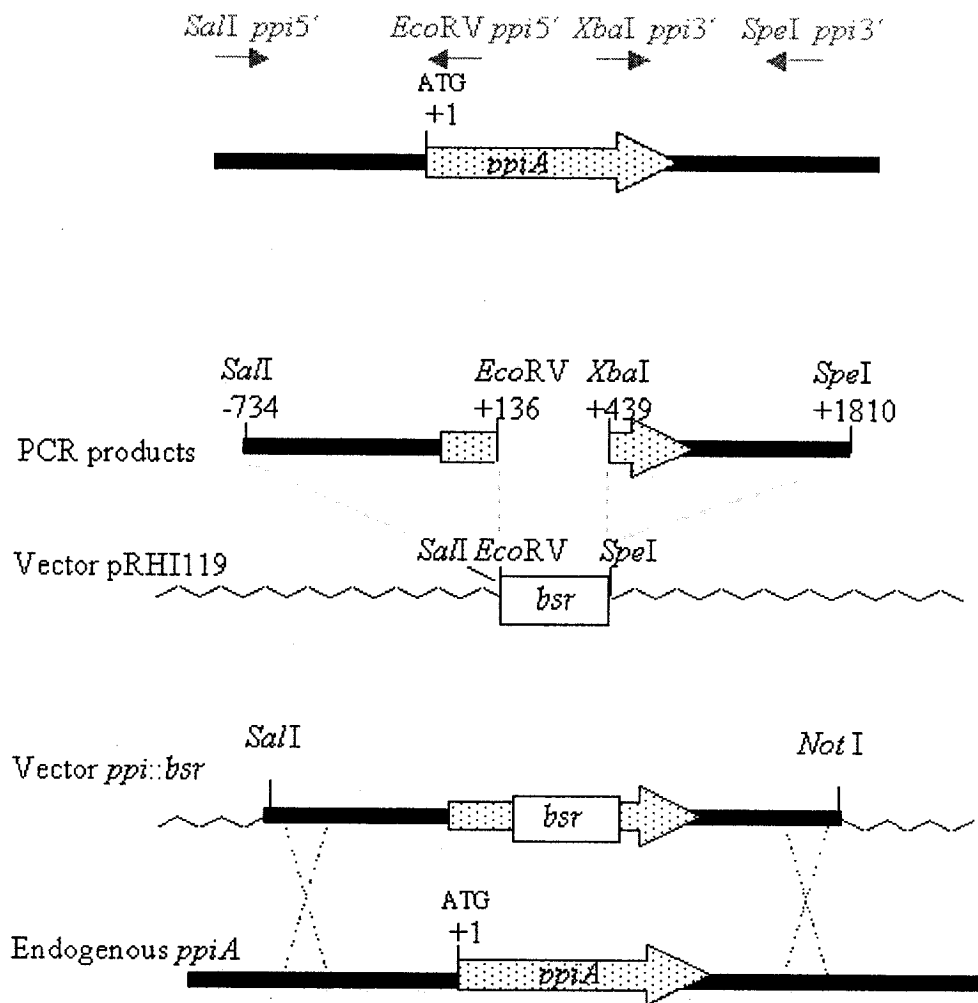
Coding region

Deleted region

Vector sequence

Flanking region

Homologous recombination

35

To confirm the presence of the *cdc2*Y21F gene in β-gal transformants, PCR reactions were performed on genomic DNA using a primer complementary to *cdc2* (Table1; primer *cdc2*Y21F sense) and a second primer designed to anneal within the backbone sequence of the vector (Table 1; pDneoII antisense).

For the transformation of cell lines harbouring the transactivator plasmid (a gift from H.K. MacWilliams), exponential phase cells were harvested by centrifugation and washed once in KK2 buffer then rewashed in ice-cold EP buffer (50 mM sucrose, 10 mM KH_2PO_4/K_2HPO_4, pH 6.1). The cell pellet was suspended in cold EP buffer at 10^8 cells/ml. The equivalent to 5×10^7 cells (0.5 ml) were combined with 30 μg of DNA and transferred to an ice-cold, sterile 4 mm electroporation cuvette. A single pulse of 0.85 kV with a capacity of 25 μF and a resistance of ∞ was applied to the cuvette containing the cell mixture. Following 5 minutes incubation on ice, 100 μl of the suspension were transferred onto a 100 mm culture dish. $CaCl_2$ and $MgCl_2$ were added to a final concentration of 1 mM each. After 15 minutes at room temperature, 10 ml of HL5 were added to the cell suspension. Cells were grown under G418 (5 μg/ml) and blasticidin (5 μg/ml) selection the following day. Colonies bearing the extrachromosomal vector appeared within seven days after transformation. To induce gene expression tetracycline (dissolved in ethanol) was added to a final concentration of 5 μg/ml. The volume of ethanol did not exceed 1 μg/ml.

For gene disruption by homologous recombination, the genomic construct, *ppi::bsr*, was cleaved with *Sal*I and *Not*I, phenol extracted and ethanol precipitated. Then, 10 μg of linearized DNA were introduced in *Dictyostelium* cells by electroporation (see above) and selected with 10 μg/ml blasticidin S (Sigma-Aldrich Canada Ltd.,

Oakville, Ontario, Canada). Transformed cell lines were cloned onto SM agar plates. Individual colonies were transferred into HL5 and propagated for Southern analysis.

2.4. Culture conditions and development of cells

Dictyostelium discoideum cells were grown axenically to mid-log phase in HL5 medium (Watts and Ashworth, 1970). Methods for initiating development on filters have been described elsewhere (Sussman, 1987). Briefly, amoebae were harvested by centrifugation at 700 g for 5 minutes and washed twice in ice-cold KK2 buffer (20 mM KH_2PO_4/K_2HPO_4, pH 6.2). Cells were plated at $1-2x10^6$ cells/cm^2 on filters or non-nutrient plates. Methods for obtaining synchronous cell cultures have been described elsewhere (MacWilliams *et al.*, 2000)

2.5. Bromodeoxyuridine immunological staining

Cells were grown in the presence of 200 μM bromodeoxyuridine directly on circular cover slips settled in 24 well plates. Samples were fixed in 70% ethanol and rehydrated in TBST (10 mM Tris-Cl, 130 mM NaCl, 0.5% Tween-20, pH 8.0). Slides were submerged in 2 N HCl for 1 hour at room temperature, transferred to a clean 24-well plate, washed four times 5 minutes in TBST and analyzed for BrdU incorporation immediately or stored at 4°C overnight. For bromodeoxyuridine detection, samples were incubated in 150 μl of a 1:100 dilution of anti-BrdU antibody (ICN, Aurora, Ohio, USA) in TBS for one hour at room temperature with constant agitation. Slides were washed three times 5 minutes in TBST and incubated for 2 hours in a 1:3000 dilution of goat anti-mouse antibody conjugated to alkaline phosphatase (ZYMED, San Francisco,

California, USA). Slides were washed three times in TBST, transferred to a clean 24-well plate, washed extensively in TBS to remove any traces of detergent and rinsed once in alkaline phosphatase buffer (100 mM Tris-Cl, 100 mM NaCl, 5 mM MgCl$_2$, pH 9.5). Slides were incubated in 0.4 mM of both BCIP and pNBT (GIBCO BRL, Burlington, Ontario, Canada) for no more than 30 minutes. Reactions were terminated with 3 mM EDTA.

2.6. Genomic DNA preparation

Exponentially growing cells were harvested by centrifugation in a cold rotor at 2000 g for 5 minutes. The cell pellet was washed twice in ice-cold 0.2% NaCl solution then resuspended in NP40 lysis buffer (50 mM HEPES, 5 mM MgCl$_2$, 10% sucrose, 2% NP40 detergent, pH 7.5 adjusted with KOH) at 5 X 10^7 cells/ml. The suspension was vortexed twice for thirty seconds at maximum speed followed by centrifugation at 300 g to remove unlysed material and cell debris. The supernatant was transferred to a clean microfuge tube and submitted to 5 minutes of centrifugation at 2000 g to harvest the nuclei. The pellet was resuspended in 100 μl of nuclei isolation buffer (10 mM Tris-Cl, 10 mM NaCl, 1 mM MgCl$_2$, 3 mM CaCl$_2$, 3.4 % sucrose, pH 7.4) and an equal volume of 2 % SDS. The sample was heated at 65°C to lyse the nuclei. Following an incubation period of 15 minutes one volume of T.E. (10 mM Tris-Cl, 1 mM EDTA, pH 9.5) was added to the mixture along with proteinase K (final concentration of 200 μg/ml). The sample was treated for 2 hours at 50°C followed by an organic extraction (Sambrook *et al.*, 1989). Nucleic acids were precipitated by the addition of two volumes of ethanol/2.5 M ammonium acetate. The mixture was stored overnight at –20°C to ensure complete

precipitation of nucleic acids. The supernatant was removed by aspiration after centrifugation at maximum speed for twenty minutes in a tabletop microfuge. The pellet was rinsed twice in ice-cold 70% ethanol and resuspended in 100 μl of HPLC-grade H_2O (Fisher Scientific, Fair Lawn, New Jersey, USA). Following RNase treatment for 1 hour at 37°C the DNA was re-extracted using phenol:chloroform.

2.7. RNA preparation

Exponentially growing cells were harvested by centrifugation at 700 g in 50 ml centrifuge tubes. Total cellular RNA was extracted from 1 X 10^8 cells using 1 ml TRIZOL reagent (Invitrogen Life Technologies, Carlsbad, California, USA). Nucleic acids were quantified by recording the absorbance reading at 260 nm and 280nm using a Cary 50 spectrophotometer (Varian, Mississauga, Ontario, Canada).

2.8. RNA and DNA blots

Ribonucleic acids (10 μg) were resolved on a formaldehyde gel as described (Sambrook *et al.*, 1989). DNA fragments were separated by slow electrophoresis (1 V/cm) through an 0.7% agarose gel. Following electrophoresis nucleic acids were transferred by capillary action onto a Nytran membrane (Millipore, Bedford Massachusetts, USA) in 10 X SSC (1.5 M NaCl, 0.15 M trisodium citrate, pH 7.0) and cross-linked by UV irradiation (UV Stratalinker 8000, Stratagene, La Jolla, California, USA).

Radiolabelled probes were synthesized by random priming using [α ^{32}P] dCTP (Amersham Biosciences, Baie d'Urfé, Québec, Canada). Unincorporated nucleotides

were removed by gel filtration through a Sephadex G-50 size exclusion column (Amersham Biosciences, Baie d'Urfé, Québec, Canada). Hybridizations were performed at 42°C in 5 X Denhardt's, 5 X SSPE (0.75 M NaCl, 0.05 M Na_2HPO_4, 0.05 M EDTA, pH 7.4), 0.5% SDS, and 100 μg/ml salmon sperm DNA and 50% formamide (Sambrook et al., 1989). Stringency washes were done at 65°C in 1 X SSC and 0.1% SDS. Northern blots were exposed to Fuji film (Fisher Scientific, Fair Lawn, New Jersey, USA) under an intensifying screen.

2.9. Distribution experiments

Strains were combined in a ratio of 1 to 9 and grown in shaken suspension overnight at 22°C on a rotary shaker (125 rpm) in the absence of antibiotics. Cells were washed free of medium and developed on pre-boiled nitrocellulose filters resting on KK2-saturated support pads at a density of 2 X 10^6 cells/cm^2. For β-gal distribution experiments a wild-type strain bearing the empty neomycin resistance vector was used as a sorting partner (Schulkes et al., 1995). At appropriate developmental stages 1/6th of the filter was immersed in fixative (60 mM Na_2HPO_4, 40 mM NaH_2PO_4 and 1 mM $MgSO_4$, pH 7.0, 2% Tween-20 and 0.5% glutaraldehyde). Samples were fixed for 10 minutes, rinsed in Z buffer and assayed for β-galactosidase activity by incubating in Dingermann's cocktail (5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 1 mM EGTA and 1 mM X-gal in Z-buffer) until staining was discernible under a light microscope (Dingermann et al., 1989). The reaction was stopped with 3% TCA. The filters were washed in water, mounted onto a microscopic slide under coverslips, and examined on a Zeiss Axioplan microscope fitted with a Kodak MDS 120 digital camera and 10 X objective.

For Gfp/Bfp distribution assays, cells were dispensed onto 1.5% purified agar/H_2O plates +/- 20 μg/ml doxycycline at a density of 2 X 10 ^{6} cells/cm^2. Plates were wrapped in aluminium foil and incubated at 22°C in a moist chamber. Distribution preferences were monitored by squashing slugs under coverslips and observing the structures under a fluorescence microscope (10X magnification).

2.10. Flow Cytometry

Cells were washed twice in KK2 buffer and resuspended in low fluorescence medium (MacWilliams, personal communication; 1 mM NH_4Cl, 20 μM $CaCl_2$, 0.1 mM $FeCl_3$, 0.4 mM $MgCl_2$, 12 μM Na_2EDTA, 8 μM $ZnSO_4$, 0.18mM H_3BO_4, 2.6 μM $MnCl_2$, 0.7 μM $CoCl_2$, 0.6 μM $CuSO_4$, 0.08 μM $(NH_4)_6Mo_7O_{24}$, 5 mM K_2HPO_4, 5g/l casein peptone, 1 g/l yeast extract). Cells were grown for approximately 2 generations on 100 mm Petri dishes to 50% confluency. An aliquot of each culture was analyzed on a PARTEC (Coulter Epics) using an argon laser to illuminate the samples at 488 nm. For each sample 100 000 events were collected and classified into two-dimensional histograms.

2.11. Sensitivity tests to the damaging agent methyl methanesulfonate

Dividing cells were tested for sensitivity to methyl methanesulfonate (Sigma Aldrich Canada Inc., Oakville, Ontario, Canada). The damaging agent was diluted to 1 M in HL5 medium and added directly to the cell suspension. To determine cell counts, an 800 μl aliquot of the culture was diluted in 10 ml of 0.9% NaCl. Cultures were counted in quadruplicate every half hour using a Coulter counter.

3. RESULTS

3.1. Overexpression of cdc2 or cdc2Y21F does not block the *Dictyostelium* cell cycle

In yeast, phosphorylation of the Y15 residue prevents cell cycle progression (Gould and Nurse, 1989). Conversely, mutants expressing a non-phosphorylatable version of cdc2 (cdc2Y15F) have a shorter cell cycle (Krek *et al.*, 1992). To determine how this mutation would affect the *Dictyostelium* cell cycle, a similar mutant was generated in *Dictyostelium* (cdc2Y21F). The growth rate of transformants bearing wild type or mutant cdc2 under the control of a tetracycline inducible promoter (TET-P_{min}cdc2 and TET-P_{min}cdc2Y21F) was monitored for 10 hours after doxycycline induction (Figure 12). Expression of the mutant gene did not appear to alter growth rate.

3.2. cdc2Y21F mutation does not affect cell cycle length

To determine the duration of the cell cycle for both the mutant (act6cdc2Y21F) and wild type strains (act6cdc2) cultures were grown in the presence of the thymidine analogue, bromodeoxyuridine, for approximately one generation. We monitored the fraction of BrdU labeled cells every hour (Figure 13). The number of BrdU positive cells rose steadily over time. The experimental data were fitted to theoretical curves (gray) representing various cell cycle time-spans (MacWilliams *et al.*, 2000). The comparative results denoted a cell cycle of 10-11 hours for both the wild type and mutant strains. The length of the cell cycle therefore remained unchanged in the Cdc2Y21F mutant.
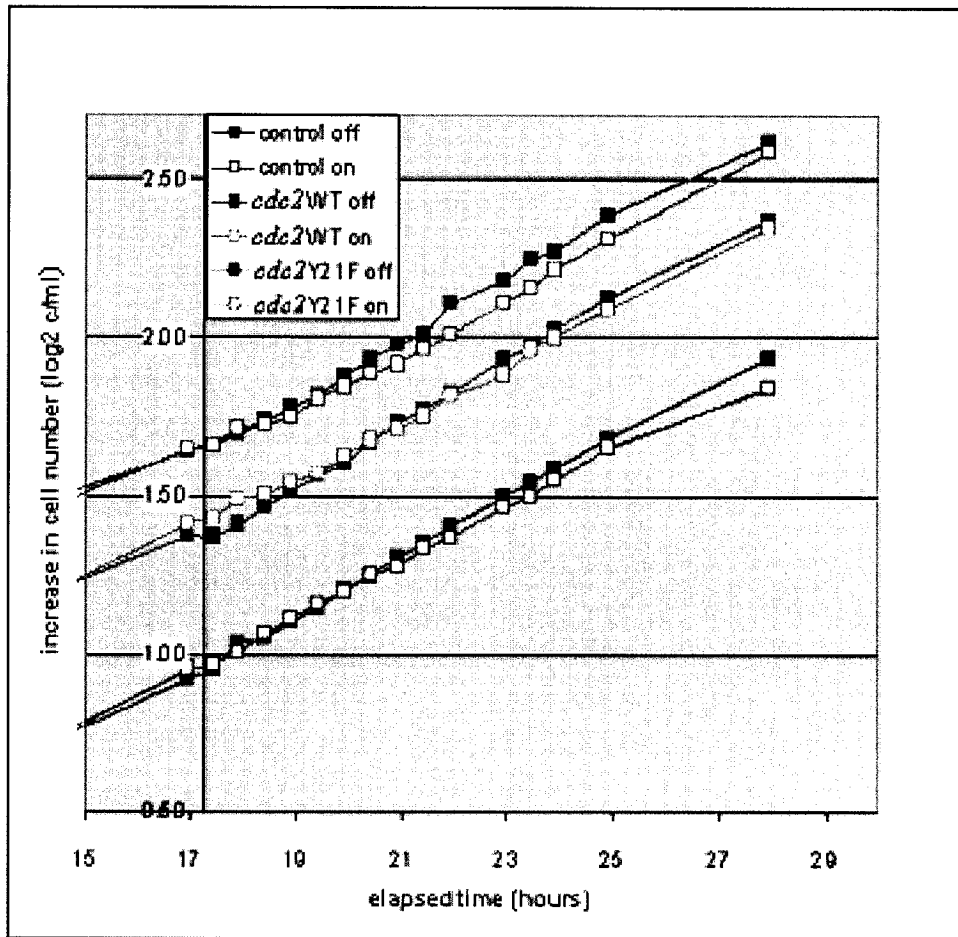
Figure 12. Inducing *cdc2*Y21F in vegetative cells.

Gene expression was induced by adding doxycycline as described in Materials and Methods. The point where the y-axis intercepts the x-axis indicates the time when doxycycline was added. Solid symbols represent non-induced cultures whereas hollow markers represent cultures that are actively transcribing the target gene.
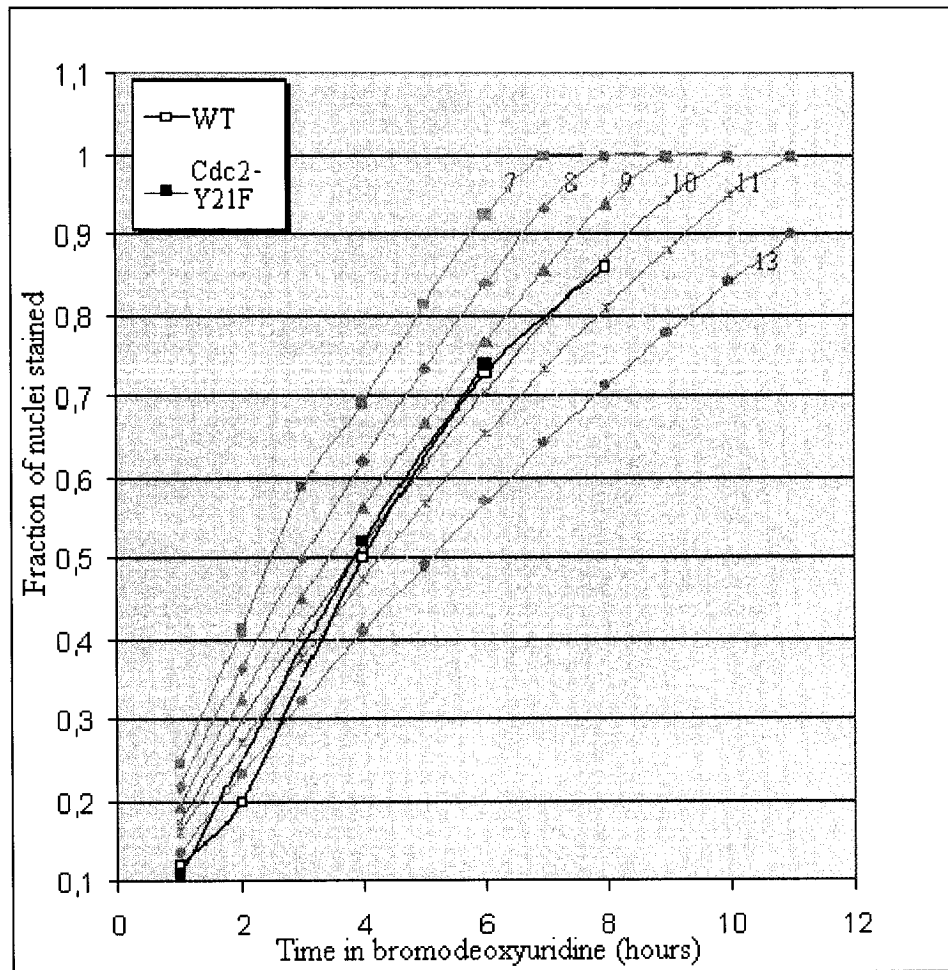
FIGURE 13. Estimation of cell cycle length using bromodeoxyuridine.

The gray curves represent theoretical cell cycles ranging from 7-13 hours (MacWilliams, et al., 2000). The experimental curves for both the wild type (blue) and Cdc2Y21F (red) cultures fall between the 10 hour and 11 hour theoretical curves indicating a 10-11 hour cell cycle.

3.3. Cdc2Y21F mutation does not affect cell size

FACS analysis was performed on vegetative cells to study the effect of Cdc2Y21F expression on cell size. Following excitation at 488 nm, forward light scatter was detected. Forward scattering patterns are correlated to cell size. We compared the FSC plots of cell constitutively expressing *cdc2*Y21F to the patterns obtained from wild-type cells and cells overexpressing *cdc2*WT. As shown in Figure 14, the histograms overlap suggesting that the mutation did not affect cell size. Similar results were obtained with doxycycline-inducible cell lines (data not shown).

3.4. Cell cycle response to methyl methanesulfonate in *Dictyostelium discoideum*

Vegetative cells were treated with the DNA damaging agent methyl methanesulfonate. Exposure to 10 mM MMS has been shown to cause effective DNA damage to *D. discoideum* cells (Gaudet and Tsang, 1999). As shown in Figure 15, we find that much lower doses caused cell cycle arrest. Addition of 0.68 mM MMS to the control and mutant cultures severely inhibited growth. The rapid cessation in cell division following treatment with MMS suggested the presence of a DNA damage checkpoint shortly before the onset of mitosis.

In view of these results wild type cells were treated with 0.68 mM MMS overnight, washed (point where the y-axis crosses the x-axis) and resuspended in conditioned medium. Aliquots of the culture were removed at regular intervals and cell counts were measured using a particle counter. Following a 6 hour lag phase there was a sharp increase in cell density (Figure 16). The observed behavior can be explained if one
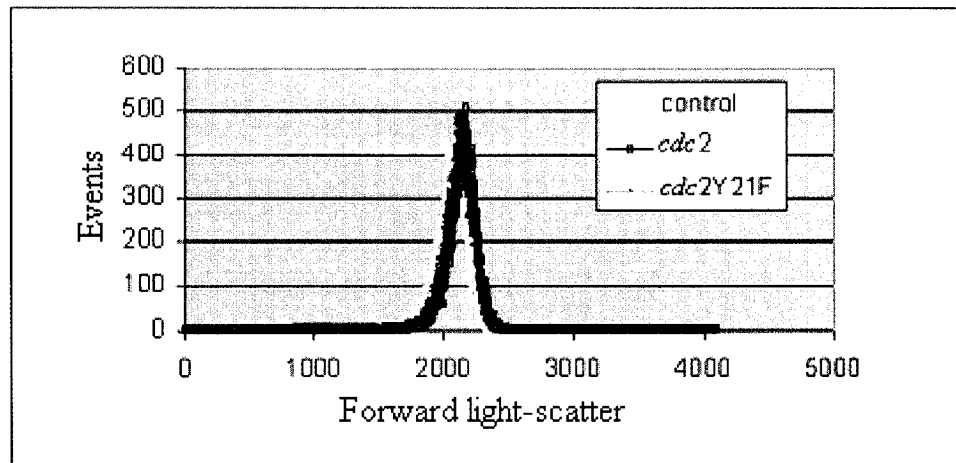
Figure 14. Effect of *cdc2*Y21F on cell size.

Histograms illustrating the forward light scatter of control cells (yellow), cells overexpressing wild-type *cdc2* (blue) and cells expressing mutant *cdc2*Y21F (red).
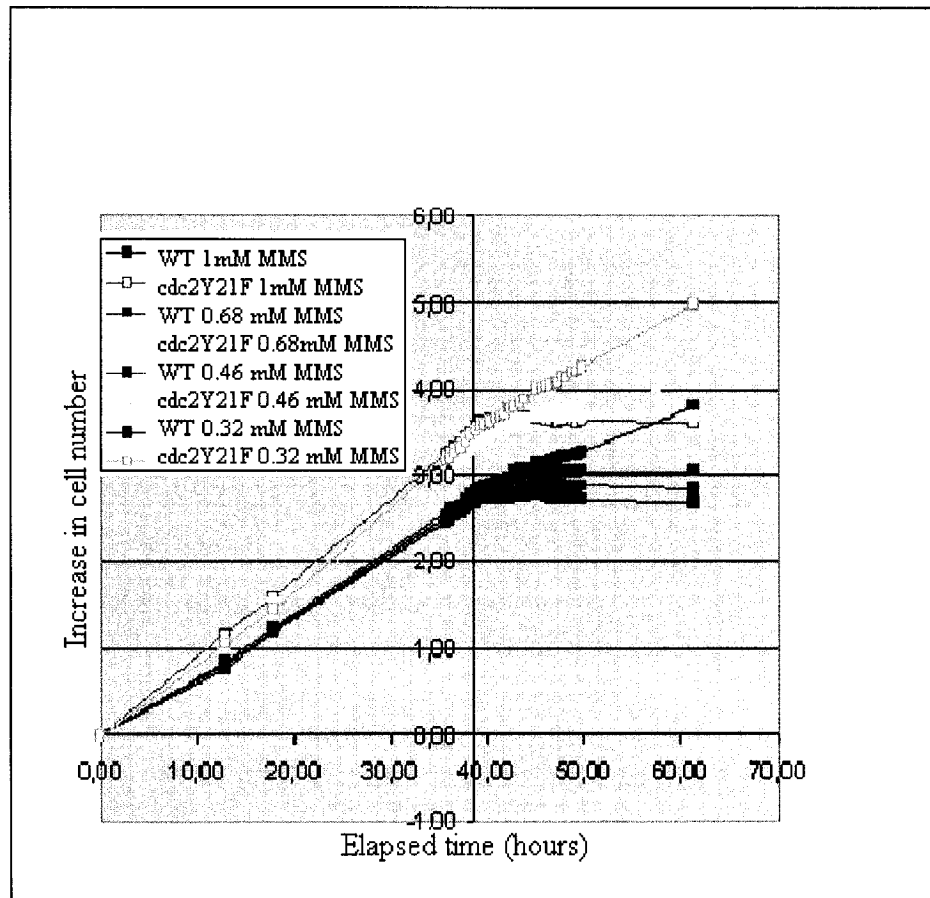
Figure 15. Effect of methyl methanesulfonate on the *Dictyostelium discoideum* cell cycle.

Wild type and Cdc2Y21F cultures were treated with 0.32 mM, 0.46 mM, 0.68 mM or 1 mM MMS. The point where the y-axis intercepts the x-axis indicates the time when the agent was added to the cultures. Samples were collected every thirty minutes and used to determine cell density.
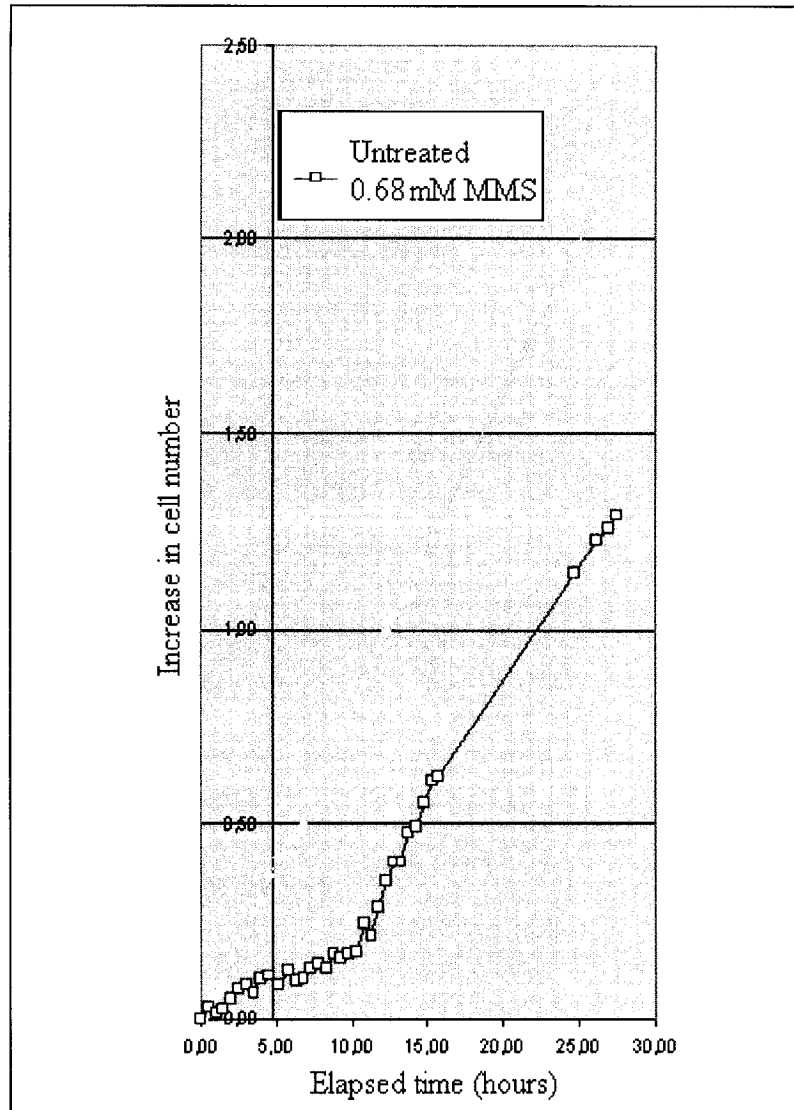
Figure 16. Cell cycle progression after transient treatment with methyl methanesulfonate.

Cells were treated with methyl methanesulfonate overnight (red curve), washed and cell growth was monitored as described in Materials and Methods. As a control, we monitored the growth rate of an untreated culture (yellow curve).

assumes that after exposure to DNA damaging agents cells temporarily arrest shortly before mitosis. Cells accumulate at this point and then reenter the cell cycle with partial synchrony. These results imply that a DNA damage checkpoint is operative in *Dictyostelium*.

3. 5. Differentiation preference of Cdc2Y21F cells

To study the cell type preference of the Cdc2 mutant, a mixture of 10% Cdc2Y21F cells bearing the β-gal marker with 90% wild type cells was made and chimeric structures were encouraged to form. At the tight aggregate, slug or early culminate stage (for developmental stages, see Figure 1) the cells were fixed and stained for β-galactosidase activity. Strain Cdc2Y21F distributed randomly throughout the mound (Figure 17, panel 1A). During later stages of development, however, strong biases became evident. The mutant sorted predominantly to the frontal prestalk region of the early culminates (Figure 17, panel 1B and 1C). Using the opposite combination (blue wild-type cells mixed into an unlabelled mutant population) there was an appreciable accumulation of wild type cells within the prespore zone of the migrating slug (Figure 17, panel 2E) and early culminate (Figure 17, panel 2F) and late culminate (Figure 17, panel 2G). Control synergy experiments eliminated the likelihood of sorting artifacts. As shown in panel 4M mutant cells in a mutant background distributed randomly throughout the entire length of the slug.

We used Gfp as a lineage marker to analyze sorting behaviours of cells expressing *cdc2*Y21F under a tetracycline-inducible promoter. In the absence of dox the promoter was virtually silent (Blauuw *et al.*, 2000). Accordingly, the cells did not display a

1 — A, B, C
2 — D, E, F, G
3 — H, I, J
4 — K, L, M

A

B

C

10% cdc2Y21F/lacZ
+90% WT

D

E

F

G

10% WT/lacZ
+90% cdc2Y21F

H

I

J

10% cdc2Y21F/lacZ
+ 90% cdc2^{OE}

K

100% lacZ

L

10% cdc2^{OE}/lacZ
+90% cdc2Y21F

M

10% cdc2Y21F/lacZ
+90% cdc2Y21F
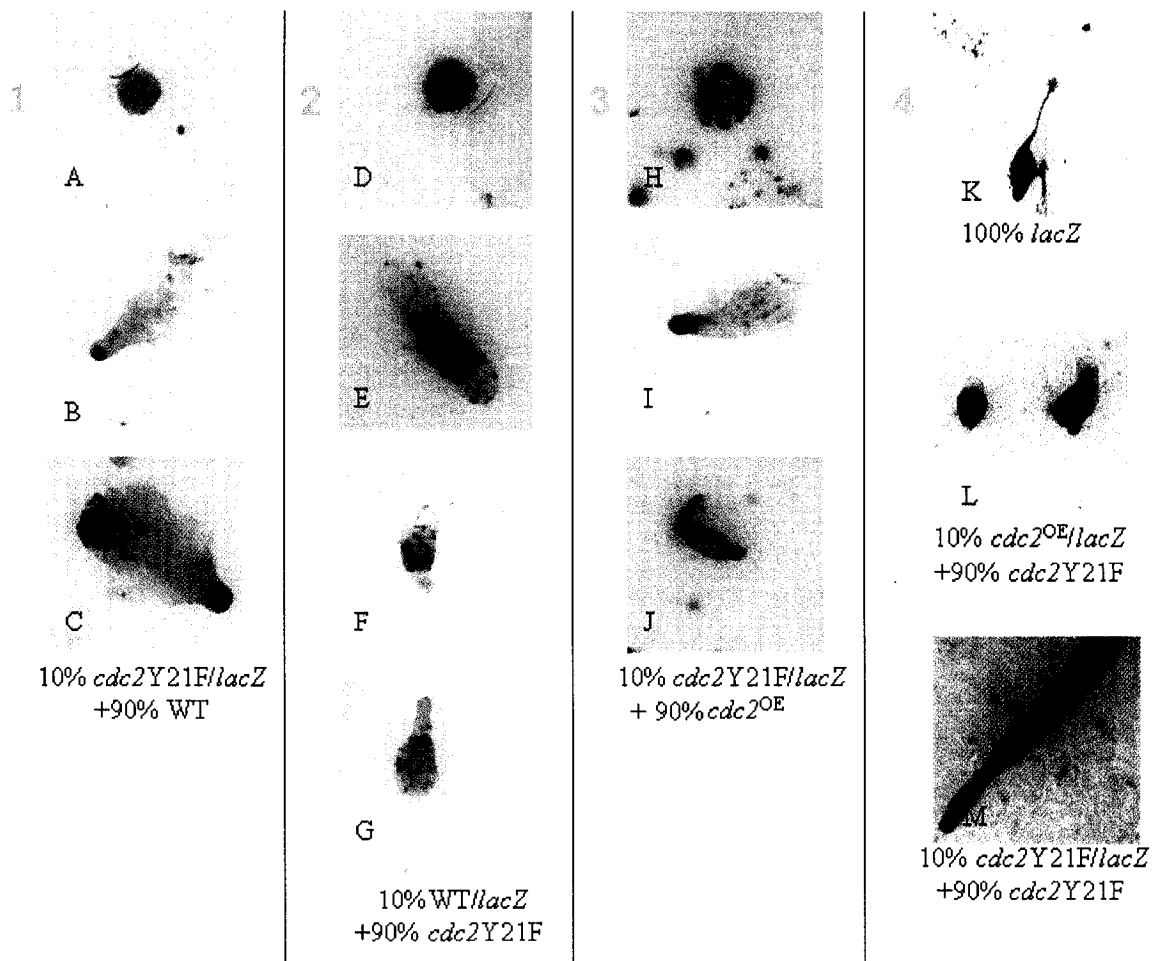
Figure 17. Sorting experiments with *Dictyostelium* cells expressing *lacZ*.

lacZ tranformants were mixed with unmarked cells as described in Materials and Methods. Cells were fixed at various stages of development and stained for β-galactosidase activity.

Figure 18. Differentiation preference of Cdc2Y21F using dox-inducible system.

Cells were developed in the presence of doxycycline as described in Materials and Methods to induce gene expression. Cells expressing *cdc2*Y21F sort to the prestalk zone when mixed with wild type cells (panel 2) while non-induced (panel 3) and wild type (panel 1) cells sort randomly in a wild type background.

1

2

3

10% Gfp wt
+
90% Bfp wt

10% Gfp/*cdc2*Y21F
induced
+
90% Bfp wt

10% Gfp/*cdc2*Y21F
not induced
+
90% Bfp wt

52
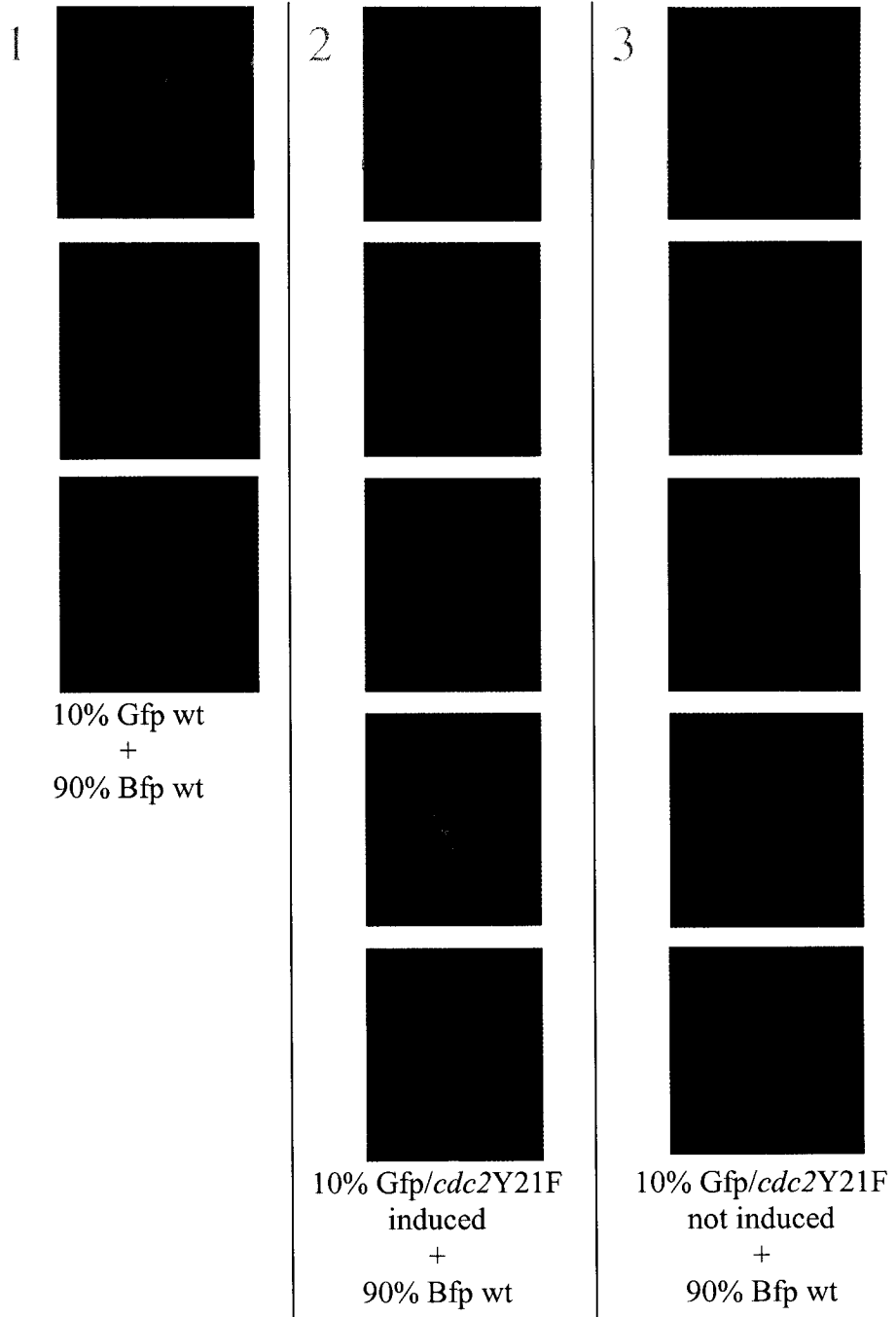
distribution preference (Figure 18, panel 3). When *cdc2*Y21F gene expression was induced with doxycycline, however, a distinct pattern emerged. During the slug stage of development, green cells (Cdc2Y21F) were found in the anterior, prestalk region. During the early culminate stage, these cells were found concentrated in the tip and in the growing stalk (Figure 18, panel 2). The prespore region was almost devoid of Gfp-labeled cells. Hence, synergy experiments using fluorescent proteins as cell markers showed that Cdc2Y21F cells are predisposed to become stalk cells. In control experiments Gfp wild-type cells mixed with a Bfp counterpart did not show a sorting preference (Figure 18, panel 1). These findings are consistent with the results obtained using β-galactosidase.

3.6. *ppiA* is expressed during the mid-G2 phase of the cell cycle

In yeast and human cells, FK506 binding protein is an enzyme that catalyses the interconversion of cis-trans isomers of prolyl residues in proteins and peptides. By sequence alignment and compilation using genomic clones from the Jena data bank (Department of genome analysis; http://genome.imb-jena.de/dictyostelium/) we identified a FKBP12 homologue in *Dictyostelium* that we termed *ppiA* (Figure 19).

To study the expression pattern of *ppiA* during the cell cycle total RNAs from cold-synchronized cells were isolated, size fractionated and analyzed by Northern blotting. The blot was probed with a 507 bp genomic fragment from the 3' end of *ppiA*. As shown in Figure 20B, the endogenous *ppiA* message peaked between 6 to 9 hours placing *ppiA* expression in mid-G2 of the cell cycle.

3.7. Disruption of the *Dictyostelium discoideum ppiA* gene

To investigate the function of *ppiA* in *D.discoideum* a gene disruption mutant was made as described in Materials and Methods. Clonal isolates were screened by PCR (data not shown) and by Southern blot analysis (Figure 22). A clone resistant to blasticidin but bearing the intact *ppiA* locus was set aside to serve as a control.

3.8. Effect of *ppiA* disruption on development

To study the role of *ppiA* in *Dictyostelium* development, wild type and *ppiA* knockout cells were washed free of nutrients and starved. The results are shown in Figure 23. Wild type cells formed discrete aggregation centers while *ppiA* knockout mutants formed large aggregates indicating that *ppiA* may play a role in early development.

Figure 19. Nucleotide and deduced amino acid sequence of the *Dictyostelium*

***discoideum ppiA* gene.**

Coding regions are indicated with a solid triangle. Letters in black indicate the cDNA

sequence. Letters in gray represent introns and flanking genomic sequences. The asterisk

indicates the stop codon (TAG). The amino acids appear in single letter code below the

cDNA sequence. The *ppiA* sequence is available on Dictybase (dictybase.org).

```
                                                           ▼
tcttccaaaa cctatatgta aaatttttaa aatcaattaa aaacatgtgg aattatatca aatctaatat
                                                         M  W   N  Y  I   K  S  N  M

gaaatatcat atccaatcaa taaatcaaaa gtcgcctcca catgttccaa aattaggggt tcaaacatta
 K  Y  H   I  Q  S   I  N  Q  K   S  P  P   H  V  P   K  L  G  V   Q  T  L

aacgttgatg aatatattga aatttgtgcg aataaccaat tcaaatgtgg aaattataac attccattgg
 N  V  D   E  Y  I  E   I  C  A   N  N  Q   F  K  C  G   N  Y  N   I  P  L

cattacatgg tgttgatgaa aatttaccat ttagatacca tagattgaca cattctcccg ataggatcaa
 A  L  H  G   V  D  E   N  L  P   F  R  Y  H   R  L  T   H  S  P   D  R  I  N

taatagaaga tcttatacca aatacaatgt taaaatatta tcaatatttt caaatgtctc taaccatcgt
 N  R  R   S  Y  T   K  Y  N  V   K  I  L   S  I  F   S  N  V  S   N  H  R
                                                                  ▼
gtatatgatc tattaccttt ttctcaaata tcctctacaa attcaaaccc aaacgtttgt tcaaataatg
 V  Y  D   L  L  P  F   S  Q  I   S  S  T   N  S  N  P   N
aaaatgataa tgaaacctat gatagtgaaa ctgataatga aaccaatcat agtgaaactg ataatagtga
tgatatgaat atgatgacaa agataataat aataataaaa acaataaatc aaacaaaaga aatcatctgg
agatcaaaaa aaaaaaaaaa aaaaaaaatg agtggggtta taaaaaaaaa taaaataaat atcctggggtt
ttttgaaaaa aattctcaga tgaggtaaaa cattaattaa aacttgaaca ccttttgatt tataaattgg
aattctttta aaagttgtgg aagatacaat tattttattt caatttgagg tattatttat tgcagccaca
ggttgatgtt gttaccaaaa aaaaaaaaag ttttttttaa ttttttttaa ttttattttt tttttttttt
tttttttttt tttttttttt tgactttttt tttttttttt ttttgacttt tttttttttt atttttccat
aacaaaataa ttcaaacttg ataaataaaa caatggtgag ttgagtttga acaacaaaac gttttgatca

atgaattaaa atctaatata tatatatttt tttttttttt aaaaaaatca gggtgttgaa atcacaatta
                                                         G  V  E   I  T  I
                                                         ▼
ttaaagaagg taaaggtaat attccaccag ttggttcaaa tgtaactgta catgtaagtt tttttttttt
 I  K  E  G   K  G  N   I  P  P   V  G  S  N   V  T  V   H

ttattatcaa ataaacaaat aaaaaaaaaa aaaaactaat tcacaatata tatatattat tctataatta

ttaataaagc atgcaggtac tttaactaat ggtaccgttt ttgattcatc aagaaagaga ggccaaccat
      H  A  G  T    L  T  N   G  T  V   F  D  S  S   R  K  R   G  Q  P
                              ▼
ttaatttttaa acttgggggct gggtatgcaa attctataat tttttttttt ttttaaactc tatcccaatc
 F  N  F  K   L  G  A   G
                                                   ▼
ataattttct aatttttttt ttttccctct ctaaaacttt atagtcaagt catcaaaggt tgggatgaag
                                                    Q  V   I  K  G   W  D  E

gtgttgctaa aatgaaagtt ggtgaaacct caaaattaac cattagccca gattttggat atggtgctag
 G  V  A  K   M  K  V   G  E  T   S  K  L  T   I  S  P   D  F  G   Y  G  A  R
                                       ▼
aggtgctggt ggtgttattc caccaaatgc aacgttagta ttccctattt taatcattcc ccttttttttt
 G  A  G   G  V  I   P  P  N  A   T

tttttttttt tttttttttt ttttaataga ctgtctttga agtagaatta atcatttata tattttttta
tagacttgtc tttgaagtag aattaatcac atttaaataa agagatgaag aattattcaa ataaatttaa
gggaattttta atattggtta acaaatatat tttctcgatt atgatttttt aaatcaacca cttttttttt
                                                                  ▼
tttatttttt tattttaaa gtctcaattt ttacaattat tattattatt atagccttgt caataatgaa
                                                                  L  V   N  N  E
            ▼
aaatagattt aattgggtga gatcaaggtt ttgaaacttg tcgttcaaaa atcttctaaa attttgttat
 K  *
```

Figure 20. **(A) Schematic representation of cell cycle phase at the time when RNA was extracted. (B) Northern blot analysis of** *ppiA* **gene expression during** *D. discoideum* **cell cycle.**

RNA was extracted from cold-synchronized cells and used for Northern blot analysis. Total RNAs were separated, blotted and probed with the 3′ end of *ppiA* as described in Materials and Methods. Molecular weight markers are indicated on the left.

A.

B.

Time of RNA extraction (hours)

(bases)   0 1 2 3 4 5 6 7 8 9
3000-
2000-
1500-
1000-   ←   *ppiA*
 500-

ppiA wild type

BcII EcoRI

AluI BcII EcoRI

AluI BcII EcoRI

4183bp

5193bp

5421bp

ppiA knockout

BcII EcoRI

AluI

AluI BcII EcoRI

bsr

2100bp

6259bp

6487bp
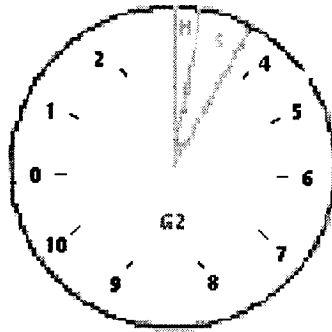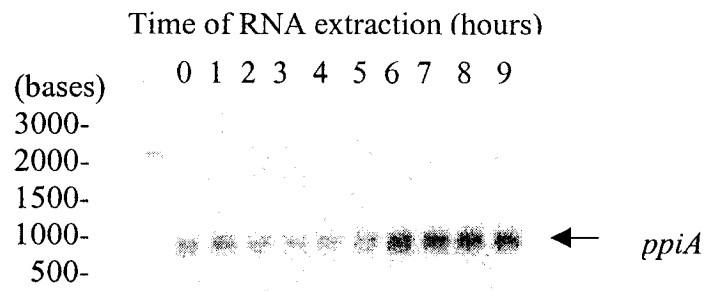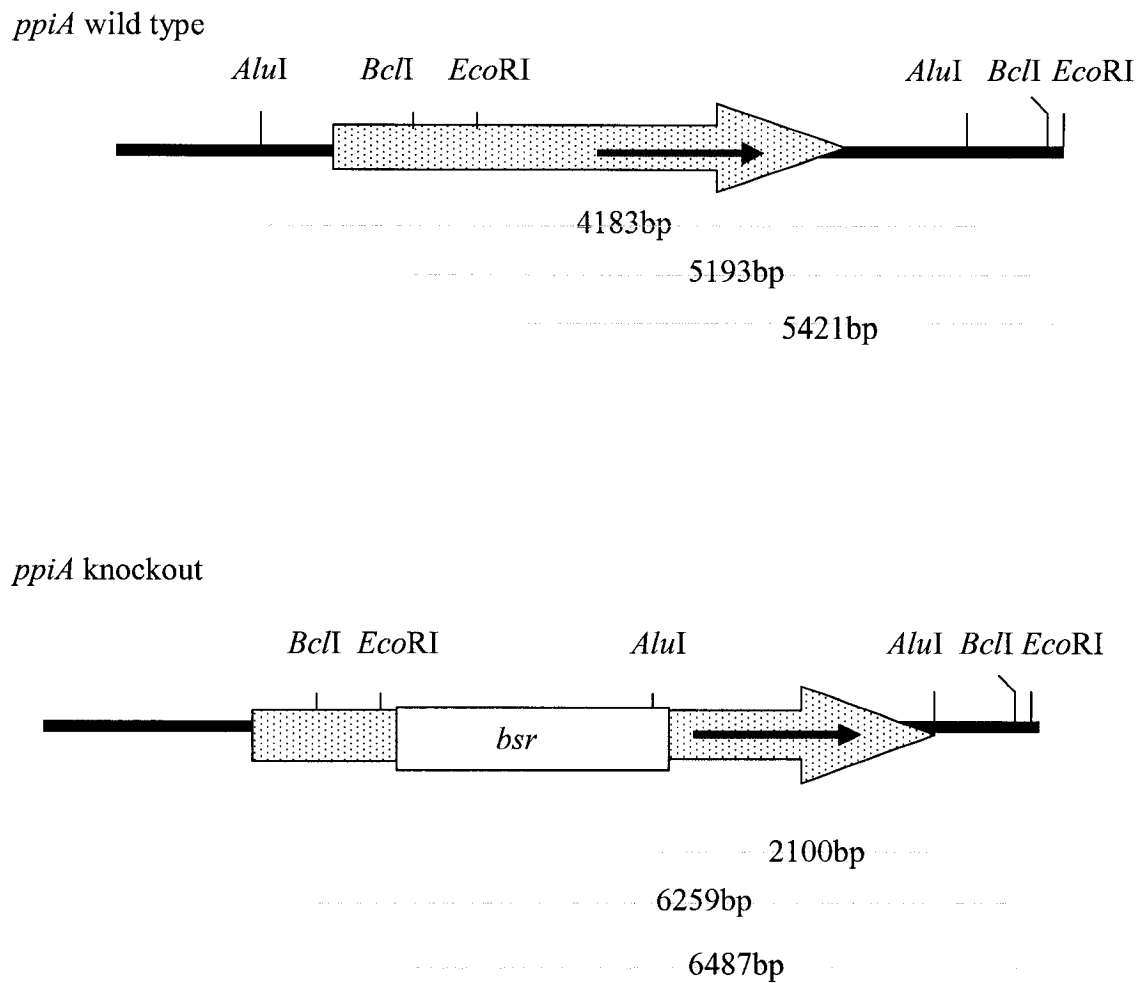
Figure 21. Restriction map of *ppiA* locus

The *ppiA* gene is shown (dotted box). The red arrow indicates the region where the probe

anneals to the gene. Restriction endonuclease sites are given. Lines below the *ppiA*

locus represent the predicted band fragments after endonuclease treatment.
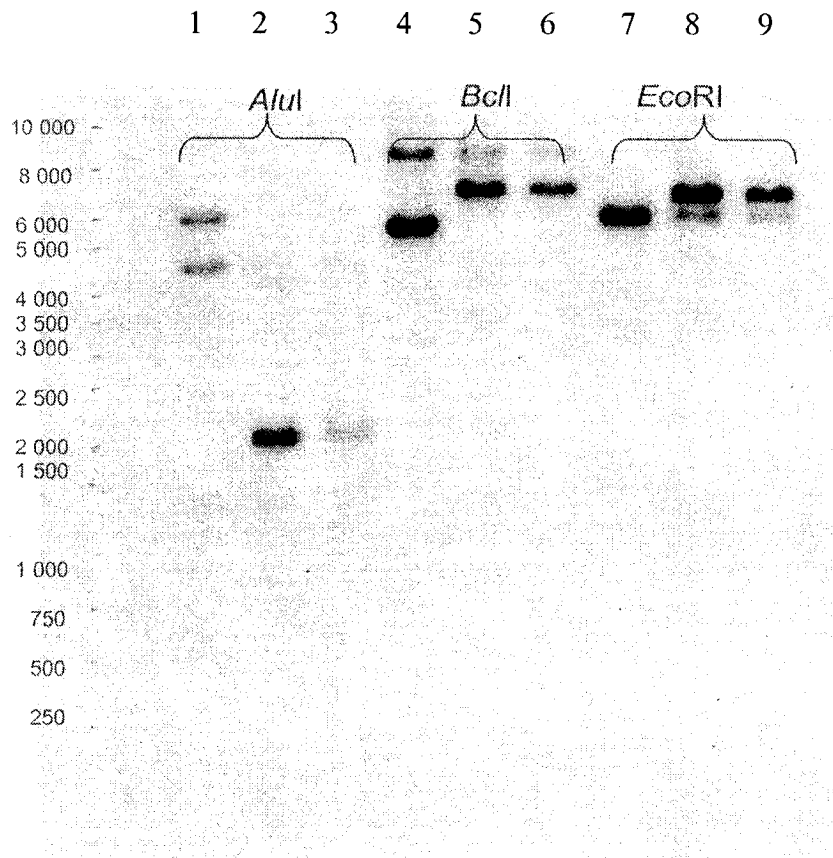
Figure 22. **Southern blot analysis of genomic DNA from wild type and** *ppiA*

disruption strains.

Genomic DNA was isolated from two independent clones in addition to the parental

strain, digested with *Alu*I, *Bcl*I and *Eco*RI, and subjected to Southern blot analysis. The

probe used was the genomic fragment from the 3′ end of *ppiA*.   The sizes of the

fragments hybridizing to the probe are indicated in base pairs. The control (lanes 1, 4 and

7) and the *ppiA* knockout mutants clone 1 (lanes 2, 5, 8) and clone 2 (lanes 3, 6, 9) show

the expected band patterns (see Figure 21) indicating that the endogenous *ppiA* gene has

been successfully disrupted.

Time after starvation = 1h   2h   3h
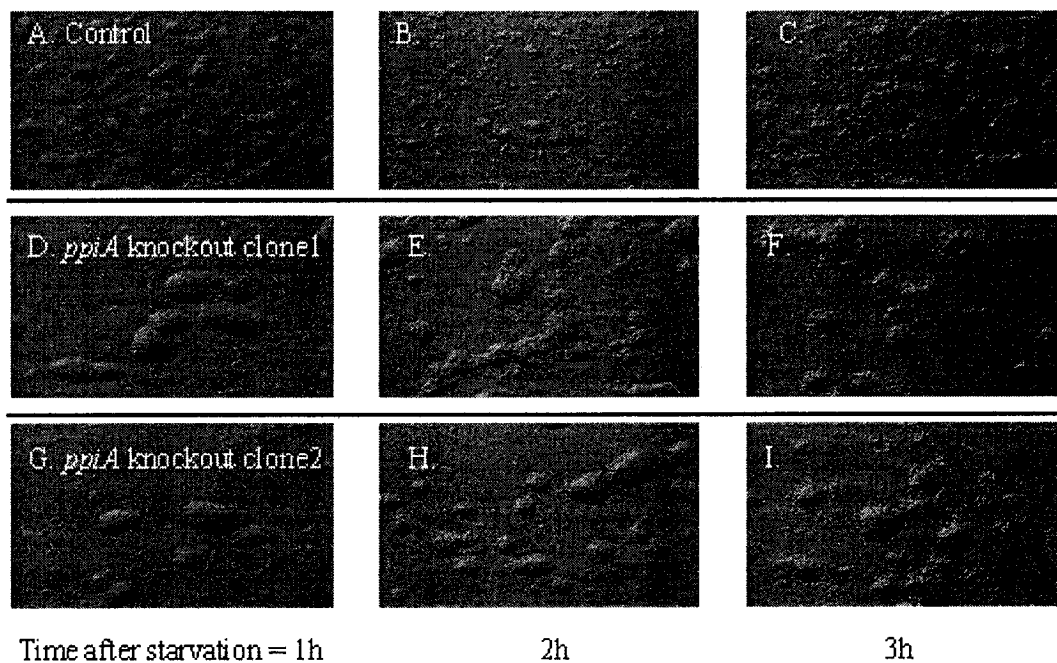
Figure 23. **Developmental phenotypes of wild type (A, B, C) and two *ppiA***

disruption mutants (D, E, F and G, H, I).

Vegetative cells were washed and plated on non-nutrient agar at 1 x 10^6 cells/cm^2.

All images were taken at the same magnification (6X) using a dissecting microscope

fitted with a Kodak MDS 120 digital camera.

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4. DISCUSSION

4.1. The correlation between *cdc2* and the cell cycle

Cdc2 plays a pivotal role in the eukaryotic cell cycle by coordinating DNA replication with mitotic division. While the protein is controlled in many ways, a major determinant of enzyme activity is the degree of Cdc2 phosphorylation. In fission yeast, preventing phosphorylation by replacing Y15 with F cripples the mechanism monitoring the replication status of the cell. The mutation results in premature mitosis in the presence of unreplicated DNA (Gould and Nurse, 1989; Rhind and Russel, 1998). In mammalian cells regulation is more stringent. Inactivation of the neighboring T phosphoacceptor site in addition to Y is required to abrogate the G2/M replication checkpoint (Draetta *et al.*, 1988; Morla *et al.*, 1989; Krek *et al.*, 1992). We wished to determine whether the *Dictyostelium* mutant Cdc2Y21F (in which the putative threonine phosphorylation site is intact but the tyrosine has been changed to a phenylalanine) would behave like yeast or mammalian cells. We therefore studied the growth effects of the mutant gene overexpressed constitutively or transiently under a tetracycline inducible promoter. Our results indicate that Cdc2Y21F and wild-type strains are similar in both growth rate and size suggesting that Y phosphorylation alone is not sufficient to delay mitosis under normal growth conditions. Thus despite the absence of a G1 phase, *Dictyostelium* appears to bear a greater resemblance to mammalian cells than yeast cells, at least in terms of Cdc2 regulation. We envision substituting the threonine residue with an alanine. It will be interesting to see whether expression of a *cdc2*(T20A)(Y21F) double mutant will have an effect on normal mitotic timing.

4.2. *ppiA* is a cell cycle regulated gene possibly involved in early development

In mammalian cells fluctuations in calcium and pH occur at the G1/S transition (reviewed by Whitaker and Patel, 1990). Similar events arise during the *Dictyostelium* cell cycle. The analogy is imperfect however because the *Dictyostelium* cell cycle is unconventional. In *Dictyostelium* cells bypass G1 altogether and advance from cytokinesis directly into S phase (Weijer *et al.*, 1984). Nevertheless periodic changes in calcium and pH levels have been reported (Azhar *et al.*, 1998; Aerts *et al.*, 1985). Transient shifts in both ionic signals occur in mid G2. Equally striking is the presence of typical G1 phase genes in *Dictyostelium* including *rnr*B (MacWilliams *et al.*, 2000) and retinoblasoma (Cercarelli and MacWilliams, in preparation) that both show an expression peak in the early portion of the cell cycle. In light of this, it has been proposed that the *Dictyostelium* cell cycle contains a Start-like transition (*Dicty*-Start) in mid G2 analogous to G1/S checkpoint (MacWilliams *et al.*, 2000). Thus the *Dictyostelium* cell cycle appears to be modified in such a way that events usually associated with entry into S phase occur instead in mid G2. In keeping with this idea, we have shown that *ppiA*, a gene whose protein product may play a role in G1/S transition, is expressed in growing cells. Specifically, the *ppiA* message peaks in mid-G2 of the *Dictyostelium* cell cycle. Gene disruption by homologous recombination results in a developmental phenotype. *ppiA* knockout strains form large aggregation territories in comparison to wild-type cells. This observation suggests that PpiA is important in the early stages of development in addition to its putative role in growth regulation. Intriguingly it has been shown that cell cycle position at the onset of starvation serves as a cue for cell type choice. PpiA may

therefore be involved in controlling the cell cycle or cell-type fate decisions made at the onset of development.

4.3. Cdc2Y21F cells are prestalk sorters

Although the mechanisms involved in generating *Dictyostelium* cell diversity are poorly understood, cell-cycle phase at starvation appears to influence cell fate. Researchers in the field have postulated that starving cells exit the growth phase approximately 2.5 hours before mitosis (putative-shift point) to enter development (Maeda *et al.*, 1989). Existing evidence, however, fails to support the single-exit point model. The main objection to the theory comes from research done by the very group that put forth the idea. When cold-synchronized cells starved shortly after the PS-point are developed in isolation, aggregation is initiated much earlier than anticipated (see introduction; Ohmori and Maeda, 1987). The interpretation, however, may still be valid. Although *Dictyostelium* cultures arrest shortly before mitosis when shifted down from 22°C to 9.5°C, prolonged incubation at sub-optimal growth temperatures causes the cells to regress in their cell cycles (Maeda, 1986). As a consequence cells released from the cold-block re-enter the cell cycle about 2.5 hours before M. Based on this observation it seems plausible that the PS-point corresponds to the G2/M replication checkpoint. Thus starving *Dictyostelium* cells block at G2/M and initiate development. Along the same line of thought, it has been proposed that starving cells exit the cell cycle not a single point (putative shift) but rather at two control points; *Dicty*-start and G2/M (MacWilliams *et al.* 2000) (Figure 7). If we accept this cell-type mechanism (based on two cell-cycle exit points) as our working model then interesting conjectures present themselves. The

model appears fit with sorting biases observed in this study. Our findings demonstrate that Cdc2Y21F preferably differentiate into prestalk cells when combined with wild type cells. To rationalize the sorting phenomenon it would suffice to assume that the alleged Cdc2 homologue controls mitotic entry in *Dictyostelium*. It is then easy to imagine that cells expressing a non-phosphorylatable form of Cdc2 could override the G2/M block. The mutant cells could, in response to nutrient depletion, drift through the cell cycle past the mitotic checkpoint and arrest instead at the subsequent checkpoint, that being *Dicty-start*. It is foreseeable that cells exiting the cell cycle at their respective checkpoint could retain cell cycle associated characteristics that could influence differentiation. For instance cells initiating development at *Dicty*-start would have low levels of cytoplasmic pH and high concentrations of free Ca^{2+}_i (Gross *et al.*, 1983; Saran, 1999). Since these attributes enhance sensitivity to DIF-1 cells in the early phases of the cell cycle would be prospective stalk cells (Sawai *et al.*, 2002). Initial cell type choice then would be contingent upon signals produced periodically throughout the cell cycle. Hence signals that control cell growth and development would converge at similar targets: the cell-cycle checkpoints.

Our inability to link *cdc2* to the cell cycle clearly makes the proposed model on cell type choice much less appealing. This begs the question- why do Cdc2Y21F cells sort? The answer may lie in how the G2/M transition is mediated. Although T14 bears an important role in the DNA replication checkpoint it does not appear to play a part in the DNA damage checkpoint of mammalian cells (Fletcher *et al.* 2002). Thus like *S. pombe* and *A. niger* DNA damage arrest in HeLa cells operates via the inhibitory phosphorylation on Y15 exclusively. Mutation of Y to a non-phosphorylatable F can

trigger premature mitosis even in the presence of DNA damage (Rhind *et al.*, 1997; Jin *et al.*, 1996). It is conceivable that *Dictyostelium* cells respond to starvation in a similar fashion. In the absence of nutrients, expression of Cdc2Y21F may compromise starvation-induced arrest. The outcome would be cells localized in the early prestalk phase of the cell cycle. Although the idea provides a simple explanation for Cdc2Y21F sorting we emphasize that it remains speculative. Knowledge of the *Dictyostelium* cell cycle is limited and presently does not allow us to judge how Cdc2 is regulated when cells are starving. Proof will require a greater understanding of Cdc2 and its interactions with other components of the cell cycle. We are currently designing experiments to address these issues. Regardless of the mechanism, however, data from this study provides a role for Cdc2 in the *Dictyostelium* differentiation.

Researchers opposed to the cell cycle theory uphold the view that cell-type choice is dependent upon cell-cell interactions (Kay, 1989; Krefft *et al.*, 1984). Irrefutable evidence supports their claim. It is well-known that isolated pieces of the slugs can adjust cell-type ratios and create small but nonetheless proportioned fruiting bodies (Sakai, 1973; Sternfeld and David, 1982). Suitably, diffusible substances capable of directing cell fate have been isolated (review by Weeks, 1991). An intercellular mechanism is manifestly in place ensuring that both cell types are present. A cell bias based on the cell cycle, however, would by no means exclude the possibility of morphogen-based regulation. Rather the choice of either pathway, influenced by the position of the cell in its cell cycle at the moment of nutrient exhaustion, would be plastic (MacWilliams and Bonner, 1979). The cell cycle would simply provide the grounds for

initial cell diversity. Morphogenic substances would then adjust the final cell type ratio and if necessary even prompt the cells to choose an alternate differentiation pathway.

The weakest aspect of the argument linking the cell cycle to differentiation comes from the observation that a fraction of the cells re-enter the cell cycle during development. Specifically prespore cells go through a second round of mitosis during the mound stage (Zada-Hames and Ashworth, 1978; Durston and Vork, 1978; Zimmerman and Weijer, 1993). Although it is conceivable that prespore cells arrested at G2/M could carry sufficient energy stores to progress through M and S these cells would in turn, according the our working model, arrest at the *Dicty*-start. Partial re-initiation of the cell cycle would thus beget an entire population of prestalk cells, which is clearly not the case. Perhaps sufficient diversity is established by mound stage and so differentiation is no longer cell-cycle dependent but relies instead on morphogenic signals secreted by the different cells (MacWilliams, 1982).

4.4. A putative DNA damage checkpoint in *Dictyostelium discoideum*

In addition to a establishing a link between Cdc2 and cell differentiation we may have uncovered a DNA damage checkpoint in *Dictyostelium*. Previous studies have shown that when cellular DNA is damaged the level of phosphorylated Cdc2 rises. HeLa cells lacking Y15 on Cdc2 but in which the phosphorylatable T residue is preserved (Cdc2TF) are unable to restrain mitotic progression in the presence of DNA damage (Fletcher *et al.*, 2002). Thus, like fungi, Y15 alone can maintain the G2/M DNA damage checkpoint. We sought to establish a link between Cdc2Y21 phosphorylation and response to DNA damage in *Dictyostelium*. As an initial experiment, vegetative cells were treated with

methyl methanesulfonate. DNA damage caused by exposure to MMS induces cell cycle arrest shortly before mitosis (Figure 15). After a brief hiatus the treated cells resume growth with partial synchrony when the agent is removed (Figure 16). Cell cycle arrest is a typical DNA-damage response, allotting time for chromosome repair. We postulate that *Dictyostelium* has a late G2 DNA damage checkpoint. To elaborate the above findings, we are currently designing experiments to examine chromosome morphology in the presence of DNA damaging compounds.

5. CONCLUSIONS AND FUTURE PROSPECTS

Although it appears that the cell cycle directs cell differentiation, the mechanisms involved have so far remained elusive. Our initial attempts at linking the cell cycle to cell differentiation offer encouraging results. Our data strengthen previous findings showing that cells in different phases of the cell cycle adopt alternate fates. Namely simulating tyrosine dephosphorylation in Cdc2, an essential cell division protein, results in a stalk cell preference.

Future research will focus on identifying other genes that are transcriptionally regulated during the *Dictyostelium* cell cycle. Global expression studies with DNA microarrays and real-time PCR will be carried out to determine cyclic changes in gene induction. Potential cell cycle markers identified will serve to further investigate the role of the cell cycle in *Dictyostelium* development. We hope that the work outlined in the present study, along with our future endeavours, will contribute towards a better understanding of cell divergence in *Dictyostelium* and perhaps even serve to demystify development in higher systems.

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