CHARACTERIZATION OF BYR4 AND CDC7 FUNCTIONAL DOMAINS AFFECTING THE SEPTATION INITIATION NETWORK IN *SCHIZOSACCHAROMYCES POMBE*

By

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LIST OF ABBREVIATIONS

aa	amino acid
Ala	alanine
APC	anaphase promoting complex
bp	base pair
BSA	bovine serum albumin
C	carboxy
CAR	cytokinetic actin ring
cdc	cell division cycle
Cdk	cyclin-dependent kinase
cDNA	complementary DNA
coIP	coimmunoprecipitation
DAPI	4', 6-diamidino-2-phenylindole
DIC	differential interference contrast
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
ECL	enhanced chemiluminescence
EDTA	ethylenediamine tetraaacetic acid
G	gap phase
GAP	GTPase activating protein
GEF	guanine nucleotide exchange factor
GFP	green fluorescent protein
GST	glutathione-s-transferase
GTP	guanosine triphosphate
HA	influenza hemagglutinin epitope
HCl	hydrogen chloride
HEPES	(N-[2-Hydroxyethyl]piperazine-N'-[2-ethansulfonic acid]

His	histidine
IgG	immunoglobulin-G
IPTG	isopropyl-β-D-thiogalactopyranoside
Kan^R	kanamycin resistance
Kd	kilo dalton
KGY	Kathy Gould yeast
l	litre
µl	microlitre (10^{-6} liter)
M	molar, mitosis
MBP	maltose binding protein
MEN	mitotic exit network
$MgCl_2$	magnesium chloride
ml	milliliter
mM	millimolar
Myc	myc epitope
N	amino
NaCl	sodium chloride
nmt	no message in thiamine
NP-40	Nonidet® P-40
^{32}P	phoshphorous-32
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pKG	plasmid Kathy Gould
RLU	relative light unit
RNA	ribonucleic acid
rpm	revolutions per minute
SB	sample buffer
SDS	sodium dodecyl sulfate
ser	serine
SIN	Septation Initiation Network

SPB	Spindle Pole Body
TAP	tandem affinity purification
Thr	Threonine
ts	temperature sensitive
Ura	uracil
YE	yeast extract
U	unit
Ub	ubiquitin
UV	ultraviolet
Δ	deletion,null
α	anti

CHAPTER I

INTRODUCTION

The Eukaryotic cell cycle

The foundation of scientific enquiry into biological questions of cells can be traced back to the late 1500's and early 1600's with the invention of the microscope. The microscope allowed early scientists to look for answers to the fundamental question 'what are all living organisms made of?' The turning point in biological research came with advent of the 'cell theory' –that suggested that all living organisms are composed of cells- the basic structural and functional unit of life. All multi-cellular organisms regardless of how complex once arose from a single cell. An understanding of how life forms grow and develop can be found in answering the basic question: What mechanisms regulate cell growth and division? Insights into understanding the regulation of cell cycle also has numerous implications for medicine and diseases such as cancer in which cells multiply unchecked.

A cell undergoing growth and division displays distinct and defined phases during which critical processes occur. These critical processes include DNA replication - where a copy of the genetic blueprint is created, mitosis- where the genetic material is faithfully segregated, and cytokinesis that results in the physical separation of daughter cells. In a eukaryotic cell these events are temporally organized in a cyclical fashion with S-phase being a period of DNA synthesis and replication and mitosis, or M-phase, during which the DNA is segregated to daughter cells. The S and M-phases are separated by two growth or gap phases G1 and G2 (Figure 1). Executing these various processes involves careful co-ordination with previous cell cycle events. The cell also has installed surveillance mechanisms or checkpoints that monitor the accurate completion of each stage and prevent cell-cycle transitions if any errors or damage is detected. A failure to temporally and spatially regulate these events is catastrophic for the cell usually resulting in aneuploidy, cell death, or cancer.

A great deal is already known about how the cell enters mitosis as well as of the checkpoints in place to ensure fidelity of early cell- cycle events (Figure 1). A variety of model organisms have been useful in dissecting out the molecular mechanisms involved in cell cycle regulation mentioned above. In particular, early genetic screens performed in fission yeast

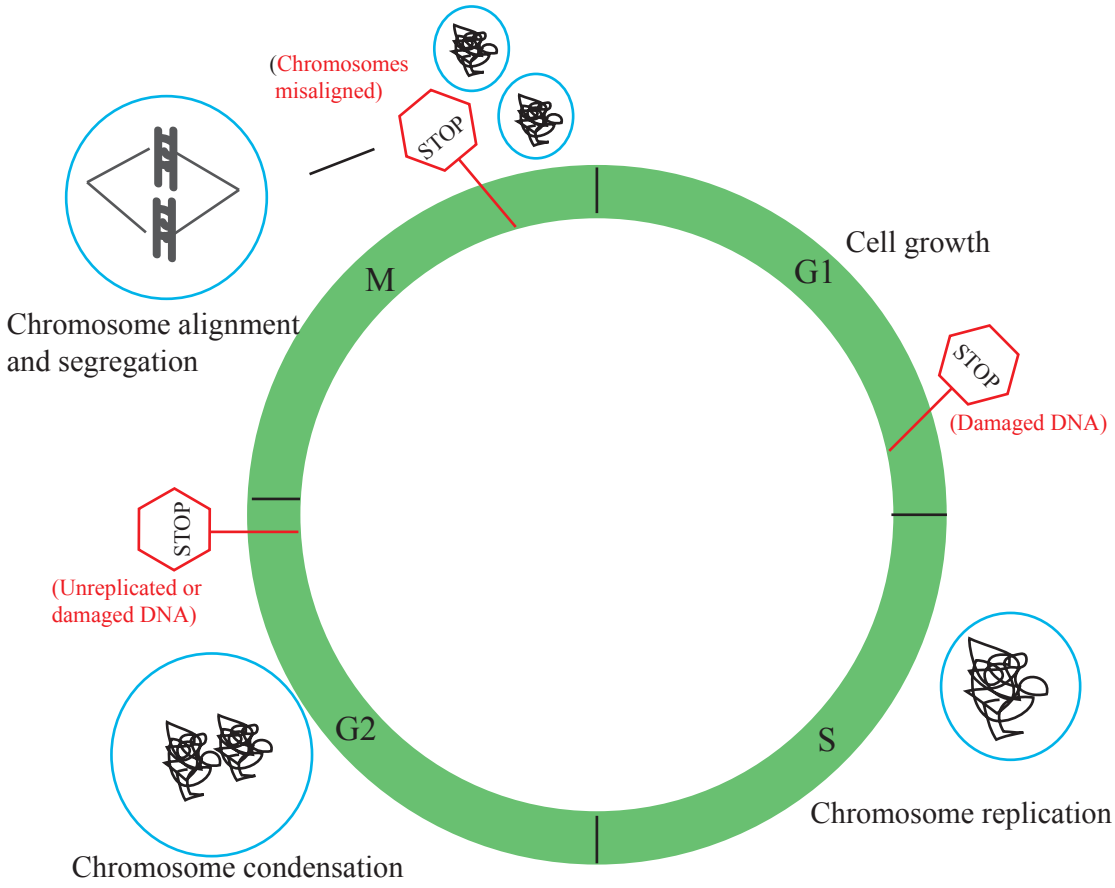


Figure 1. The eukaryotic cell cycle. The eukaryotic cell cycle is divided into distinct phases, M,G1, S and G2. M phase is usually followed by cytokinesis. S phase is the period were DNA replication occurs. The cell grows in size throughout interphase (G1,S and G2 combined). Checkpoints moniter and arrest cells at the indicated stages in response to damaged or unreplicated DNA or misaligned chromosomes.

9A). Loss of function was not due to degradation of unstable protein at 36^0C, since protein levels of HA-Cdc7(360-518) were comparable to those of HA-Cdc7 (Figure 9B)

In order to test whether Cdc7 could oligomerize *in vivo,* I created a diploid strain in which one allele of *cdc7* was tagged with the Myc_{13} epitope and the other tagged with the HA_3 epitope. Anti- HA immunoprecipitates from the dually tagged diploid strain, but not single tagged strains, contained Cdc7-Myc_{13} and vice versa (Figure 9C). These results indicated that Cdc7 did indeed exist as an oligomer *in vivo*. To estimate what oligomeric state Cdc7 was in, lysates from asynchronously growing Cdc7-Myc_{13} and control untagged strains were prepared under native conditions and sedimented on sucrose gradients. After sedimentation fractions were collected and Cdc7-Myc_{13} was immunoprecipitated using an antibody against the myc epitope from each fraction. Immunoprecipitates were then analyzed by immunoblotting for myc. Molecular size standards were run in parallel on an identical sucrose gradient. We found that the majority of Cdc7-Myc, co-sedimented in fractions 10 and 11 with the molecular weight marker, Phosphorylase B, which forms a trimer of 292.8 kDa.

Since the predicted molecular weight of a Cdc7-Myc_{13} dimer is approximately 285 kDa, this suggests that Cdc7 exists primarily as a dimer. Some Cdc7-Myc_{13} sediments lower in the gradient and this could represent Cdc7 in complex with other proteins (such as Spg1) and/or in higher order complexes with itself. We also investigated whether Spg1 was able to associate with itself. The results of the yeast two-hybrid and co-immunoprecipitation from *spg1-HA_3/ spg1-myc_{13}* diploid strain showed that Spg1 is not associated with itself (Figure 10A). This suggests that either Spg1 is not present in Cdc7 complexes or that a Cdc7 dimer binds to one molecule of Spg1. Consistent with the latter hypothesis overexpression of Spg1 failed to disrupt Cdc7 self association (Figure 10B). These data taken together with the observation that Cdc7-Myc_{13} was not observed in any fraction that would be consistent with a monomeric form (140 kDa) suggest that Cdc7 exists predominantly as a dimer *in vivo* and associates with Spg1 as a dimer.

Schizosaccharomyces pombe identified the *cdc2* gene product, the founding member of cyclin-dependent kinase (CDK) family (Fantes 1977; Fantes and Nurse 1978; Thuriaux, Nurse et al. 1978; Nurse and Thuriaux 1980; Carr, MacNeill et al. 1989; MacNeill and Nurse 1989). Cdc2 functions as a master regulator of the cell cycle in all eukaryotic organisms examined to date (Beach, Durkacz et al. 1982; Simanis and Nurse 1986; Lee and Nurse 1987; Lee and Nurse 1988; Lee, Norbury et al. 1988; Jimenez, Alphey et al. 1990). *In vivo*, in all systems studied, the kinase activity of Cdc2 peaks at the G2/M boundary while Cdc2 protein levels remain constant through the cell cycle (Booher and Beach 1986; Simanis and Nurse 1986; Draetta and Beach 1988; Dunphy, Brizuela et al. 1988; Labbe, Lee et al. 1988; Felix, Pines et al. 1989; Gautier, Matsukawa et al. 1989; Moreno, Hayles et al. 1989). The key means of regulating Cdc2 activity is through periodic association with its cyclin subunit. As indicated by the name cyclins are only expressed at specific times during the cell cycle.

Cdk in complex with cyclin leads to its activation and subsequent phosphorylation of downstream targets. Positive and negative regulatory phosphorylations, cyclin-dependent inhibitors (CKI) and ubiquitin-mediated proteolysis of cyclin subunits all contribute in regulating the activity of CDKs. In contrast to *S.pombe* and *S.cerevisiae*, in which Cdc2/Cdc28 alone drives the cell cycle, higher eukaryotes utilize multiple different CDKs, each of which associate with their specific cyclin regulatory partners and are specialized to promote specific cell cycle transitions.

Fission yeast, *Schizosaccharomyces pombe*, as a model organism

As described above, a number of seminal discoveries pertaining to cell cycle regulation were identified using model organisms like fission yeast, *S.pombe.* Because of the remarkable conservation of cell cycle control mechanisms from yeasts to humans, studies performed in *S.pombe* have held true for and complement analyses of cell cycle control performed in higher eukaryotes. *S.pombe* cells spend a majority of their time (approximately 70%) in G2. Hence *S.pombe* has classically served as an excellent model organism for studying G2/M regulation. Additionally, fission yeast is highly amenable to molecular genetic, physiological and biological analyses. *S.pombe* cells are cylindrical in shape, grow by tip elongation and divide by medial fission at a constant cell size. This feature has been extremely useful in screening for mutants in

components of the cell cycle machinery, for cells which continue to grow but fail to divide become highly elongated; or cells which divide at a reduced cell size display a "*wee*" phenotype.

Until recently a less studied aspect of cell division had been the process of cytokinesis. *S.pombe* cells divide in a manner similar to higher eukaryotes and utilize a number of conserved molecules that function in assembling the cleavage apparatus and regulating constriction. Hence, *S.pombe* has served as an invaluable model organism to elucidate the mechanics of cytokinesis and its temporal regulation (discussed in greater detail below).

Cytokinesis in eukaryotes

Cytokinesis, the physical separation of two new daughter cells, marks the end of cell division. This process like all the prior events of the cell cycle must be temporally and spatially regulated such that cytokinesis takes place at the right time and in the correct place. Only in the last decade or so are molecular mechanisms involved in this complex process beginning to be unraveled. Work done in several model organisms and recently through genome-wide RNA interference (RNAi) screens has identified many proteins essential for cytokinesis (Somma, Fasulo et al. 2002; Goshima and Vale 2003; Echard, Hickson et al. 2004; Eggert, Kiger et al. 2004). Not surprisingly, proteins required for the completion of cytokinesis have a diverse range of functions, such as cytoskeletal remodeling, vesicular trafficking and membrane fusion. The precise molecular events involved in cytokinesis may vary in degrees of complexity and organization based on where on the evolutionary scale they fall (Bi 2001). However, the emerging theme is that the basic components remain conserved from yeast to humans, with adaptations for the type of cell or mode of division. All dividing cells need to a) determine a site of division, b) establish the division apparatus, often called the cleavage furrow or cytokinetic actin ring (CAR) and c) constrict the division apparatus coupled with new membrane addition that leads to the creation of two daughter cells.

Determining the site of division

In animal cells anaphase onset triggers the formation of the central spindle between the segregating chromosomes. The central spindle is believed to be the marker for assembly of the division apparatus (Adams, Tavares et al. 1998; Giansanti, Bonaccorsi et al. 1998). However in certain cells, such as *C.elegans* embryos, the astral microtubules determine cleavage furrow

formation (Dechant and Glotzer 2003). How the central spindle or astral microtubules signal to actively promote furrow ingression is not known. In contrast to animal cells, budding yeast establish their division site early in the cell cycle, and it is marked by the location of the previous division site. A small GTPase signaling module recognizes the site of division and initiates the formation of the new bud (reviewed in Casamayor and Snyder 2002). Additionally, the activation of another small GTPase Cdc42 results in the formation of a filamentous ring made of septins (GTP binding proteins) at the bud neck or site of division. The septin ring marks the site of division and plays an essential role in the formation of the cytokinetic actin ring. Fission yeast, like metazoans divide symmetrically. However the position of the division site is determined by the position of the nucleus in the cell. In wild type cells the nucleus is maintained in the middle through opposing forces generated by microtubules (Tran, Doye et al. 2000; Tran, Marsh et al. 2001). Mid1, an anillin family protein, and Plo1 kinase play a role in establishment of CAR position by linking the interphase nucleus to the cell division site. Mid1 is a nuclear protein that upon entry into mitosis relocates to form a cortical band overlying the nucleus and promotes the recruitment of actomyosin ring components to a band like structure overlying it (Motegi, Mishra et al. 2004). This localization pattern is dependent on the function of Plo1 kinase (Chang, Woollard et al. 1996; Sohrmann, Fankhauser et al. 1996; Bahler, Steever et al. 1998). Plo1 may have additional functions in determining actomyosin ring placement that is independent of its role in controlling Mid1 localization (Paoletti and Chang 2000). Other proteins such as the protein kinase, Pom1 and *pos* genes are involved in signaling for ring placement although their precise roles haven't been clearly elucidated (Bahler and Nurse 2001).

Actomyosin ring assembly and constriction

Once the division site is established the contractile apparatus assembles at the cell cortex. Both yeasts and animal cells divide with the help of a contractile ring that is made of actin and myosin amongst several other proteins. The mechanical force for furrow ingression is most likely provided by myosin II. At the end of anaphase the ring constricts and brings about the division of the cytoplasm. This is usually accompanied by the formation of new membrane and/or cell wall material. The basic components of the ring are the same in all eukaryotic organisms although there are differences in the timing and order of addition of various components.

In animal cells the activation of another small GTP binding protein RhoA leads to actin polymerization and myosin II activation (Kimura, Tsuji et al. 2000; Kosako, Yoshida et al. 2000; Maddox and Oegema 2003; Yoshizaki, Ohba et al. 2004). The disassembly of the actomyosin ring likewise is promoted by the down regulation of Rho activity at the cleavage furrow. GTPase activating protein MgcRacGAP localizes to the spindle midzone during cytokinesis and it's GAP activity has been implicated as being important for cytokinesis (Hirose, Kawashima et al. 2001; Kitamura, Kawashima et al. 2001; Lee, Kamijo et al. 2004). It is still unclear what the exact function or the GTPase target of MgcRacGAP is *in vivo*, with Rho, Rac and Cdc42 being implied as potential candidates.

The final step of cytokinesis, membrane fusion, requires delivery of membrane vesicles that fills into the space remaining after full ingression of the contractile ring (Low, Li et al. 2003). The machinery involved in membrane insertion during cytokinesis includes syntaxins, syntaxin associated proteins, coatomer complex members, rab family GTPases, and subunits of the exocyst complex (Reviewed in (Schweitzer and D'Souza-Schorey 2004; Albertson, Riggs et al. 2005)). Unlike animal cells, fission yeast cells begin to assemble their CAR early in M-phase. In fact, as mentioned earlier, a number of ring components accumulate in a broad cortical band around the nucleus in a Mid1 dependent manner. By live cell imaging of GFP-tagged ring components a sequence of events leading to the formation of the CAR can be described. The earliest proteins to arrive at the cell division site include type II myosin heavy chain Myo2, IQGAP related protein Rng2, PCH domain protein Cdc15 and formin Cdc12. Cdc15 interacts directly with both the Arp2/3 complex activation machinery and the formin Cdc12 to orchestrate early events in CAR formation (Fankhauser, Reymond et al. 1995; Carnahan and Gould 2003). In the final stages, F-actin, tropomyosin Cdc8, and a number of other actin cross-linking proteins together form a structured actomyosin ring.

Unlike animal cells, fission yeast also synthesizes a septum that is placed between the two cells. Deposition of septal material occurs concurrently with actomyosin ring constriction and membrane synthesis. Once the primary septum is laid, two secondary septa are synthesized flanking the primary septum. Cell separation is accomplished upon the dissolution of the primary septum. The process of septum deposition directly regulates the process of ring constriction. Much like animal cells exocyst components, syntaxins and Rab GTPases are important for cytokinesis in *S.pombe* (Liu, Wang et al. 1999; Cortes, Ishiguro et al. 2002; Wang, Tang et al.

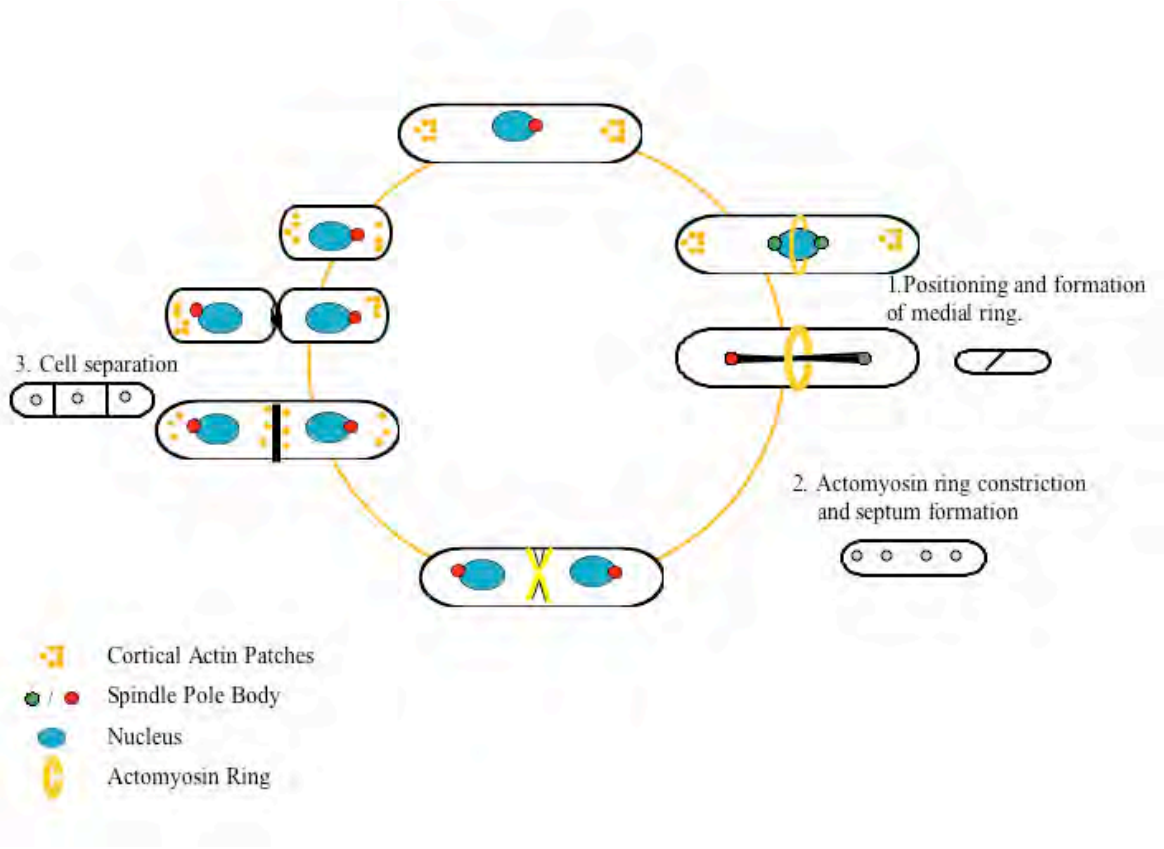


Figure 2. Steps in fission yeast cytokinesis. During interphase, F-actin is observed in cortical patches at the tip of the cell. During early mitosis the patches relocalize to the middle of the cell to form the medial ring over the nucleus. During anaphase, the actomyosin ring constricts and the primary septum forms. Digestion of the septum liberates the newly formed cells. The cartoons represent the expected phenotypes of mutants defective at a particular stage.

2002; Wang, Tang et al. 2003). One of the main unanswered questions has been how all of these processes are coupled at the molecular level. How is the mitotic and nuclear division cycle co-ordinated with cytokinesis?

Temporal regulation of cytokinesis

The cells are ready to physically divide once mitosis is complete and genetic material has been segregated. Therefore there must be regulatory coupling between the exit of mitosis and the onset of cytokinesis. In a variety of eukaryotic organisms inactivation of mitotic CDKs is a prerequisite for cells to exit mitosis and undergo cytokinesis (Guertin and McCollum 2001; McCollum and Gould 2001; Echard and O'Farrell 2003). Inactivation of mitotic CDKs is achieved largely through ubiquitin-dependent proteolysis of the mitotic cyclins via the APC/C (reviewed in (Zachariae and Nasmyth 1999)). Studies on the mechanisms that coordinate exit from mitosis with cytokinesis have revealed the presence of a conserved GTPase-driven signaling pathway in both the fission yeast *S. pombe* and the budding yeast *S. cerevisiae*. Coined the SIN (Septation Initiation Network) in fission yeast and MEN (Mitotic Exit Network) in budding yeast both pathways, although differing in certain details, primarily serve to coordinate mitotic exit with cytokinesis. Interestingly both SIN and MEN components localize to the spindle pole body (SPB), the centrosome equivalent in yeast, either constitutively or at some stage in the cell cycle. The SPB has emerged as an important signaling and organizing center for the assembly of SIN and MEN components.

The Septation Initiation Network, SIN

The ability to visually score S.*pombe* cells for specific defects in various aspects of the cell division process enabled the identification of mutants that are specifically defective in one aspect of the cell division process but not another (Figure 2). A group of mutant genes, collectively referred to as *sid* (septation initiation defective) genes, were identified as being defective in actomyosin ring constriction and septum deposition (Marks, Fankhauser et al. 1992; Balasubramanian, McCollum et al. 1998). Loss of function mutations in *sid* genes result in long multinucleate cells due to the continuation of nuclear division and cell growth but a failure to deposit septal material. Through the cloning of all the genes involved and biochemical analyses of their protein products led to the identification of the Septation Initiation Network. The SIN

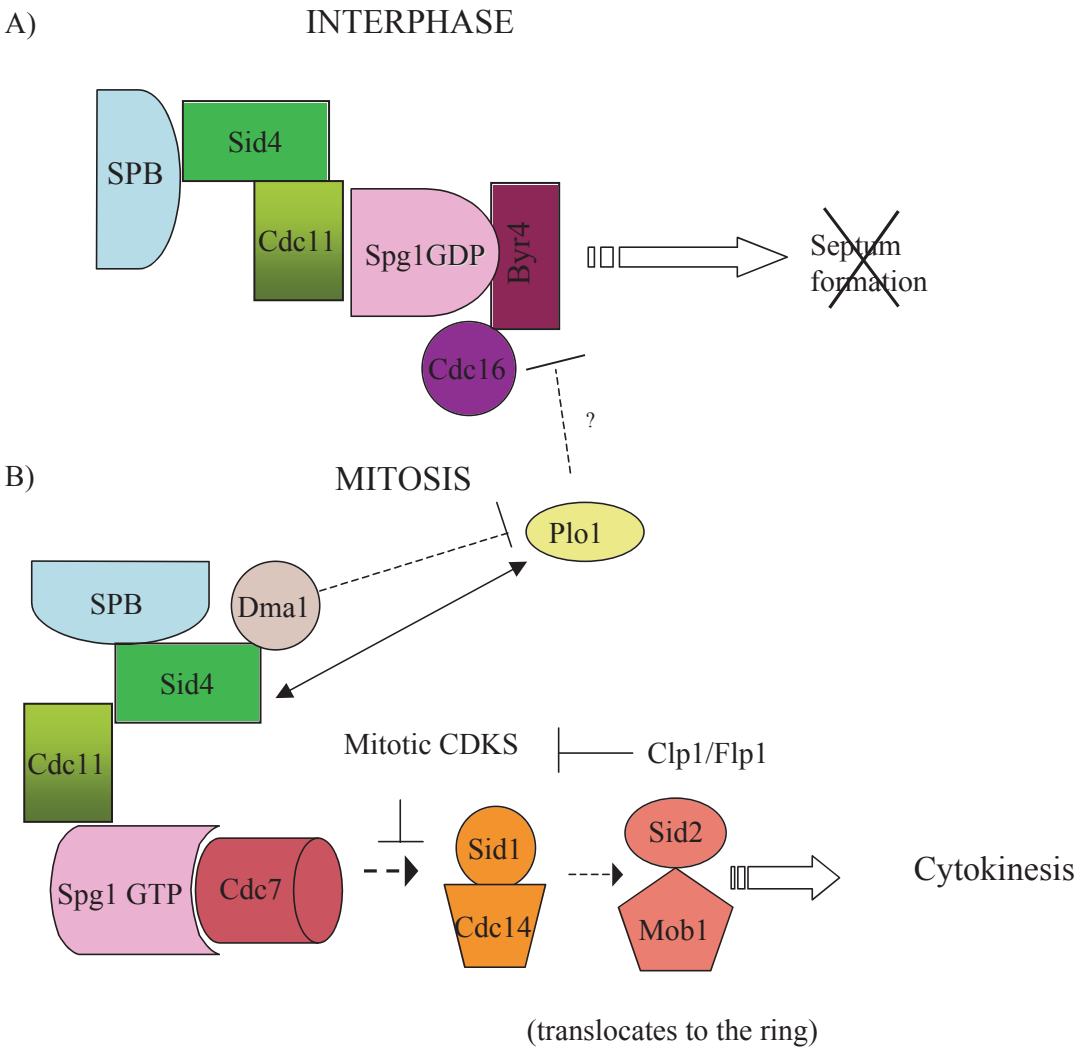


Figure 3. Current model for the septation initiation network (SIN) in *S. pombe.* (A) The interphase SPB is occupied by the scaffold proteins Cdc11-Sid4 and the GAP complex Byr4-Cdc16. Spg1 is maintained in its inactive GDP bound state to prevent inappropriate septum formation. (B) Upon entry into mitosis Byr4-Cdc16 leaves the SPB, Spg1 is converted to its GTP bound state; Cdc7 kinase is recruited to the active SPB. Inactivation of Cdc2 allows for subsequent recruitment of Sid1-Cdc14 following which Sid2-Mob1 kinase complex translocates to the ring. The Plo1 kinase may directly affect Spg1 or inhibit the GAP complex. The asymmetric localizations of the SIN components to the SPBs or the location of other regulators have not been shown.

includes a GTPase Spg1 (Schmidt, Sohrmann et al. 1997), a two-component GAP for Spg1p composed of Byr4 and Cdc16 (Furge, Wong et al. 1998) and 4 protein kinases: Plo1 (Ohkura, Hagan et al. 1995), Cdc7 (Fankhauser and Simanis 1994), Sid1 and its regulatory subunit Cdc14 (Guertin, Chang et al. 2000) and Sid2 and its regulatory subunit Mob1 (Sparks, Morphew et al. 1999; Hou, Salek et al. 2000; Hou, Guertin et al. 2004). Additionally a number of structural components have been identified that influence SIN signaling in various ways (Figure 3) (Chang and Gould 2000; Krapp, Schmidt et al. 2001; Tomlin, Morrell et al. 2002).

The SIN Components

Cdc11-Sid4 scaffold

All known SIN proteins reside on the SPB at some point during the cell cycle. Their localization requires Sid4 function and thus, Sid4 is considered to be a SPB anchor for the SIN (Chang and Gould 2000). Sid4 links to downstream SIN components through another protein called Cdc11 (Chang and Gould 2000; Krapp, Schmidt et al. 2001; Tomlin, Morrell et al. 2002). Cdc11 is the *S. pombe* homolog of the *S. cerevisiae* SPB component Nud1 (Gruneberg, Campbell et al. 2000). Cdc11 physically associates with Sid4 via its C-terminus, while its N-terminus makes a wide variety of direct protein–protein interactions with SIN components including Spg1, Sid2, and Cdc16. Interestingly Cdc11 also associates with the mitotic CDK Cdk1-Cdc13 (Morrell, Tomlin et al. 2004). FRAP (Flourescence Recovery After Photobleaching) experiments show that Sid4 and Cdc11 are extremely stable at the SPB, a property ideal for proteins that function as a platform to assemble and organize signaling components. Not surprisingly, the signaling components of the SIN dynamically associate with the SPB. The Cdc11-Sid4 scaffold associates with at least two other regulators of the SIN, Dma1 and Plo1. Thus, all the components of the SIN are ideally positioned in close proximity to one another to allow rapid signaling and tight co-ordination of late mitotic events (Morrell, Tomlin et al. 2004).

The GTPase cycle and its regulation

Central to the activation of the SIN pathway is the GTPase Spg1. Spg1 was originally identified as a multicopy suppressor of a dominant negative mutant of the Cdc7 kinase (Schmidt, Sohrmann et al. 1997). A temperature sensitive allele of Spg1 was also isolated in a genetic

screen that identified genes involved in cytokinesis (Balasubramanian, McCollum et al. 1998). The closest relative of Spg1 is its *S. cerevisiae* homolog Tem1. Spg1 also shares limited sequence conservation with N-Ras (Schmidt, Sohrmann et al. 1997). Spg1 was subsequently shown to have GTPase activity *in vitro* (Furge, Wong et al. 1998). Underscoring the key role of Spg1 in SIN signaling was the observation that increased production of Spg1 leads to the formation of septa from any point in the cell cycle (Schmidt, Sohrmann et al. 1997).

The Cdc7 kinase functions as an effector for Spg1 and is therefore an activator of SIN signaling (Schmidt, Sohrmann et al. 1997; Sohrmann, Schmidt et al. 1998). Interestingly, Cdc7 kinase activity remains constant throughout the cell cycle and its kinase activity is not dependent on Spg1 function (Sohrmann, Schmidt et al. 1998). Spg1 and Cdc7 localize to the SPB. Hence although the kinase activity appears to be constant throughout the cell cycle, it may only target its downstream substrates when localized to the pole. Spg1 localizes to the SPB throughout the cell cycle (Sohrmann, Schmidt et al. 1998). However during interphase it is inactive and exists in a GDP-bound state at the single SPB. During metaphase, Spg1 becomes 'activated' or GTP-bound at both SPBs. It is then inactivated at one of the poles during anaphase B, giving rise to an asymmetric state. Cdc7 is only recruited to the pole that is occupied by the GTP-bound Spg1p; hence it also exists at both poles during metaphase, and then is asymmetrically localized to one pole during anaphase. The asymmetric localization of SIN components appears to be important for proper SIN regulation since Cdc7 is associated with both SPBs during anaphase in cells with deregulated septum formation (Mulvihill, Petersen et al. 1999; Tanaka, Petersen et al. 2001). While the apparent need for asymmetry in a symmetrical organism is puzzling, the mechanisms involved in SIN asymmetry are beginning to surface. The red fluorescent protein dsRed takes several hours to fold into an actively fluorescing molecule and hence is a useful tool to differentiate between an old SPB versus and a newly formed SPB. The *S. pombe* SPB component Pcp1-RFP was used to track the SPB after starvation. Pcp1-RFP is asymmetrically localized, associating with the 'old' SPB but not the 'new' (Grallert, Krapp et al. 2004). Using a dually labeled strain, Pcp1-RFP Cdc7-GFP, it was determined that the active SIN is housed on the new SPB (Grallert, Krapp et al. 2004). This finding begs the question- how does the SIN get activated at only the 'new' and not the 'old' SPB? Additionally are there components of the SPB that get specifically recruited to the old versus the new SPB and influence SIN activity?

Since Spg1 activation is critical in establishing the onset of cytokinesis, regulating the timing of Spg1 activation is key. The balance of maintaining a GTPase in an active or inactive state is typically regulated by GAPs (GTPase Activating Protein) and GEFs (GTP Exchange Factor) To date however no GEF for Spg1 has been identified. A unique two component GAP, comprised of Byr4 and Cdc16 has been identified that functions to keep Spg1 inactive during interphase and hence prevent septum formation at an inappropriate time (Minet, Nurse et al. 1979; Fankhauser, Marks et al. 1993; Song, Mach et al. 1996; Furge, Wong et al. 1998; Jwa and Song 1998). Inactivating either GAP component leads to the formation of multiple septa. The Cdc16 component bears structural similarity to proteins known to have GAP activity, most closely resembling the Ypt-GAP family (Fankhauser, Marks et al. 1993; Will and Gallwitz 2001). However, Cdc16 is unable to hydrolyze GTP bound to Spg1 in an *in vitro* assay in the absence of Byr4 (Furge, Wong et al. 1998; Furge, Cheng et al. 1999). Byr4 associates directly with both Spg1 and Cdc16 and one function predicted for Byr4 is to position Cdc16 in proximity to its cognate GTPase (Furge, Cheng et al. 1999). Byr4 was also shown to interact with NIMA related kinase Fin1, and Fin1 requires Byr4 for its recruitment to the SPB (Grallert, Krapp et al. 2004).

As would be expected, the Byr4-Cdc16 GAP complex also localizes to the SPB (Cerutti and Simanis 1999; Li, Furge et al. 2000). During interphase it is present on the single SPB, keeping Spg1 inactive. During anaphase Cdc16-Byr4 localizes to only one SPB, the one without Cdc7. In fact the asymmetric localization of Cdc16-Byr4 appears to occur prior to that of Cdc7 suggesting that the complex may prevent the recruitment of Cdc7 to that SPB (Cerutti and Simanis 1999; Li, Furge et al. 2000). It is interesting to note that in one-half of the population of cells Fin1 is associated with both SPBs until septation is completed (Grallert, Krapp et al. 2004). However, Fin1 association with the SPB requires Byr4 suggesting that - contrary to previous observations- Byr4 persists on both SPBs until septation (Cerutti and Simanis 1999; Li, Furge et al. 2000). Since the localization of Byr4 was not examined simultaneously with that of Fin1 we do not know if the latter holds true. It is likely that Fin1 requires additional components besides Byr4 for retainment at the SPB.

The kinases and a link to the site of division

Two protein kinase complexes function downstream of Cdc7 in the SIN. During anaphase the Sid1 kinase in a complex with Cdc14 gets recruited to the pole housing active Spg1 and Cdc7 (Guertin, Chang et al. 2000). *sid1*$^{+}$ encodes an essential kinase that belongs to the PAK/GC family, specifically to group II GCKs (Guertin, Chang et al. 2000; Guertin and McCollum 2001). The kinase activity of Sid1 appears to be cell cycle regulated, peaking in late anaphase (Guertin, Chang et al. 2000). However, neither the protein levels of Sid1 nor its interaction with Cdc14 changes during the cell cycle. Cdc14 is essential for Sid1 localization to the SPB and for full Sid1 catalytic activity (Guertin and McCollum 2001). Interestingly, recruitment of Sid1-Cdc14 to the SPB depends on the inactivation of Cdc2 (Guertin, Chang et al. 2000). Hence in creating this dependency between mitotic CDK inactivation and SIN signaling a system is in place to ensure that septum formation does not take place during mitosis, when CDK activity is high.

The recruitment of Sid1-Cdc14 to the SPB triggers the accumulation of another protein kinase complex Sid2–Mob1 at the medial ring. The Sid2-Mob1 complex is present constitutively at SPBs and is the only known component of the SIN to also localize at the site of division. The exact mechanism by which Sid2-Mob1 arrives at the division site is still a mystery, although the localization does require the microtubule network (Sparks, Morphew et al. 1999). At the division site, Sid2-Mob1 likely transmits signals originating from the SPB to downstream targets. Consistent with a role for Sid2 downstream in the SIN is the observation that Sid2 kinase activity depends on all the other SIN components and peaks at the time of cytokinesis (Sparks, Morphew et al. 1999; Hou, Salek et al. 2000). Sid2 is predicted to phosphorylate a substrate(s) that would lead to medial ring constriction and septation. Interestingly Sid2 localization to the ring depends on the ring component Cdc15, a known phosphoprotein (Fankhauser, Reymond et al. 1995; Sparks, Morphew et al. 1999). Hence, it is tempting to speculate that Cdc15 might be a target of Sid2.

Sid2-Mob1 is subject to multiple modes of regulation. Sid2 phosphorylation on two conserved residues and its physical association with Mob1 are important for its activity (Hou, Guertin et al. 2004). Additionally, Sid2 homodimerizes and is inactive in that state (Hou, Guertin et al. 2004). The regions of Sid2 implicated to be important for self- association are also required for Mob1 binding. The presence of excess Mob1 in the cell results in disruption of Sid2 dimers

(Hou, Guertin et al. 2004). The above observations lead to a model in which Sid2 exists in two complexes, one with Mob1 and one without. Both complexes would be inactive in interphase. During mitosis the Sid2 component of an inactive Mob1-Sid2 complex at the SPB gets phosphorylated in a SIN dependent manner. The phosphorylation event alters Mob1 binding to Sid2 such that it allows for Sid2 kinase activation (Hou, Guertin et al. 2004).

Other regulators of the SIN

Genetic epistasis analysis places all of the SIN components mentioned above in a linear pathway. While demonstrating the linearity of the pathway awaits more biochemical characterization there is increasing evidence that a number of other players influence SIN signaling at some stage.

Plo1 kinase of *S. pombe* belongs to the POLO family of protein kinases. As documented in various eukaryotic organisms, Plo1 is involved in multiple aspects of mitosis and cytokinesis (Ohkura, Hagan et al. 1995; Golsteyn, Lane et al. 1996; Tanaka, Petersen et al. 2001; Mulvihill and Hyams 2002). Given the multiple roles of Plo1, trying to ascertain a direct and precise role for Plo1 in the SIN has proven to be difficult. The evidence that Plo1 does indeed influence the SIN comes from the observation that Plo1 overproduction results in transient recruitment of Cdc7 to the SPB and subsequent production of multiple septa. Plo1 itself associates with the SPB during mitosis where it interacts with the scaffold protein and SIN component Sid4 (Mulvihill, Petersen et al. 1999; Tanaka, Petersen et al. 2001; Mulvihill and Hyams 2002; Morrell, Tomlin et al. 2004). Furthermore, in a screen for mutants designed to identify Plo1 substrates, a number of SIN component alleles were identified; these mutants depend on high Plo1 activity for viability (Cullen, May et al. 2000).

In higher eukaryotes the activation of CDK leads to the activation of a number of downstream kinases such as the polo-family and the NIMA-related kinases. The *S.pombe* NIMA kinase Fin1 plays an important role in mitotic spindle formation and spindle pole body maturation (Krien, Bugg et al. 1998; Grallert and Hagan 2002). Although Fin1 associates with the SIN inhibitor Byr4, Fin1 also localizes to the new SPB that hosts the active SIN, and thus Fin1 must have another binding partner at the SPB. Loss of Fin1 function leads to recruitment of Cdc7 to both SPBs and promotes septation suggesting that Fin1 plays a role in attenuating SIN signaling at the old SPB (Grallert, Krapp et al. 2004).

Another protein, Dma1, prevents septation by hindering the binding of Plo1 to the SPB. Dma1 itself also localizes to the SPBs through association with Sid4 (Murone and Simanis 1996; Guertin, Venkatram et al. 2002). One prediction is that Dma1 may directly compete with Plo1 for a SPB binding partner and thus prevent Plo1 from localizing to the SPB (Guertin, Venkatram et al. 2002).

Two other putative negative regulators of SIN signaling have been identified - *zfs1* (a zing-finger containing protein) and *scw1* (a RNA-binding protein). Their exact mode of action is unclear. Mutations in *scw1* may play a role in stabilizing microtubules and therefore indirectly affect the SIN (Beltraminelli, Murone et al. 1999; Jin and McCollum 2003).

Mutations in B' regulatory subunits of protein phosphatase 2A, Par1 and Par2 can rescue certain SIN mutants. This observation and other genetic interactions suggest that Par1 and Par2 may be involved at various levels in the SIN (Jiang and Hallberg 2000; Jiang and Hallberg 2001; Le Goff, Buvelot et al. 2001). Consistent with some association with SIN components, Par1 localizes to the SPB and then to the ring during mitosis (Le Goff, Buvelot et al. 2001).

Regulating SIN activity

A number of proteins are involved in SIN signaling. An important unanswered question is what are the signals that are ultimately responsible for delivering the 'time to turn SIN on / off' message. Mitotic CDK inactivation is a prerequisite for SIN to be 'on'. Coupling SIN activation to mitotic CDK inactivation serves to prevent septum formation prior to mitosis. The Sid1-Cdc14 complex appears to be a sensor for the state of mitotic CDK activity because only when Cdc2 activity has dropped below a certain threshold does the complex localize to the SPB. Consistent with the idea of entraining the SIN with the level of CDK activity is the observation that hyperactivation of the SIN via inactivation of Cdc16 leads to ectopic formation of septa only during interphase or late stages of mitosis—each a stage when the mitotic CDK activity is low (Fankhauser, Marks et al. 1993).

The cell also needs a mechanism to prevent premature SIN inhibition in situations where cytokinesis is delayed. The cytokinesis checkpoint serves to fulfill that requirement by ensuring that CDK activity is kept low and SIN remains 'on' until cytokinesis is completed. The checkpoint was uncovered through the identification of mutations in the *cps1*$^{+}$/*drc1*$^{+}$ gene, a subunit of 1,3 β-glucan synthase. These mutants arrest with two interphase nuclei that complete

another round of DNA replication after completing mitosis but fail at septum formation and cytokinesis (Le Goff, Woollard et al. 1999; Liu, Wang et al. 1999; Liu, Wang et al. 2000). The block depends on the presence of F-actin structures and a functional SIN (Liu, Wang et al. 2000; Mishra, Karagiannis et al. 2004). Inactivating the CDK inhibitor Wee1 or Clp1/Flp1, the *S. pombe* homolog of the *S. cerevisiae* Cdc14 phosphatase, also alleviates the block (Trautmann, Wolfe et al. 2001). Clp1/Flp1 is normally sequestered in the nucleolus during interphase or on the SPB. It is released from the nucleolus and re-localizes to the spindle, kinetochores and actomyosin ring when cells enter mitosis. The release from the nucleolus is SIN independent but SIN signaling must cease for Clp1 to re-localize to the nucleolus at the end of mitosis (Cueille, Salimova et al. 2001; Trautmann, Wolfe et al. 2001). In cells arrested in G2 by the cytokinesis checkpoint, activated Clp1 is found in the cytoplasm. Taken together, these data suggest that the cytokinesis checkpoint functions to maintain Clp1 in the cytoplasm where it can inhibit Cdc2 activity. One way by which Clp1 phosphatase achieves this inhibition is by dephosphorylating and thus inactivating the Cdc2 activator -Cdc25 phosphatase (Esteban, Blanco et al. 2004; Wolfe and Gould 2004). Keeping Cdc2 activity low allows the SIN to remain active until cytokinesis is complete; the SIN in turn keeps Clp1 out of the nucleolus when cytokinesis is delayed to allow for cytokinesis to be completed. While the molecular details of these events remain elusive, this feedback mechanism ensures that SIN activity is temporally regulated.

Conservation of the SIN

In *S. cerevisiae* proteins of the MEN network control the inactivation of CDK-Cdc28 to allow for mitotic exit and cytokinesis. The MEN network is also part of a checkpoint that monitors spindle position in anaphase (reviewed in Segal and Bloom 2001; McCollum 2002; Smeets and Segal 2002). In addition proteins of the MEN network play a direct role in regulating cytokinesis that is distinct from their role in mitotic exit. The MEN, like the SIN, is a GTPase regulated protein kinase signaling cascade. It consists of four protein kinases (Cdc5, Cdc15, Dbf2 and Dbf20), a GTPase (Tem1), a GEF (Lte1), a two- component GAP (Bfa1-Bub2), a protein phosphatase Cdc14, and a scaffold protein Nud1 (Table1 and Figure 4).

The MEN brings about CDK inactivation by keeping the Cdc14 phosphatase out of the nucleolus and away from its inhibitor Net1/Cfi1. Like Clp1/Flp1, Cdc14 is normally sequestered in the nucleolus during interphase (Shou, Seol et al. 1999). It is released from Net1/Cfi1 during

anaphase where it dephosphorylates CDK substrates. The resulting wave of dephosphorylation leads to inactivation of Cdc28 activity through a variety of different mechanisms (Reviewed in Stegmeier and Amon 2004). Although MEN activity is required to keep Cdc14 out of the nucleolus, mutations in *net1* permit Cdc14 release in the absence of MEN function and uncover another function for the MEN in cytokinesis (Visintin, Hwang et al. 1999; Visintin and Amon 2000).

The GTPase Tem1 appears to act at the top of the MEN pathway in a role analogous to that of Spg1 in the SIN (Shirayama, Matsui et al. 1994; Asakawa, Yoshida et al. 2001). Tem1 and its cognate GAP Bfa1-Bub2 (analogous to Byr4-Cdc16 in *S.pombe*) localize preferentially to the spindle pole body destined for the daughter cell (Fraschini, Formenti et al. 1999; Bardin, Visintin et al. 2000; Pereira, Hofken et al. 2000). Lte1, the presumed GEF for Tem1 is spatially restricted to the bud cortex of the daughter cell (Prinz and Amon 1999; Bardin, Visintin et al. 2000; Pereira, Hofken et al. 2000). As the daughter bound SPB moves into the bud it comes into contact with Lte1 and Tem1 gets activated. Tem1 is held inactive by GAP activity of Bfa1-Bub2 until it encounters Lte1 in the bud (Wang, Hu et al. 2000; Geymonat, Spanos et al. 2002; Geymonat, Spanos et al. 2003). Tem1 activation clearly requires inactivation of the Bfa1-Bub2 complex that is situated at the same SPB. Bfa1 is phosphorylated in a cell -cycle dependant manner, by the kinase Cdc5. The phosphorylation inhibits the GAP activity of the complex (Hu, Wang et al. 2001; Lee, Jensen et al. 2001; Geymonat, Spanos et al. 2002).

Table 1. Core SIN and MEN components

S.pombe	**Protein function**	***S.cerevisiae***	***Higher Eukaryotes***
Sid4	SPB scaffold protein	Unknown	Kendrin?
Cdc11	SPB scaffold protein	Nud1	Centriolin
Plo1	Kinase	Cdc5	Polo kinase family
Spg1	GTPase	Tem1	?
Byr4	Part of GAP complex	Bfa1	?
Cdc16	Part of GAP complex	Bub2	GAPCenA
Unknown	GEF?	Lte1	?
Cdc7	Kinase	Cdc15	?
Sid1	Kinase	Unknown	?
Cdc14	Binds to Sid1	Unknown	?
Sid2	Kinase	Dbf2	Warts/LATS1 (human)
Mob1	Sid2/Dbf2 binding partner	Mob1	hMob1, mMob1

Phosphorylated forms of Bfa1 cannot interact with Tem1 as demonstrated by a failure of mitotic Bfa to co-immunoprecipitate with Tem1 (Hu, Wang et al. 2001). Bub2 is also phosphorylated during mitosis but the effect of the phosphorylation event on Bub2 function is not clear. Interestingly, Bfa1 also gets dephosphorylated and thus reactivated by Cdc14 in telophase, keeping the window during which MEN is active limited (Hu, Wang et al. 2001; Pereira, Manson et al. 2002). Tem1 activation leads to the recruitment and activation of Cdc15, homologous to *S.pombe* Cdc7 kinase. Like Cdc7, Cdc15 protein levels and kinase activity remain constant through the cell cycle. Cdc15 localizes to the SPB during anaphase where it is presumed to target downstream components of the MEN (Cenamor, Jimenez et al. 1999). The protein kinase complex of Dbf2-Mob1, equivalents of *S.pombe* Sid2-Mob1, is downstream of all MEN components (Lee, Frenz et al. 2001; Mah, Jang et al. 2001). Similar to Sid2 in *S. pombe*, Dbf2 protein levels remain constant but its kinase activity is cell cycle regulated peaking in late mitosis (Toyn and Johnston 1994).

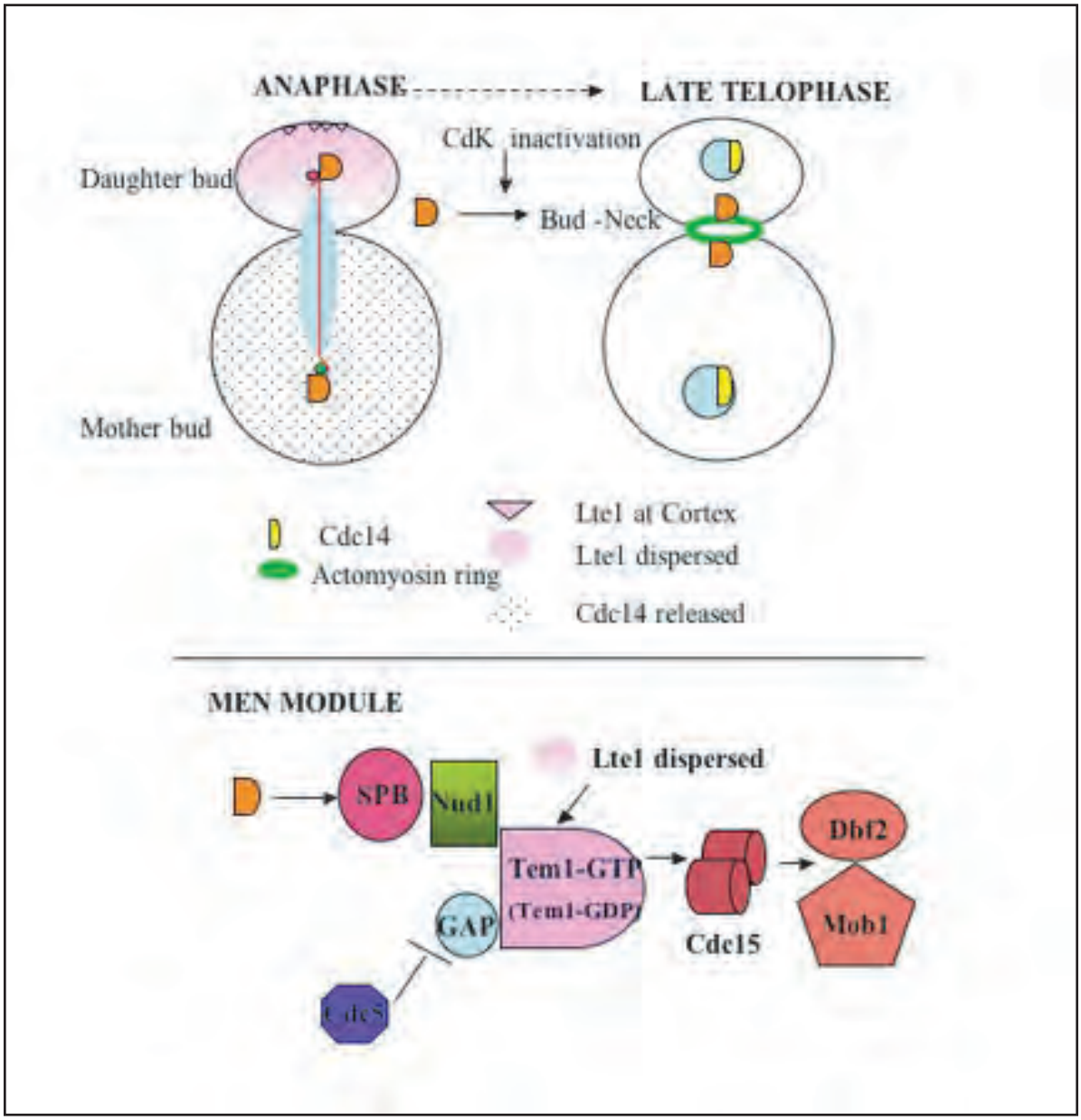


Figure 4. Current model of MEN regulation of mitotic exit in *S.cerevisiae.* During anaphase the SPB destined for the daughter cell carries with it MEN components that consist of Tem1 GTPase Bub2--Bfa1 GAP and Nud1. Lte1 is present in the bud and activates Tem1. The components Cdc15 and Dbf2-Mob1 are recruited to the SPB. The exact nature and timing of Cdc15 and Dbf2 localization are unclear. The activation of the MEN causes complete release of Cdc14 from the nucleolus, which promotes mitotic exit. Upon Cdc28 inactivation the MEN module is cleared from the SPB and translocated to the bud neck region where it functions to promote cytokinesis.

Mob1 is essential for viability and *mob1* temperature sensitive alleles are defective in Dbf2 binding, suggesting that the interaction with Dbf2 is important for Mob1 function (Komarnitsky, Chiang et al. 1998; Luca, Mody et al. 2001). Dbf2 is localized to the SPB throughout most of the cell cycle; however, Mob1 localizes to the SPB only during anaphase (Frenz, Lee et al. 2000; Luca, Mody et al. 2001; Yoshida and Toh-e 2001). Cdc15 can phosphorylate and activate Dbf2 *in vitro* however only in the presence of Mob1 (Mah, Jang et al. 2001). This suggests that Cdc15 can activate Dbf2 only during anaphase, when Mob1 is associated with it at the SPB. Interestingly, like *S. pombe* Sid2, Dbf2 and Mob1 re-localize to the division site in late anaphase. This re-localization event requires the function of MEN and the septins (Frenz, Lee et al. 2000; Luca, Mody et al. 2001; Yoshida and Toh-e 2001). The exact role of Dbf2-Mob1 at the bud neck and in cell division is not clear but it is likely that they will serve to phosphorylate substrates that play roles in cytokinesis.

MEN components are anchored to the SPB via the scaffold protein Nud1. Nud1 associates with Bub2 and Bfa1 in yeast two-hybrid analysis and co-immunoprecipitation (Gruneberg, Campbell et al. 2000). The MEN seems to be essential for regulating mitotic inactivation whereas the SIN regulates the formation of the septum after mitotic CDK inactivation. However, a number of MEN mutants are defective in cytokinesis suggesting the MEN has additional roles in cytokinesis (Jimenez, Cid et al. 1998; Luca, Mody et al. 2001; Menssen, Neutzner et al. 2001; Song and Lee 2001). When the mitotic exit defect in *tem1-1* allele is alleviated by a mutation in *net1*, it forms chained cells, an indicator of a cytokinetic defect (Shou, Seol et al. 1999). The MEN mutant *mob1-77* overproducing the CDK inhibitor Sic1 can bypass the mitotic exit defect but still fails at cytokinesis (Luca, Mody et al. 2001). These observations imply that the role of MEN in cytokinesis is direct and not a consequence of delayed mitotic exit.

The MEN proteins Cdc5, Cdc15, Dbf2 and Mob1 localize to the bud-neck in late mitosis (Frenz, Lee et al. 2000; Song, Grenfell et al. 2000; Xu, Huang et al. 2000). Mitotic CDK inactivation is required for cytokinesis in *S. cerevisiae* as it is in *S. pombe* (Hwa Lim, Yeong et al. 2003). Untimely inactivation of CDK activity leads to premature translocation of Dbf2/Dbf20 to the bud neck (Hwa Lim, Yeong et al. 2003). Once at the bud-neck the MEN may play a role in regulating assembly or contraction of the actomyosin ring. For instance, Tem1 has been

shown to be required for proper actomyosin and septin ring dynamics (Lippincott, Shannon et al. 2001).

A growing number of possible mammalian homologs of SIN and MEN components are being identified (Cuif, Possmayer et al. 1999; Hirota, Morisaki et al. 2000) (Table 1). The human homolog of Sid2/Dbf2 is the warts/LATS1 kinase, which localizes to the mitotic apparatus and is phosphorylated specifically during mitosis (Hirota, Morisaki et al. 2000). Cells lacking the human homolog of Cdc14-hCdc14A have cytokinetic defects. hCdc14A also localizes to the centrosome (Kaiser, Zimmerman et al. 2002). A mammalian homolog of Nud1 and Cdc11—Centriolin localizes specifically to the mother centriole and then is found adjacent to the mid-body. Depletion of centriolin by RNAi leads to cytokinetic failure (Gromley, Jurczyk et al. 2003). The cytokinetic function of centriolin is attributed to a domain within the protein that bears homology to Nud1 and Cdc11. Interestingly the same domain also binds Bub2 (Gromley, Jurczyk et al. 2003). Elucidating the functions of known mammalian homologs as well as identifying other homologs should reveal whether similar mechanisms or pathways exist in higher eukaryotes.

Summary

Cytokinesis is a complex process that involves careful co-ordination in both space and time, of a plethora of processes that are as varied as membrane synthesis to ring constriction. The study of cytokinesis in fission yeast *S.pombe* has revealed a signaling network that plays a critical role in controlling actomyosin ring constriction and septum formation. The timing of cytokinesis and septum formation hinges upon the activation of the GTPase Spg1. Hence understanding the regulation of Spg1 is critical for elucidating how cytokinesis is temporally regulated. However, very little is known about how Spg1 is regulated or about its downstream effector kinase Cdc7. In these studies I have investigated the role of Cdc7 and have identified numerous functional domains within the SIN kinase that are essential for its function (Chapter III). Second, I have investigated the regulation of the GAP component Byr4 by phosphorylation as a means of regulating Spg1activation and further characterized its interaction with Spg1 and Cdc16 (Chapter IV and V).

CHAPTER II

MATERIALS AND METHODS

S.pombe strains and media

S.pombe strains (Table 3) used in this study were grown in YE medium or EMM minimal medium with addition of appropriate nutritional supplements as described in (Moreno, Klar et al. 1991). Yeast transformations were performed by electroporation (Prentice 1992) or a lithium acetate method (Keeney and Boeke 1994). Expression of constructs under control of the thiamine- repressible promoter *nmt* was carried out as previously described (Basi, Schmid et al. 1993; Maundrell 1993). Cell synchrony was achieved by using a *cdc25-22* mutant arrest and release (Fankhauser, Reymond et al. 1995) or by centrifugal elutriation as previously described (Chang, Morrell et al. 2001).

Molecular biology methods

Standard genetic and recombinant DNA methods were used except where noted. PCR amplifications from S. pombe genomic DNA or a cDNA library were carried out to obtain various gene fragments. PCR amplifications were performed using either TaqPlus Precision polymerase (Stratagene) or Vent polymerase (New England Biolabs) according to manufacturers instructions. Oligonucleotides were synthesized by Integrated DNA Technologies, Inc. Site-specific mutations were created using the Stratagene Chameleon or Quickchange site-directed mutagenesis kit in accordance with manufacturers instructions. Sequencing was done at the Vanderbilt Sequencing Facility. All plasmids used in this study are listed in Table 4.

In vivo tagging and gene deletions

Strains expressing carboxy -terminal HA_3, Myc_{13}, EGFP, CFP or YFP tagged versions of proteins were constructed using a PCR-based approach as described by (Bahler, Wu et al. 1998). Accurate integration and expression of protein was confirmed by PCR and either microscopic or

immunoblot inspection, as appropriate. All tagged strains were viable at temperatures ranging from 25 to 36^{o}C.

Gene replacements were accomplished by replacing the coding sequences with the *ura4*$^{+}$ gene at the locus of interest by homologous recombination in a diploid strain as described (Bahler, Wu et al. 1998). Ura^{+} transformants were confirmed by PCR amplification using primers within the *ura4*$^{+}$ gene and primers outside the disruption cassette. Heterozygous diploid strains were sporulated followed by tetrad dissection to determine if these genes were essential for viability.

Yeast two-hybrid analysis

The yeast two-hybrid system used in this study was described previously (James, Halladay et al. 1996). Various regions of *cdc7*$^{+}$, *byr4*$^{+}$, *cdc16*$^{+}$ and *spg1*$^{+}$ cDNAs were amplified by PCR with primers containing 5' and 3' restriction sites and cloned in frame into either the bait plasmid pGBT9 and/or prey plasmid pGAD424 (Clonetech, Palo Alto, CA). Plasmids were sequenced to verify the maintenance of the correct reading frame and absence of any PCR-induced mutations. Protein interactions were tested by co-transforming the bait and prey plasmids into *S.cerevisiae* strain PJ69-4A. Leu^{+} and Trp^{+} transformants were scored for positive interactions by plating on synthetic dextrose medium lacking adenine and histidine. β-Galactosidase reporter enzyme activity in the two-hybrid strains was measured using the Galacto-Star ^{TM} chemiluminescent reporter assay system according to the manufacturer's instructions (Tropix Inc., Bedford MA), with the exception that cells were lysed by glass bead disruption. Each sample was measured in triplicate.

Cytology and Microscopy

All strains producing GFP –tagged proteins were visualized live unless otherwise specified. Microscopy was carried out on an UltraView LCI confocal microscope equipped with a 488nm argon-ion laser (for GFP and YFP excitation) and a 442nm Helium Cadmium laser (for CFP excitation). Images were captured on an Orca-ER charge-coupled device (CCD) camera (Hamamatsu, Japan) using Ultra-View software (PerkinElmer Life Sciences). Images were processed using Volocity 2.0 software (Improvision, Lexington, MA). Z-series optical sections were taken at 0.5 μM spacing.

To visualize cell walls and septa, 1ml of cells from the culture were fixed in ethanol and resuspended in 1ml of 1:100 dilution of methyl blue stock solution (100mg/ml) (Sigma Aldrich, St. Louis, MO). The cells were incubated with the dye for 15-30 minutes and subsequently washed three times with sterile PBS. The cells were resuspended in an appropriate volume of PBS and mounted on a slide with DAPI to visualize the nuclei. To visualize the nuclei in live cells, 1 ml of cells was incubated with a 1:1000 dilution (final) of Hoechst 33258 (Sigma Aldrich, St. Louis, MO) and allowed to incubate for 20-30 seconds. The cells were washed with PBS and visualized under the scope.

DAPI, Aniline blue stained cells and Hoechst images were obtained, processed and analyzed with OpenLab 2.1.3 software (Improvision, Lexington, MA) on a Zeiss microscope (Axioskop 2;Carl Zeiss,Thornwood, NY) equipped with a Plan APOCHROMAT 100 x /1.4 NA objective lens (Carl Zeiss) and a GFP and DAPI filter set (Chroma technology).

Protein lysates, immunoprecipitations and immunoblotting

Whole cell pombe lysates were prepared in NP-40 buffer (Gould, Moreno et al. 1991), and immunoprecipitations were carried out using either anti-HA (12CA5), anti-Myc (9E10) or polyclonal anti-GFP antibodies as described (McDonald, Ohi et al. 1999). Denatured lysates were prepared as outlined in (Burns, Ohi et al. 2002). Proteins were resolved by either SDS-PAGE or 4-12% NuPAGE gel in MOPS buffer. Were noted 3-8% or 7% Tris-Acetate gels in Tris- acetate buffer was also used. Proteins were transferred by electroblotting to a PVDF membrane (Immobilon P, Millipore Corp., Bedford MA) Immunoblotting was done with anti-HA (12CA5; 2 mg/ml), anti-Myc (9E10; 2 mg/ml), anti-Cdc2 (PSTAIRE 1:5000 Sigma, St. Louis, MO), and rabbit polyclonal antibodies to Byr4 (1:2000 dilution of serum), rabbit polyclonal to Cdc16 (1:1000 dilution of serum) or anti-GFP (1:1000 dilution of serum) antibodies. Primary antibodies were detected with horseradish peroxidase–conjugated goat anti-mouse or goat anti-rabbit secondary antibodies (0.4 mg/ml; Jackson ImmunoResearch Laboratories, West Grove, PA) at a dilution of 1:50,000, visualized by ECL using SuperSignal (Pierce, Rockford, IL).

Immunoprecipitation/Phosphatase assay

Following immunoprecipitation as described above, beads were washed two times with NP-40 buffer and 3 times with phosphatase buffer (25 mM HEPES-NaOH pH 7.4, 150mM NaCl, 0.1 mg/ml BSA) (REF). Beads were split into two samples, pelleted washed and resuspended in 9 µl of 1X phosphatase buffer (New England Biolabs) + 2mM $MnCl_2$ and 1 µl of either lambda phosphatase (New England Biolabs) or buffer alone (mock). Beads were incubated at 30^{o}C for 30-45 minutes followed by 2 washed in NP-40 buffer and resuspended in 2X Sample buffer. Lambda phosphatase or mock treated immunoprecipitates were resolved and immunoblotted as described above.

Sucrose gradients

A 200 µl volume of clarified protein lysate was layered onto a 5 ml 5-20% sucrose gradient (in NP-40 buffer) with .25 ml, 50% sucrose plug at the bottom. Gradients were ultracentrifuged at 40,000 rpm and 4^{o}C for 19h in a Beckman SW50.1 rotor (Berkeley, CA). Sedimentation markers were fractionated on gradients prepared and spun in parallel. 20 x .25 ml fractions were collected from the bottom of the gradient. Fractions were resolved on a 10% SDS-PAGE gel and transferred by electroblotting to PVDF membrane as noted above. For immunoprecipitations 1.5 µg of primary antibody was added to each fraction and processed as described above.

In vivo labeling cells with ^{32}P orthophosphate, phospho amino acid analysis and tryptic peptide mapping

Cells were grown to mid-log phase in minimal media plus any necessary supplements. They were then washed in phosphate free media and allowed to grow in the same with supplements and 50 mM NaH_2PO_4 for around 16-18 hours. Cells were collected resuspended in 10 mls phosphate free media and incubated for another 4 hours in the presence of ^{32}P orthophosphate label (NEN, New Jersey). Cells were then harvested and lysed to prepare lysates that are resolved by SDS-PAGE. Proteins were transferred by electroblotting to a PVDF membrane (Immobilon P, Millipore Corp., Bedford, MA) and detected by autoradiography. For phospho-amino acid analysis the ^{32}P-labeled protein subjected to partial acid hydrolysis with 6N

HCl while still bound to the PVDF membrane, and the phospho-amino acids were separated in two dimensions by thin-layer electrophoresis at pH 1.9 and pH 3.5 along with a mix of phospho-serine, phospho-threonine and phospho-tyrosine standards. The phosphoamino acid standards were visualized upon staining with Ninhydrin (0.25% in Acetone) whereas the labeled phosphoamino acids in the protein were visualized by autoradiography. For tryptic peptide maps the protein subject to digestion by Trypsin in ammonium bicarbonate buffer while still bound to PVDF for a total of 6 hours. The peptides were then separated in two dimensions first by thin-layer electrophoresis in buffer pH1.9 followed by chromatography (Boyle, van der Geer et al. 1991).

Preparation of recombinant proteins and *in vitro* binding assays

Glutathione-S-transferase (GST), Maltose-Binding Protein (MBP) or His6-fusion proteins were produced in *Escherichia coli* BL21-codonplus-RIL cells (Stratagene) from pGEX (Stratagene), pMAL-2C (New England Biolabs) or pET15b (Novagen) expression vectors, respectively. The induction of recombinant protein expression was achieved with 0.4mM isopropyl-β-D-thiogalactopyranoside (IPTG; Fisher Scientific, Pittsburgh, PA) at temperatures ranging from 32^{o}C to 25^{o}C. The proteins were purified under native conditions on Ni-NTA agarose (from Qiagen, for His_6-fusions), glutathione-agarose beads (for GST) or amylose resin (for MBP). The proteins were eluted from the beads by incubating in excess amounts of imidazole (for His), glutathione (for GST) or maltose (for MBP). The eluted fractions were then dialyzed against binding buffer (20mM Tris-HCl, pH 7.0, 150 mM NaCl, 2 mM EDTA and 0.1% NP-40). For binding reactions appropriate amounts of two eluted proteins were incubated together and the volume was made up to 1 ml with binding buffer. The appropriate resin was added to pull down one of the proteins in the reaction. The beads were washed extensively and bound proteins were resolved by SDS-PAGE followed by Coomassie blue staining to visualize bound proteins.

***In vitro* kinase assays**

His_6 fusion proteins of Byr4 or MBP-Byr4 were made in *E.coli* as described above, except the proteins were left bound to beads. The beads were washed with HB15 Buffer (60 mM β -glycerophosphate, 25 mM MOPS [pH 7.2], 15 mM p-nitrophenylphosphate, 15 mM EGTA,

15 mM $MgCl_2$, 1 mM DTT, and 0.1 mM $NaVO_3$) and resuspended in a final volume of 20 μl HB-15 buffer. 10 μl of HB-15 buffer containing 50 μM ATP, 3 μCi of ^{32}P-γ-ATP, and 100 ng of insect cell produced Cdk1p-Cdc13p was added to the beads. The beads were then incubated at 30^{o}C for 30 min in an Eppendorf-shaker, which allowed for constant shaking and resuspension of beads in buffer. 20 μl of 2X Sample buffer was added to terminate the reaction. The samples were separated by SDS-PAGE and either detected by Coomassie staining or transferred onto PVDF membrane. Coomassie stained gels were dried with a BioRad 583 Gel-Dryer and exposed to film to detect the incorporation of the radiolabel. The PVDF membrane was also exposed to film following which radiolabeled protein was subject to phosphoaminoacid analysis or tryptic peptide mapping as described above.

Table 3. Strains used in this study

Strain	Genotype
KGY246	*h⁻ ade6-M210 ura4-D18 leu1-32*
KGY170	*cdc10-129 h+*
KGY352	*nuc2-663 h-*
KGY444	*cdc25-22 leu1-32 ura4-D18 ade6-M210 h+*
KGY466	*cdc25-22 rad1::ura4 leu1-32 h-*
KGY516	*rad1::ura4 leu1-32 ura4-D18 ade6-M210 h-*
KGY702	*cdc16-116 leu1-32 ura4-D18 ade6-M210 h+*
KGY4071	*h⁻ spg1-myc::Kan^R ade6-M210 ura4-D18 leu1-32*
KGY3842	*h⁻ cdc7-myc::Kan^R ade6-M210 ura4-D18 leu1-32*
KGY2061	*h⁻ cdc7-24 ade6-M21X ura4-D18 leu1-32*
KGY2678	*h⁺ spg1-GFP::Kan^R ade6-M210 ura4-D18 leu1-32*
KGY2167	*h⁺spg1-3XHA::ura4 ura4-D18 ade6-M210 leu1-32*
KGY1790	*h⁺spg1-3XHA::ura4 ura4-D18*
KGY5401	*h⁻ cdc7-myc::KanR spg1HA::ura4 ade6-M210 leu1-32 ura4-D18*
KGY4426	*cdc7-myc::Kan^R/cdc7-3XHA::ura4 ade6-M210/ade6-M216 ura4-D18/ura4-D18 leu1-32/leu1-32*
KGY2628	*sid4-GFP::Kan^R ura4-D18 ade6-M210 leu1-32 h-*
KGY2678	*spg1-GF::Kan^R ura4-D18 ade6-M210 leu1-32 h+*
KGY2933	*cdc7-GFP::Kan^R ade6-M210 leu1-32 ura4-D18 h-*
KGY2945	*sid2-GFP::Kan^R leu1-32 h+*
KGY3341	*cdc11-GFP::Kan^R ura4-D18 leu1-32 ade6-M210 h-*
KGY4206	*mts3-1 rad1::ura4 ura4-D18 leu1-32 ade6-M210 h-*
KGY4267	*spg1-myc::Kan^R h-*
KGY4430	*cdc7-myc::Kan^R cdc25-22 ade6-M210 ura4-D18 leu1-32 h+*
KGY4626	*rad1::ura4 cdc16-116 leu1-32 ura4-D18 ade6-M210 h?*

Table 2. Plasmids constructed for this study

Plasmid Number	Vector	Insert
pKG16	pREP1	Byr4(aa100-665)
pKG19	pREP1	Byr4(aa400-665)
pKG125	pREP1	Byr4(aa1-600)
pKG144	pREP1	Byr4(aa300-665)
pKG2447	pREP1	Byr4(aa600-665)
pKG2620	pREP1	Byr4(aa100-300)
pKG2636	pREP1	Byr4(aa475-595)
pKG30	pREP81-GFP	Byr4(aa100-665)
pKG37	pREP81-GFP	Byr4(aa400-665)
pKG2618	pREP1-GFP	Byr4(aa600-665)
pKG2619	pREP81-GFP	Byr4(aa600-665)
pKG2621	pREP1-GFP	Byr4(aa100-300)
pKG2622	pREP81-GFP	Byr4(aa100-300)
pKG141	pREP81-GFP	Byr4(aa1-600)
pKG156	pREP81-GFP	Byr4(aa300-665)
pKG2637	pREP1-GFP	Byr4(aa475-595)
pKG2638	pREP81-GFP	Byr4(aa475-595)
pKG2730	pREP81-GFP	Byr4
pKG722	pET15b	Byr4(aa475-595)
pKG2185	pGAD	Byr4
pKG2186	pGAD	Byr4(aa479-592)
pKG2187	pGAD	Byr4(aa1-479)
pKG2241	pGAD	Byr4(aa479-665)
pKG2242	pGAD	Byr4(aa535-665)
pKG2243	pGAD	Byr4(aa400-665)
pKG2245	pGAD	Byr4(aa660-665)
pKG2246	pGAD	Byr4(aa1-100)
pKG2247	pGAD	Byr4(aa1-300)
pKG2248	pGAD	Byr4(aa100-665)
pKG2249	pGAD	Byr4(aa1-535)
pKG2272	pGAD	Byr4(aa308-665)
pKG2273	pGAD	Byr4(aa1-600)
pKG2612	pGAD	Byr4(aa100-300)
pKG2634	pMAL	Byr4
pKG2679	pGAD	Byr4(aa475-595)
pKG2717	pGEX4T1	Byr4(aa100-300)
pKG2718	pGEX4T1	Byr4(aa475-595)
pKG3364	pET15b	Byr4(aa100-665)
pKG3369	pREP81-GFP	Byr4S4A
pKG3565	pET15b	Byr4(100-665)S4A
pKG3592	pET15b	Byr4(100-665)S5A
pKG13	pREP81-GFP	Cdc7(aa1-535)
pKG482	pREP81-GFP	Cdc7(aa250-535)

Table 2, cont.

Plasmid Number	Vector	Insert
pKG513	pREP1-GFP	Cdc7(aa250-535)
pKG517	pREP1-GFP	Cdc7(aa300-535)
pKG3031	pREP81-GFP	Cdc7(aa360-535)
pKG3127	pREP81-GFP	Cdc7
pKG1335	pREP81-GFP	Cdc7(aa360-518)
pKG3194	pREP81-GFP	Cdc7(aa1-250)
pKG3195	pREP81-GFP	Cdc7(aa1-260)
pKG3196	pREP81-GFP	Cdc7(aa535-1062)
pKG3197	pREP81-GFP	Cdc7(K38R)
pKG3198	pREP81-GFP	Cdc7(aa1-500)
pKG3281	pREP81-GFP	Cdc7(aa250-1062)
pKG3282	pREP81-GFP	Cdc7(aa360-1062)
pKG3283	pREP81-GFP	Cdc7(aa261-800)
pKG3285	pREP81-GFP	Cdc7(aa261-840)
pKG3295	pREP81-GFP	Cdc7(aa361-800)
pKG3296	pREP81-GFP	Cdc7(aa361-840)
pKG3489	pREP81-GFP	Cdc7(aa1-990)
pKG3490	pREP81-GFP	Cdc7(aa1-920)
pKG3491	pREP81-GFP	Cdc7(aa1-900)
pKG3492	pREP81-GFP	Cdc7(aa360-900)
pKG3494	pREP81-GFP	Cdc7(aa360-870)
pKG1336	pREP81-GFP	Cdc7(Δ360-518)
pKG724	pGAD	Cdc7(aa250-535)
pKG726	pGAD	Cdc7(aa300-535)
pKG2188	pGAD	Cdc7(aa535-1062)
pKG2189	pGAD	Cdc7(aa1-535)
pKG2250	pGAD	Cdc7
pKG2251	pGAD	Cdc7(aa1-250)
pKG2253	pGAD	Cdc7(aa1-500)
pKG2254	pGAD	Cdc7(aa1-634)
pKG2724	pGAD	Cdc7(aa1-350)
pKG2725	pGAD	Cdc7(aa350-535)
pKG1192	pGAD	Cdc7(aa360-518)
pKG1360	pGAD	Cdc7(Δ360-518)
pKG1361	pGBT9	Cdc7(Δ360-518)
pKG1191	pGBT9	Cdc7(aa360-518)
pKG731	pET15b	Cdc7(aa250-535)
pKG1442	pREP41	Cdc7
pKG1510	pREP41-HA	Cdc7(Δ360-518)
pKG1511	pREP41-HA	Cdc7(aa360-518)
pKG1512	pREP41-HA	Cdc7
pKG3541	pREP1-NTAP	Cdc7(aa250-535)
pKG2184	pGAD	Spg1

Table 2, cont.

Plasmid Number	Vector	Insert
pKG2180	pGBT9	Spg1
pKG796	pMAL-2C	Spg1Q69L
pKG803	pGEX	Spg1Q69L
pKG813	pMAL-2C	Spg1T42A
pKG1096	pGEX4T-1	Spg1T42A
pKG1826	pMAL-2C	none
pKG1699	pGEX4T-1	none
pKG1743	pGAD424	none
pKG1744	pGBT9	none

CHAPTER III

IDENTIFICATION OF FUNCTIONAL DOMAINS WITHIN SIN KINASE CDC7

Introduction

The Septation Initiation Network (SIN) serves to coordinate cytokinesis with mitotic exit in the fission yeast *Schizosaccharomyces pombe*. SIN components Spg1, a ras-like GTPase and Cdc7 together play a central role in regulating the onset of septation and cytokinesis. Activation of the SIN pathway is triggered by activation of Spg1 and increased production of Spg1 leads to the formation of septa from any point in the cell cycle (Schmidt, Sohrmann et al. 1997). Interestingly, Spg1 activation at the SPB is asymmetric. It exists in the GDP- bound state during interphase at the single SPB, during metaphase it is GTP-bound at both SPBs only to be 'inactivated' or GDP bound at one SPB during anaphase B (Sohrmann, Schmidt et al. 1998).

Cdc7 is a member of the STE11/MEKK family of kinases (http://alpha.kinasenet.org/pkr/) (Hanks, Quinn et al. 1988; Smith, Shindyalov et al. 1997; Smith 1999; Petretti and Prigent 2005). Most kinases belonging to the MEKK family share a similar structure with the kinase domain at the C-terminus and a regulatory domain at the N-terminus (Hagemann and Blank 2001). They also bind to small G-proteins (Hagemann and Blank 2001). Cdc7 differs from typical members of the family in that the kinase domain of Cdc7 resides at its N-terminus. However, like other family members, it binds to and is an effector for a GTPase, in this case Spg1 (Schmidt, Sohrmann et al. 1997; Sohrmann, Schmidt et al. 1998). Cdc7 is only recruited to the pole that is occupied by GTP-bound Spg1; hence it also is asymmetrically localized to one pole during anaphase. The asymmetric localization of SIN components appears to be important for proper SIN regulation since Cdc7 is associated with both SPBs during anaphase in cells with deregulated septum formation (Mulvihill, Petersen et al. 1999; Tanaka, Petersen et al. 2001).

Cdc7 kinase plays a crucial role in propagation of the SIN as it serves to relay the activation signal from Spg1-GTP to its downstream effectors. In order to understand how Cdc7 is regulated we have carried out a detailed structure/ function analysis of Cdc7. We find that a region adjacent to the kinase domain is responsible for Spg1 association and have identified an

overlapping but distinct SPB localization domain. Cdc7 self-associates and we provide evidence that this association is critical for Cdc7 function.

Results

Cdc7 associates with Spg1 only during mitosis

Cdc7 only associates with GTP-bound Spg1 and presumed to function as its effector. Most G-protein effector kinases are activated upon induction or binding to their cognate G-protein (Coso, Chiariello et al. 1995; Zhao, Leung et al. 1995). Despite the observation that Spg1 is in its GTP bound form at the SPB only during mitosis, the kinase activity of Cdc7 has remains constant throughout the cell cycle (Sohrmann, Schmidt et al. 1998). Given this anomaly I decided to address whether Cdc7 was always associated with Spg1. We generated a synchronous population of cells by centrifugal elutriation that contained endogenously tagged Cdc7-Myc_{13} and Spg1-HA_3. Samples were collected at regular intervals and examined for septation and nuclear division as indicators of the cell-cycle stage (Figure 5A). Protein lysates were prepared and Spg1 was immunoprecipitated by anti-HA antibodies and assessed for the presence of Cdc7-Myc_{13} by immunoblotting (Figure 5B, bottom panel). Control *spg1-ha$_3$* strain lacking endogenously tagged Cdc7 and wild type (WT) untagged strain were used as controls. Cdc7-Myc_{13} was present in Spg1-HA_3 immunoprecipitates prepared from mitotic but not interphase cells. This is also the stage at which Spg1 is in its GTP bound form (Sohrmann, Schmidt et al. 1998). In uninucleate cells that have exited from mitosis (at minutes 90, 105 and 120), no Cdc7-myc_{13} was detectable. Lack of association was not due to lower amounts of Spg1-HA_3 in the immunoprecipitate or overall protein levels (Figure 5B, top and middle panels). These results indicate that unlike other G-protein associated kinases the binding to Spg1 does not influence Cdc7 kinase activity.

The N-terminus of Cdc7 contains an Spg1 binding site

Given that Cdc7 association with Spg1 is the likely regulatory step in SIN activation we first addressed what regions of Cdc7 are responsible for binding to Spg1. I analyzed a series of *cdc7* fragments in the two-hybrid assay. Residues 250-535 were found to contain a strong interaction domain (Figure 6A). Since Cdc7 only associates with Spg1 in its GTP bound form

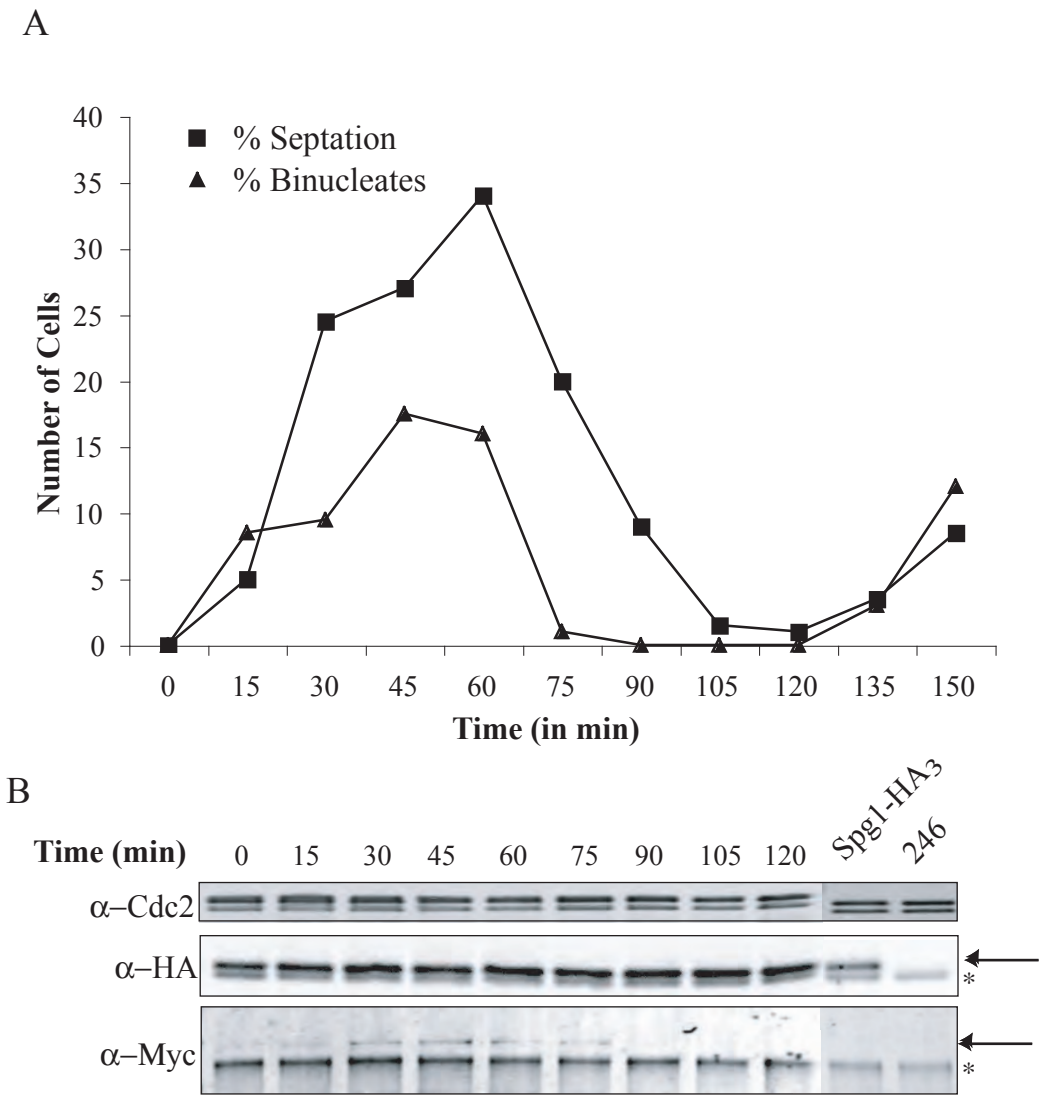


Figure 5. Cdc7 associates with Spg1 only during mitosis. *cdc7-myc$_{13}$ spg1-ha$_3$* strain (KGY5401) was grown to mid-log phase and synchronized in G2 by centrifugal elutriation. Samples were taken at regular time intervals and processed for DAPI staining (Binucleates) and Septation index as a measure of cell cycle progression (A). (B) Protein lysates were prepared from each sample and Spg1 was immunoprecipitated using anti-HA antibodies from lysates that were normalized to contain equal amount of protein as determined by immunoblotting lysates for Cdc2 with anti-PSTAIR antibodies (top panel). The amount of Spg1-HA$_3$ immunoprecipitated was detected by immunoblotting with 12CA5 antibodies (middle panel, * indicates a background band that is present in 246 control, arrow points to Spg1-HA$_3$ band). Cdc7-Myc$_{13}$ (indicated by the arrow) in Spg1-HA$_3$ immunoprecipitates was detected by immunoblotting with 9E10 antibodies (bottom panel, * indicates background band that is present in Spg1-HA$_3$ and wild-type KGY246 controls).

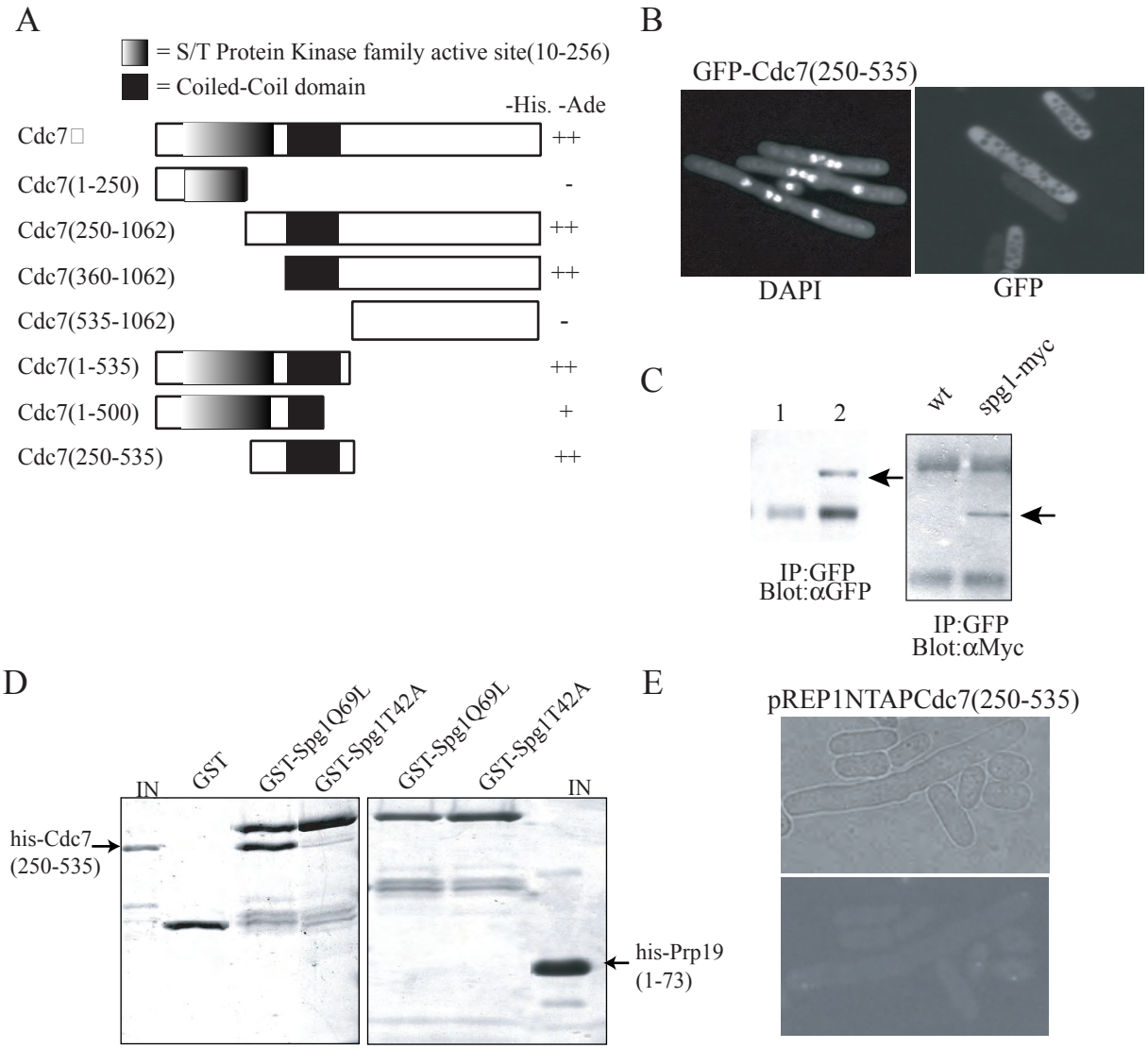


Figure 6. Amino acids 250-535 of Cdc7 are sufficient to bind Spg1 (A) *spg1* bait plasmid was co-transformed with various *cdc7* or control prey plasmids. Transformants were grown on medium lacking histidine and adenine to score for positive interactions. *cdc7* constructs and their ability to grow with the *spg1* construct are indicated. (B) Wild-type cells (KGY246) expressing GFP-Cdc7(250-535) were grown to mid log phase in the absence of thiamine at 32^{O}C for 18 hours. Cells were fixed in methanol and stained with DAPI to visualize the nuclei (left panel). To visualize GFP-Cdc7(250-535) images of live cells were captured (right panel). (C) The *spg1-myc*$_{13}$ strain (KGY4071) was transformed with pREP1 vectors producing GFP-Cdc7(250-535). Transformants were grown in the presence (lane1) and absence of thiamine (lane2) and protein lysates were subject to immunoprecipitation with polyclonal anti-GFP serum as were lysates from control untransformed wild type (KGY246) cells. Immunoprecipitates were run on a 4-12% Bis-Tris Gel and immunoblotted with 9E10 monoclonal anti-myc antibody or polyclonal anti-GFP antibody (D) Approximately equal amounts of fusion proteins GST-Spg1Q69L and GST-Spg1T42A were incubated with recombinant His$_6$-Cdc7(250-535) or control protein His$_6$-Prp19(1-73). His$_6$-Cdc7 (250-535) associated with GST-Spg1Q69Land not GST or GST-Spg1T42A when pulled down with glutathione agarose beads, as visualized by coomassie staining. His$_6$-Prp19(1-73) did not associate with either. (E) Spg1-GFP cells (KGY2678) expressing NTAP-Cdc7(250-535) under the control of the *nmt1*promoter in pREP1 were grown as in B above. Images of live cells were captured to visualize GFP (bottom panel) as well as DIC (top panel).

(Sohrmann, Schmidt et al. 1998), I tested if His_6-Cdc7 (250-535) bound Spg1 in a nucleotide dependent manner. For this I examined the ability of bacterially produced His_6-Cdc7 (250-535) to interact with recombinant Spg1 mutants *in vitro*. His_6-Cdc7 (250-535) was able to bind GST-Spg1Q69L, a constitutively active mutant (Schmidt, Sohrmann et al. 1997), but not GST-Spg1T42A an effector domain mutant (Schmidt, Sohrmann et al. 1997) (Figure 6D). We also tested whether Cdc7(250-535) and Spg1-Myc_{13} could co-immunoprecipitate from *S. pombe* protein lysates. To this end, GFP tagged Cdc7(250-535) was overproduced in an *spg1-myc*$_{13}$ strain. Anti-GFP immunoprecipitates contained Spg1-Myc_{13} (Figure 6C), confirming the ability of these proteins to associate *in vivo*.

We noticed that when GFP-Cdc7 (250-535) was overproduced, it generated a SIN phenotype (Figure 6B). This could be because it prevented endogenous Cdc7 from localizing to the poles and thus from binding Spg1 or because it titrated Spg1 from the SPBs. To distinguish between these two possibilities, I first examined the localization of GFP-Cdc7(250-535) when it was overproduced. Inconsistent with the first possibility, GFP-Cdc7(250-535) localized to the cytosol (Figure 6B). Furthermore overproduction of Cdc7(250-535) displaced endogenous Spg1-GFP from the SPB (Figure 6E). These data taken together indicate that amino acids 250-535 of Cdc7 are sufficient for binding to Spg1 *in vitro* and *in vivo*, and when present in sufficient quantity, to sequester Spg1 away from SPBs. Further, these results indicate that Cdc7 residues outside of its Spg1 binding domain are required for Cdc7 to localize to the SPB.

Identification of SPB localization domain within Cdc7

To determine which regions of Cdc7 are required to target it to the SPB, I expressed N and C-terminal truncations of Cdc7 fused to GFP. Since Cdc7 is an essential gene I tested these truncations in cells that also contained endogenous Cdc7. Full-length GFP-Cdc7 localized to the SPB in a cell cycle dependent manner, however unlike endogenous Cdc7 it localizes to the SPB symmetrically during anaphase as has been noted previously when Cdc7 is overexpressed (Sohrmann, Schmidt et al. 1998).

A kinase dead mutant of Cdc7, GFP-Cdc7K38R, also localized to the SPB symmetrically during anaphase. GFP-Cdc7 (260-1062) and GFP-Cdc7 (360-1062) constructs lacking the kinase domain (Figure 7A, 7B) also were capable of localizing to the SPB. Hence neither the kinase

A

Cdc7 truncations	*cdc7-24* rescue	Localization
Cdc7 (1-250)	ND	Cytoplasmic
Cdc7 (1- 535)	Yes,partial	Cytoplasmic
Cdc7 (1- 900)	Yes	SPB
Cdc7 (250-1062)	NO	SPB, symmetric
Cdc7 (360-1062)	NO	SPB, symmetric
Cdc7 (535-1062)	NO	Cytoplasmic, nuclear
Cdc7 (250-535)	ND	Cytoplasmic
Cdc7 (360-535)	ND	Cytoplasmic
Cdc7 (360-870)	ND	SPB, symmetric
Cdc7 K38R	NO	SPB, symmetric

B

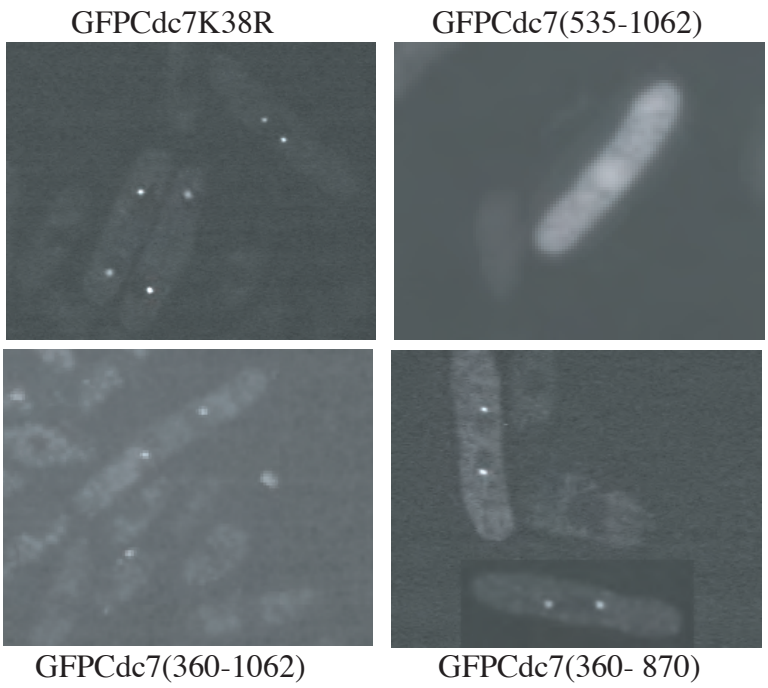


Figure 7. SPB localization localization domain of Cdc7. (A) Characterization of Cdc7 truncation mutants. To test for rescue of the growth defect of *cdc7-24* mutants, cells carrying vector alone or pREP41HACdc7 truncations were grown on selective plates without thiamine at permissive temperature (25^{O}C) and then shifted to restrictive temperature 36^{O}C. Colony formation at restrictive temperature was indicative of rescue. Deletion mutants of Cdc7 were tagged with GFP and expressed from the low-strength *nmt81* promoter of pRE81GFP vector. Cells containing these plasmids were grown in the presence of thiamine overnight, washed in media without thiamine and allowed to grow to mid log phase in the absence of thiamine at 32^{O}C for 18 hours. GFP-Cdc7 localization was observed in live cells.

domain nor kinase activity is required for Cdc7 SPB localization. I then tested C-terminal deletions to narrow the region responsible for Cdc7 SPB localization. While GFP-Cdc7 (360-870) could localize to the SPB, further loss of N or C-terminal residues resulted in abrogation of SPB localization (Figure 7A). Hence the region that is responsible for SPB association is larger than that required for Spg1 binding and resides within residues 360-870 (Figure 10C).

To correlate the ability of Cdc7 constructs to localize and bind Spg1 with overall Cdc7 function, N-terminal GFP Cdc7 fusion proteins were tested for the ability to rescue the growth defect of *cdc7-24* cells (Figure 7A). I found that the full length and C-terminal truncations (Cdc7 1-535, Cdc7 1-900) were able to rescue (Figure 7A), and as expected, all Cdc7 fragments lacking the kinase domain did not. The minimal rescuing fragment (Cdc7 1-900) contains both the SPB localization and Spg1 association domains and hence includes all the necessary elements that are critical for Cdc7 function. These results indicate that C-terminal residues 900-1062 are dispensable for Cdc7 function.

Cdc7 self associates *in vivo*

In the course of our yeast two- hybrid analysis to define Spg1 binding regions within Cdc7, we discovered that Cdc7 interacts with itself in the two-hybrid assay (Figure 8A). The minimal domain required for this interaction was narrowed to aa360-518 (Figure 8A). This region is embedded within the identified minimal Spg1 binding domain (residues 250-535, Figure 10C) and also contains a coiled-coil motif, a common protein –protein interaction domain. Residues 360-518 did not interact with Spg1, however deleting these residues from the protein abolished both Cdc7 self –interaction (Figure 8A) and interaction with Spg1 in the yeast two hybrid assay (Figure 8C). This suggests that Cdc7 interacts with itself via this domain and this self-interaction might be required for Spg1 binding. As expected, then, deletion of this domain affected Cdc7 function; pREP41HA-Cdc7(Δ360-518) was unable to rescue the temperature sensitive lethality of *cdc7-24* allele even under low-level of expression (+T) (Figure 9A). Loss of function was not due to degradation of unstable protein at 36^0C, since protein levels of HA-Cdc7(360-518) were comparable to those of HA-Cdc7 (Figure 9B)

In order to test whether Cdc7 could oligomerize *in vivo,* I created a diploid strain in which one allele of *cdc7* was tagged with the Myc_{13} epitope and the other tagged with the HA_3 epitope. Anti- HA immunoprecipitates from the dually tagged diploid strain, but not single

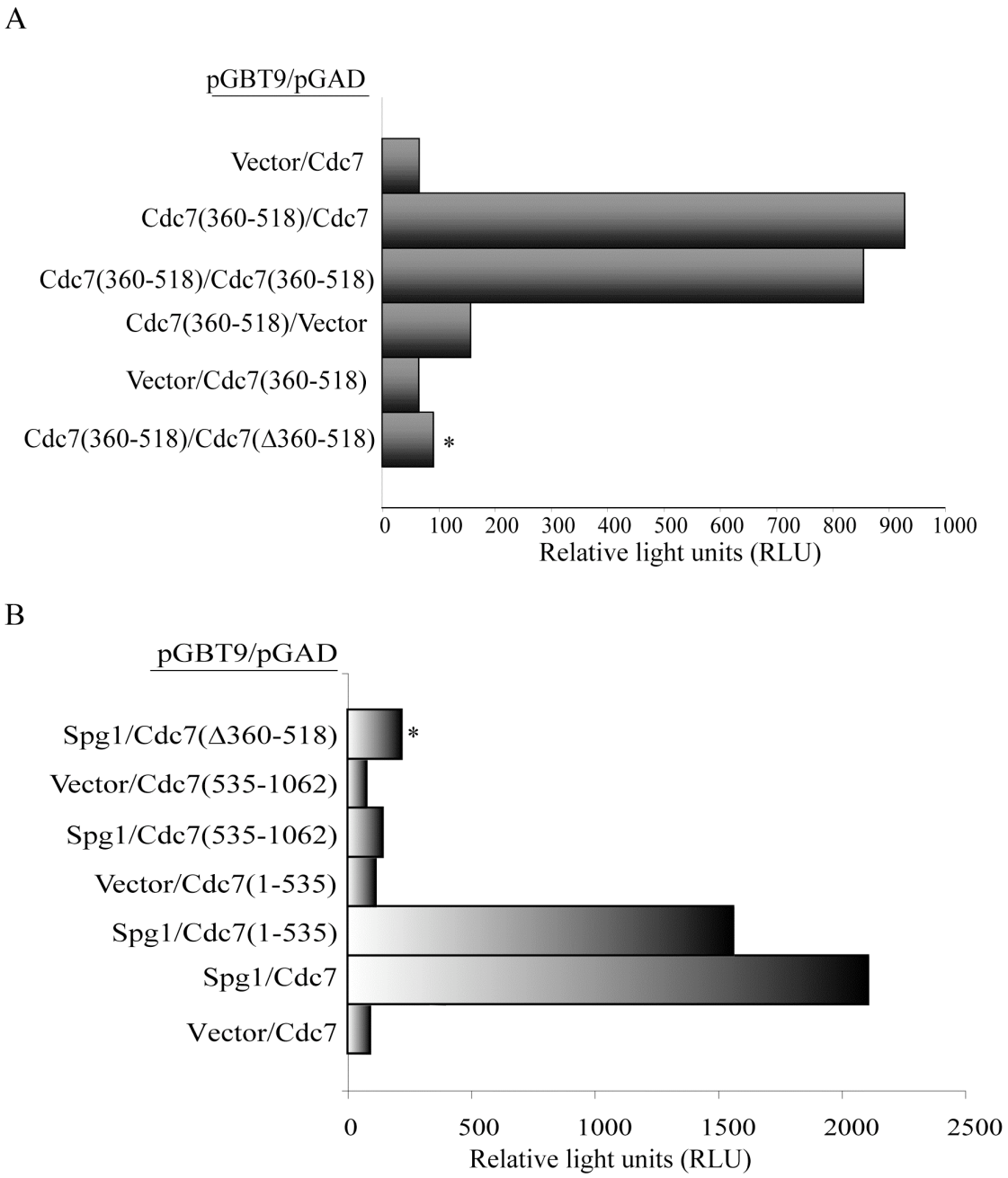


Figure 8. Cdc7 interacts with itself. A) *cdc7* or control bait plasmids were co-transformed with various *cdc7* or control prey plasmids. Transformants were grown on medium lacking histidine and adenine to score for positive interactions. Transformants were assayed for β-galactosidase assays and their interactions are indicated in relative light units. (B) Same as in A, except *spg1* or control bait plasmids were co-transformed with various *cdc7* prey plasmids. Asterix indicates loss of self-interaction as well as interaction with Spg1 upon deletion of residues 360-518.

tagged strains, contained Cdc7-Myc_{13} and vice versa (Figure 9C). These results indicated that Cdc7 did indeed exist as an oligomer *in vivo*. To estimate what oligomeric state Cdc7 was in, lysates from asynchronously growing Cdc7-Myc_{13} and control untagged strains were prepared under native conditions and sedimented on sucrose gradients. After sedimentation fractions were collected and Cdc7-Myc_{13} was immunoprecipitated using an antibody against the myc epitope from each fraction. Immunoprecipitates were then analyzed by immunoblotting for myc. Molecular size standards were run in parallel on an identical sucrose gradient. We found that the majority of Cdc7-Myc, co-sedimented in fractions 10 and 11 with the molecular weight marker, Phosphorylase B, which forms a trimer of 292.8 kDa.

Since the predicted molecular weight of a Cdc7-Myc_{13} dimer is approximately 285 kDa, this suggests that Cdc7 exists primarily as a dimer. Some Cdc7-Myc_{13} sediments lower in the gradient and this could represent Cdc7 in complex with other proteins (such as Spg1) and/or in higher order complexes with itself. We also investigated whether Spg1 was able to associate with itself. The results of the yeast two-hybrid and co-immunoprecipitation from *spg1-HA_3/ spg1-myc_{13}* diploid strain showed that Spg1 is not associated with itself (Figure 10A). This suggests that either Spg1 is not present in Cdc7 complexes or that a Cdc7 dimer binds to one molecule of Spg1. Consistent with the latter hypothesis overexpression of Spg1 failed to disrupt Cdc7 self association (Figure 10B). These data taken together with the observation that Cdc7-Myc_{13} was not observed in any fraction that would be consistent with a monomeric form (140 kDa) suggest that Cdc7 exists predominantly as a dimer *in vivo* and associates with Spg1 as a dimer.

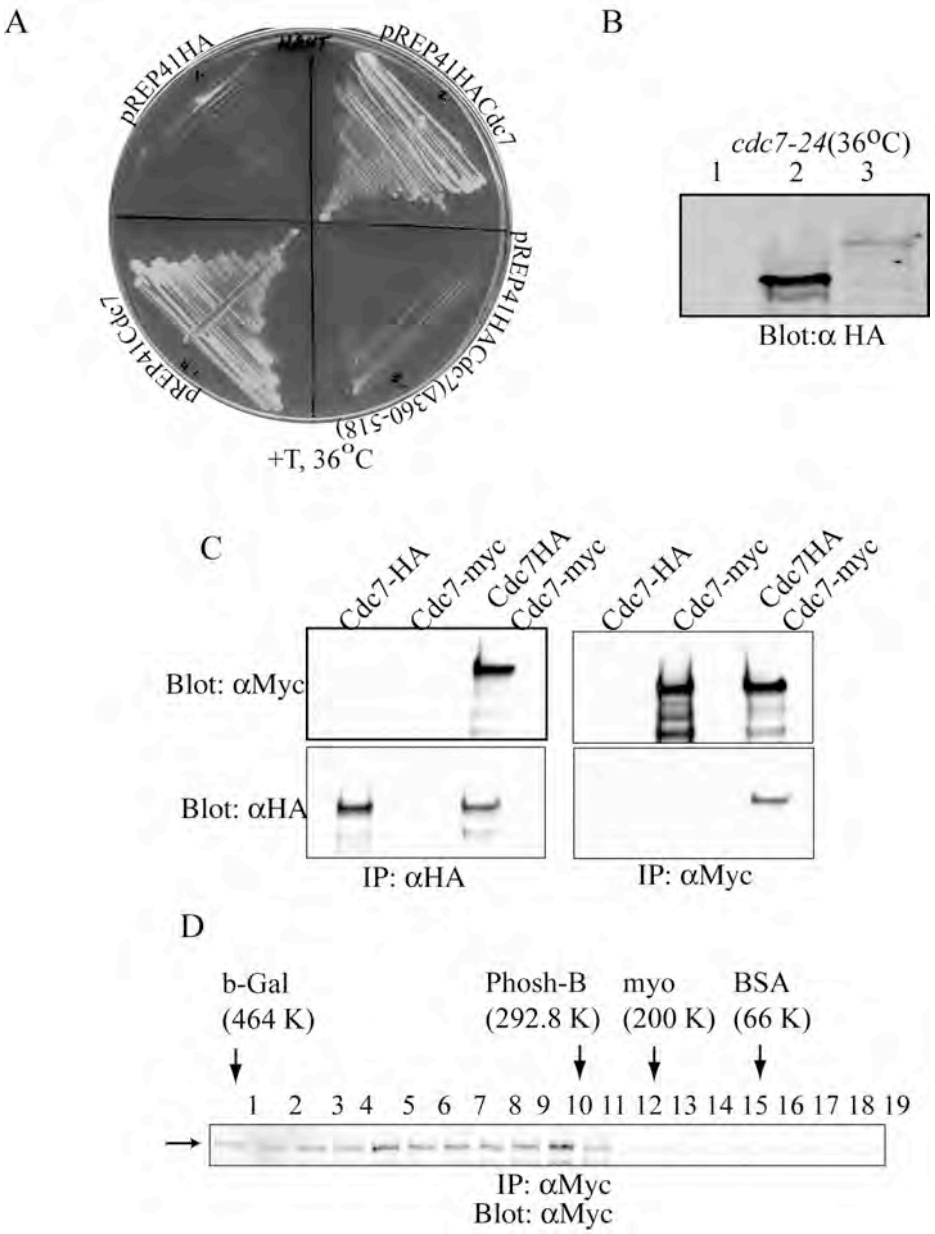


Figure 9. Cdc7 self associates *in vivo*. (A) *cdc7-24* mutant cells carrying vector alone or pREP41HACdc7 truncations were grown on selective plates with thiamine at permissive temperature (25°C). Cells were then patched to single colonies on plates with and without thiamine and shifted to restrictive temperature of 36°C. (B) *cdc7-24* mutant cells carrying vector alone (1), pREP41HACdc7(Δ360-518) (2) or pREP41HACdc7(3) were grown as in (A) except in liquid media. Protein lysates were prepared from cells shifted to restrictive temperature and run on an SDS-PAGE gel. Lysates were immunoblotted with anti-HA antibodies. (C) anti-Myc (right) and anti-HA (left) immunoprecipitates from the indicated strains were blotted with anti-Myc (upper panels) and anti-HA (lower panel) antibodies. (D) Native lysates were prepared from *cdc7-myc*$_{13}$ (KGY 3842) cells and fractionated over a sucrose gradient. Cdc7-Myc$_{13}$ was immunoprecipitated from gradient fractions using anti-Myc mAB 9E10 and analyzed by SDS-PAGE. Molecular size standards were run in parallel on an identical sucrose gradient.

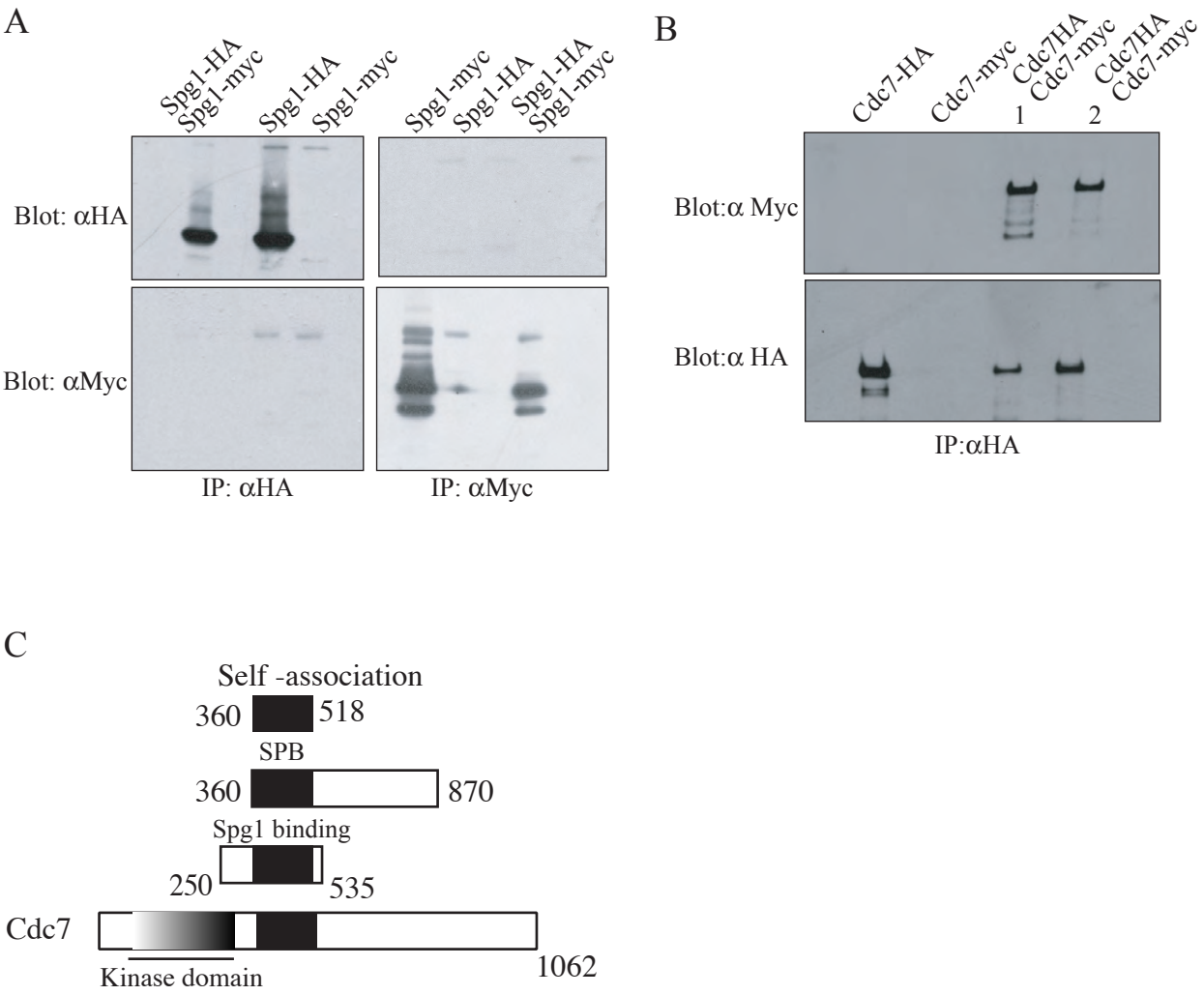


Figure 10. Overproduction of Spg1 is unable to disrupt Cdc7 self association. A)anti-Myc (right) and anti-HA (left) immunoprecipitates from the indicated strains were blotted with anti-Myc (lower panels) and anti-HA (upper panel) antibodies .B) c*dc7-HA*$_3$ */cdc7-myc*$_{13}$ diploid strains carrying pREP3XSpg1(lane2) or empty vector (lane 1) were grown to mid-log phase without thiamine at 32^{O}C for 20 hours. Protein lysates were prepared and anti-HA immunoprecipitates from lysates were blotted with anti-Myc and anti-HA antibodies.
(C) Organization of various functional domains within Cdc7.

Conclusions

Cdc7 is a protein kinase essential for cytokinesis in fission yeast. Prior to this work very little functional information about Cdc7 was known. It was predicted to be an effector kinase for Spg1 and shown to bind only active or GTP bound Spg1. Cdc7 kinase activity however remains constant throughout the cell cycle. I show that, as predicted, Cdc7 only binds to Spg1 during mitosis and hence its kinase activity is most likely unaffected by binding to Spg1. In order to elucidate Cdc7 regulation I have identified various functional domains within Cdc7. I show that a region adjacent to the kinase domain is responsible for association with Spg1 but insufficient for SPB localization.

In addition I find that Cdc7 self associates which is required for binding to Spg1. Our findings indicate that there are potentially multiple modes of regulating Cdc7 function and hence the SIN.

CHAPTER IV

IDENTIFICATION OF FUNCTIONAL DOMAINS WITHIN GAP COMPONENT BYR4

Introduction

The onset of cytokinesis and septum formation in *S. pombe* is regulated via the Septation Initiation Network (SIN) signaling pathway. The key to controlling the onset of septation lies in the activity of Spg1, a small Ras -superfamily GTPase that is present at the SPB throughout the cell cycle (Schmidt, Sohrmann et al. 1997; Sohrmann, Schmidt et al. 1998). Spg1 alternates between its inactive GDP-bound form (during interphase) and its active GTP-bound form (during metaphase). Later in anaphase it becomes inactivated at one pole to generate a poorly understood asymmetric state. The effector for Spg1, Cdc7 kinase, also localizes asymmetrically to the SPB associating only with the active form of Spg1 (Sohrmann, Schmidt et al. 1998). Acting to prevent Spg1 activation and septation during interphase is a two-component GAP comprised of Byr4 and Cdc16 (Furge, Wong et al. 1998). During anaphase Cdc16-Byr4 localizes to only one SPB, the one without Cdc7. In fact the asymmetric localization of Cdc16-Byr4 appears to occur prior to that of Cdc7 suggesting that the complex may prevent the recruitment of Cdc7 to that SPB (Cerutti and Simanis 1999; Li, Furge et al. 2000). Cdc16 is related to Ypt/Rab specific GAPs but despite this homology, Cdc16 requires its partner Byr4 for this activity *in vitro* (Neuwald 1997; Furge, Wong et al. 1998). While Byr4 does not appear to contain motifs of known function, Byr4 may function in part to tether Cdc16 near Spg1 since it, but not Cdc16, binds stably to Spg1 through several independent domains.

Although Byr4 functions with Cdc16 to reduce Spg1-GTP levels, overproduction of Byr4 generates a SIN phenotype that is eventually lethal (Song, Mach et al. 1996; Jwa and Song 1998). In this study, we have pursued an explanation to this apparent paradox. Since co-production of Spg1 can suppress the lethal effects of Byr4 overproduction it is possible that high levels of Byr4 inhibit the ability of Spg1-GTP to bind its effector, the Cdc7 kinase. Here, I have examined the consequence of Byr4 overproduction phenotype on SIN component localization. I

have also carried out an analysis of Spg1and Cdc16 binding domains within Byr4 and demonstrate that the association with Cdc16 is required for SPB localization of Byr4.

Results

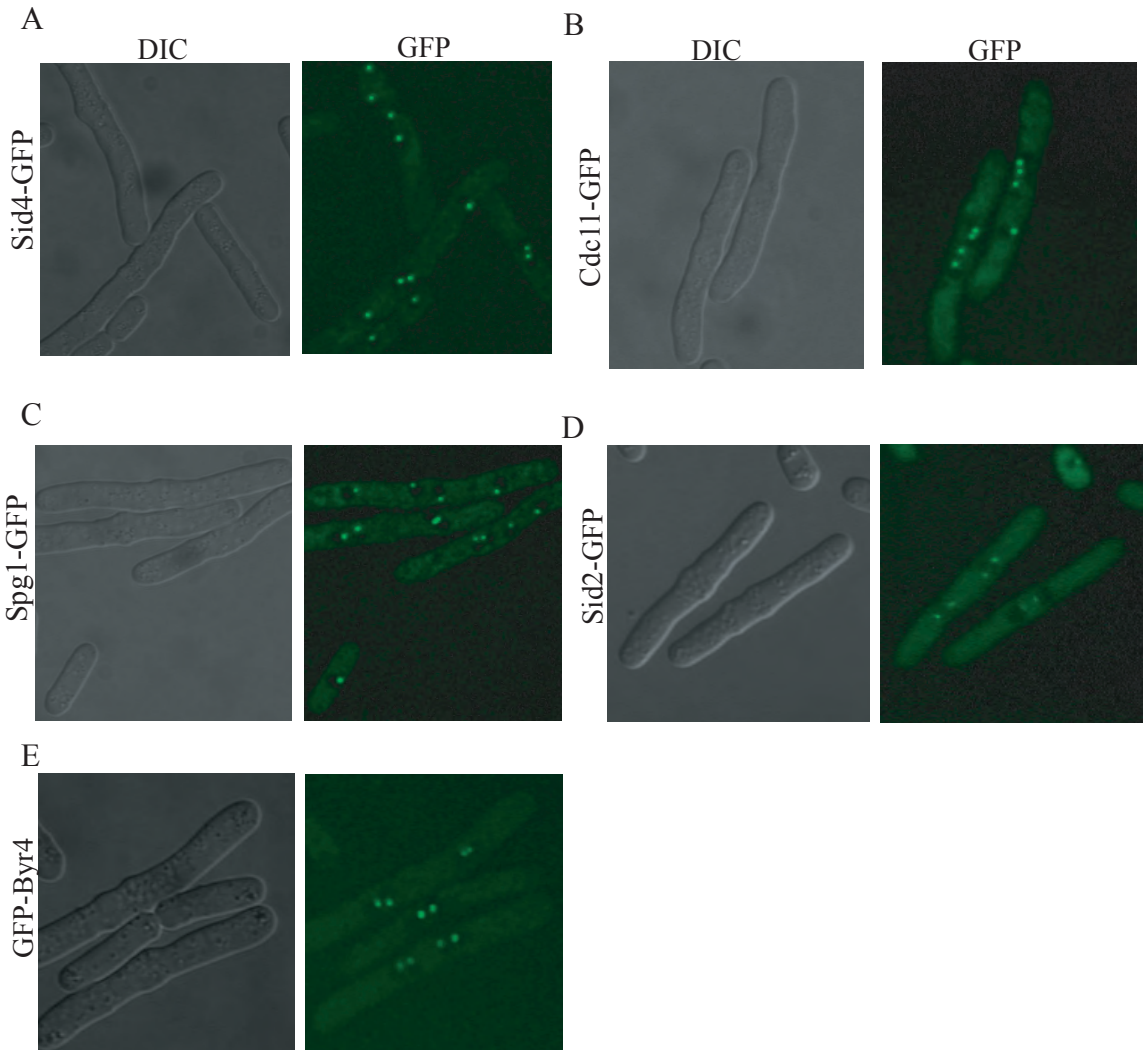
Byr4 overproduction interferes with SIN component localization

To determine if excess Byr4 interfered with the SPB localization of any of the SIN components, their localization patterns were examined in Byr4-overproducing cells. We first examined the localization of Sid4-GFP and Cdc11-GFP, the SPB scaffolds for the SIN, and found that they were maintained at the poles when Byr4 was overproduced (Figure 11A, B). The next component of the pathway we examined was Spg1-GFP. Spg1 is normally present at SPBs throughout the cell cycle and this localization is dependent on the integrity of the Cdc11-Sid4 scaffold. We observed that Spg1 is still maintained at the poles in Byr4-overproducing cells (Figure 11C). This finding is contrary to what has previously been published (Li, Furge et al. 2000).

Cdc7 kinase is a known effector for Spg1 and is recruited to the SPB by active Spg1 (Sohrmann, Schmidt et al. 1998; Li, Furge et al. 2000). At low levels of *byr4* expression, about 9% of the cells (87/989) were binucleate. Of these, 85% had Cdc7 on either one or both SPBs. As Byr4 levels increased due to de-repression of the *nmt41* promoter, the number of cells with two or more nuclei increased. When 35% of the cells were bi- or multi-nucleate only 2% of cells had detectable Cdc7-GFP signal at a SPB (Table in Figure 11). These data indicate that Byr4 overproduction displaces Cdc7 from the SPB.

We also examined the localization of Sid2, a SIN component that is present constitutively at SPBs and transiently recruited to the medial ring during anaphase if the SIN pathway is functioning (Sparks, Morphew et al. 1999). We found that Sid2 remained at SPBs overproducing Byr4, but was never detected at rings (Figure 11D). Hence Byr4 overproduction seems to specifically displace Cdc7 from SPBs leading to a block in SIN signaling.

To determine whether Byr4 itself could localize to SPBs when overproduced, Byr4p was fused to GFP and placed under control of the low-strength *nmt81* promoter. Under inducing conditions, GFP-Byr4 localized to SPBs and generated a SIN phenotype (Figure 11E).



Byr4 Expression Levels	%Binudeates	%Binucleates with Cdc7-GFP staining at one or 2 SPBs
Low level (+T) High level (-T)	9% (87/879) 35% (bi and multinucleate)	85% (strong) 2% (weak but detectable)

Figure 11. Overproduction of Byr4 interferes with SPB localization of Cdc7. The (A) *sid 4-GFP* (KGY2628), (B) *cdc11-GFP* (KGY3341), (C) *spg1-GFP* (KGY2678), and (D) *sid2-GFP* strain (KGY2945) strains expressing pREP41Byr4 were grown in the absence of thiamine at 29^{O}C for 18 hours.
(E)Wild-type cells (KGY246) expressing GFP-Byr4 under the control of *nmt81* promoter in pREP81 were grown to mid log phase in the absence of thiamine at 29^{O}C for 18 hours. Images of live cells were captured.
Table. Number of binucleate cells displaying Cdc7-GFP in SPBs upon Byr4 overproduction.

N-terminus of Byr4 contains 2 binding sites for Spg1

The domains of Byr4 that bind Spg1 were broadly characterized using an *in vitro* binding assay (Furge, Cheng et al. 1999). These were found to lie in 4 different regions that spanned the entire length of the protein (Furge, Cheng et al. 1999). We decided to accurately determine what regions of Byr4 were required for interacting with Spg1 by using the yeast two- hybrid system. By testing a series of *byr4* fragments in the two-hybrid assay, at least two independent regions were identified that interacted well with Spg1 (Figure 12A). By further deletion analysis, the best interaction in the amino terminus was observed with a Byr4 fragment that spanned amino acids 100-300 (Figure 12A and B). Because amino acids 1-200 also interact with Spg1, it is possible that an Spg1 interaction domain is present within Byr4 amino acids 100-200 (Figure 12A and B). The second strong interaction domain contained the two imperfect direct repeats of Byr4, amino acids 475-595 (Figure 12A and B).

To extend our analysis of these domains, I overproduced them in cells to observe their effect on Spg1 localization and also tested their ability to interact with Spg1 *in vitro*. Overproduction of Byr4 fragments 100-300 or 475-595 *in vivo* (Figure 13A) generated a SIN phenotype similar to full length Byr4 (Figure 13B). Unlike full-length Byr4, however, these fragments prevented SPB localization of Spg1 (Figure 13B, top panel). They did not interfere with the SPB localization of Sid4, Cdc11, or Sid2 (Figure 13B, lower three panels). These results are consistent with the possibility that these fragments either saturate the SPB binding site for Spg1 or titrate Spg1 from the spindle poles. Consistent with the latter possibility, GFP tagged versions of these fragments localize to the cytosol and are not detected at SPBs (Figure 13C). I also tested whether these fragments and Spg1-myc could co-immunoprecipitate from *S. pombe* protein lysates. To this end, GFP tagged Byr4(100-300) and Byr4(475-595) were overproduced in a *spg1-myc* strain. Anti-GFP immunoprecipitates contained Spg1-myc (Figure 13D), confirming the ability of these proteins to associate *in vivo*.

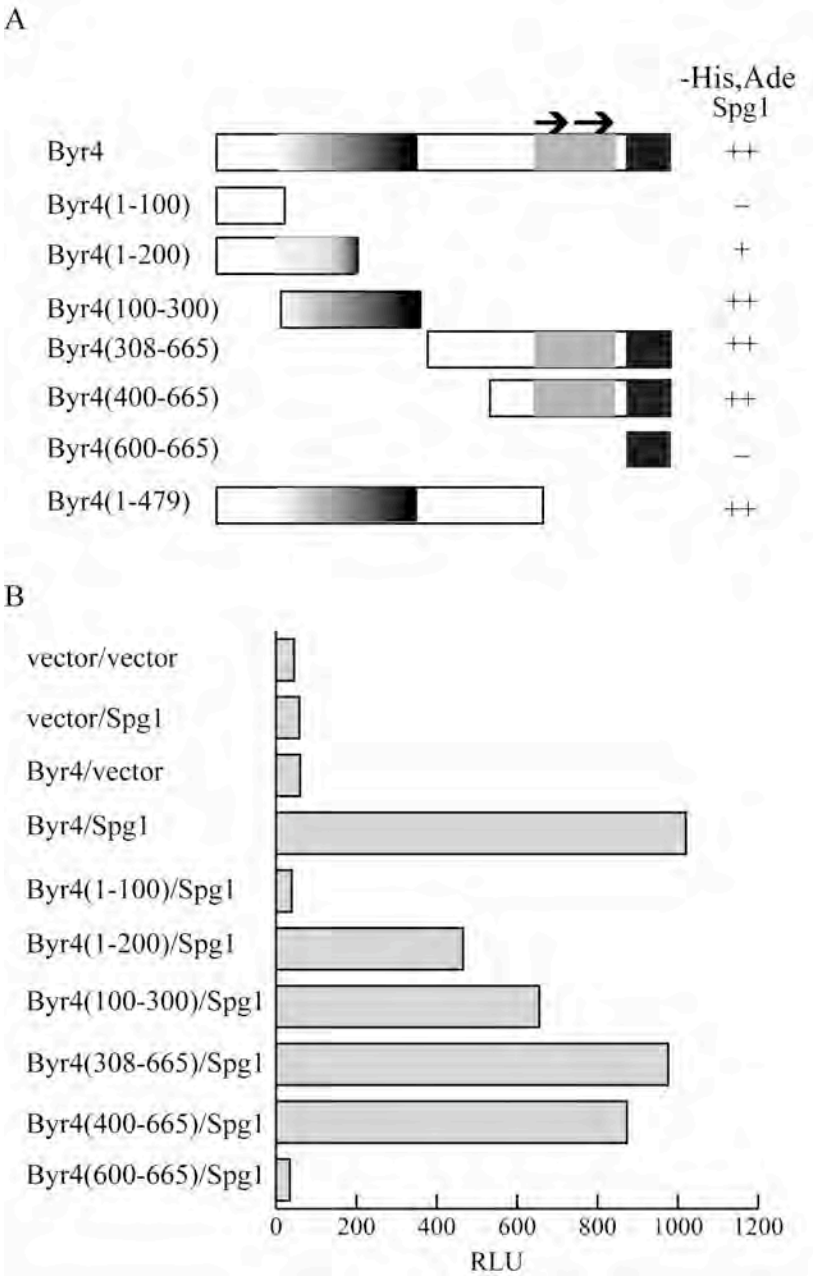


Figure 12. Delineation of Spg1 interaction domains within Byr4. *spg1* or control bait plasmids were co-transformed with various *byr4* or control prey plasmids. A) Transformants were grown on medium lacking histidine and adenine to score for positive interactions. *byr4* constructs and their ability to grow well with *spg1* constructs are indicated. (B) Transformants were also assayed for β-galactosidase assays and their interactions are indicated in relative light units.

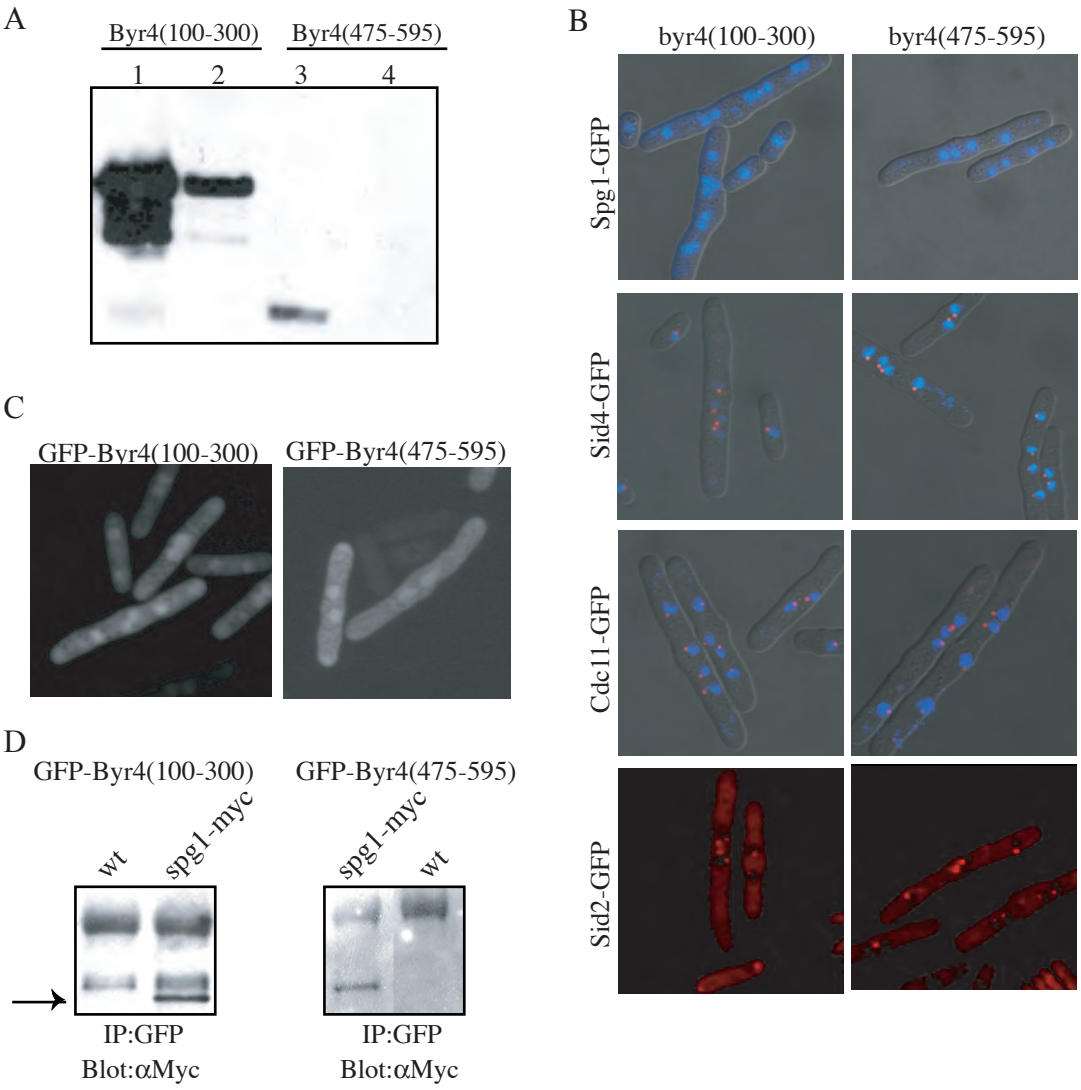


Figure 13. Overproduction of Byr4 domains that interact with Spg1 displace Spg1 from SPBs. (A) Protein lysates prepared from wild type cells producing Byr4(100-300) (lanes 1 and 2) and Byr4 (475-595) (lanes 3 and 4) in the presence (lanes 2 and 4) and absence (lanes 1 and 3) of thiamine were run on a 4-12% Bis-Tris gel and blotted with anti-Byr4 serum. (B) Live images of *spg1-GFP, sid4-GFP, cdc11-GFP* and *sid2-GFP* strains overproducing Byr4(100-300) or Byr4(475-595). GFP has been false colored red and cells in the top three panels were also stained with Hoechst that is in blue. (C) Wild-type cells (KGY246) expressing GFP-Byr4(100-300) under the control of *nmt1*promoter in pREP1 (upper panel) or GFP-Byr4(475-595) under the control of *nmt81* promoter in pREP81 (lower panel) were grown to mid log phase in the absence of thiamine at 32^{O}C for 14hours and 18 hours respectively. Images of live cells were captured. (D) The *spg1-myc*$_{13}$ strain (KGY4071) was transformed with pREP1 vectors producing GFP-Byr4(100-300) (upper panel) or GFP-Byr4(475-595) (lower panel). Transformants were grown in the absence of thiamine and protein lysates were subject to immunoprecipitation with polyclonal anti-GFP serum as were lysates from control untransformed wild type (KGY246) cells. Immunoprecipitates were run on a 4-12% Bis-Tris Gel and immunoblotted with 9E10 monoclonal anti-myc antibody.

C-terminus of Byr4 binds Cdc16

In addition to binding Spg1, Byr4 forms a complex with Cdc16 (Furge, Wong et al. 1998). The Byr4 domain responsible for this interaction was mapped previously using purified proteins and found to reside at the very C-terminus of Byr4, residues 595-665 (Furge, Cheng et al. 1999). We also found that Byr4 amino acids 600-665 interacted with Cdc16 but not Spg1 in the yeast two-hybrid system (Figure 14A). Because Byr4 localization to the SPB requires Cdc16 function (Furge, Wong et al. 1998), I tested whether this fragment alone contained an SPB-binding domain. Consistent with this domain being the SPB targeting region in Byr4, this fragment alone was sufficient to localize GFP to the SPB although cytoplasmic flourescence was also observed (Figure 14B, middle panel). A Byr4 fragment lacking this domain was unable to localize to the SPB, whereas all N-terminal deletions of Byr4 containing the Cdc16 binding domain localized to the SPB (Figure 14B left and right panels). Thus Byr4 association with the SPB is strictly dependant on its association with Cdc16, despite containing two Spg1 binding sites. Overproduction of Cdc16-binding fragment of Byr4 *in vivo* results in multiseptate cells suggesting it prevents full length Byr4 from accessing Cdc16 (Figure 14B). To confirm that this fragment can interact with Cdc16 *in vivo*, GFP-Byr4(600-665) was overproduced and immunoprecipitated using anti-GFP immune or pre-immune sera. Anti-GFP (but not pre-immune) immunoprecipitates contained Cdc16 as detected by Cdc16 specific polyclonal anti-sera (Figure 14C).

Conclusions

Byr4 – Cdc16 is a unique two-component GAP system that maintains Spg1 in an inactive state during interphase. In order to understand how Byr4 may contribute to SIN regulation I have identified different functional domains within Byr4 and addressed their roles in cytokinesis by overexpression studies. I show that Byr4 has two regions that are responsible for binding to Spg1. Further the C-terminal 65 amino acids are required for binding to Cdc16.

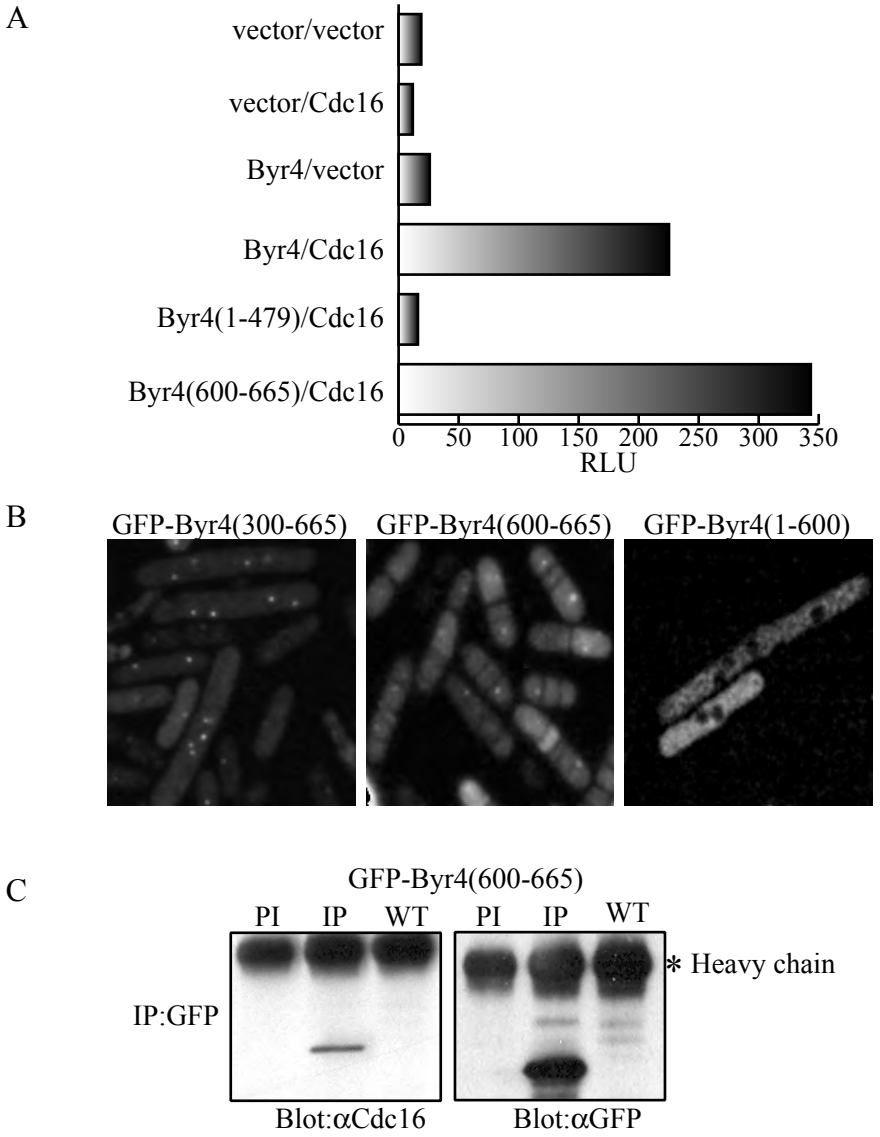


Figure 14. Characterization of Cdc16 binding domain. A) *cdc16* or control bait plasmids were co-transformed with various byr4 or control prey plasmids. Transformants were assayed for β-galactosidase assays and their interactions are indicated in relative light units. (B) Wild type cells (KGY246) expressing the indicated GFP-Byr4 fusion proteins from the *nmt81* promoter were grown to mid log phase in medium lacking thiamine at 32^{O}C for 18 hours and live images of cells were captured to visualize GFP. (C) Wild type cells (KGY 246) expressing the GFP-Byr4(600-665) fusion proteins from the *nmt1* promoter were grown as in B, except protein lysates were prepared and subject to immunoprecipitation with either polyclonal anti-GFP serum or pre-immune serum. Immunoprecipitates were resolved on a 4-12% Bis-Tris Gel and immunoblotted with anti-Cdc16 serum or anti-GFP serum.

CHAPTER V

ROLE OF PHOSPHORYLATION IN BYR4 REGULATION

Introduction

Spg1 activation is critical in establishing the onset of cytokinesis. Therefore, regulating the timing of Spg1 activation is key. The balance of maintaining a GTPase in an active or inactive state is typically regulated by GAPs (GTPase Activating Protein) and GEFs (GTP Exchange Factor) To date however no GEF for Spg1 has been identified. A unique two component GAP, comprised of Byr4 and Cdc16 has been identified that functions to keep Spg1 inactive during interphase and hence prevent septum formation at an inappropriate time (Minet, Nurse et al. 1979; Fankhauser, Marks et al. 1993; Song, Mach et al. 1996; Furge, Wong et al. 1998; Jwa and Song 1998). Inactivating either GAP component leads to the formation of multiple septa. The Cdc16 component bears structural similarity to proteins known to have GAP activity, most closely resembling the Ypt-GAP family (Fankhauser, Marks et al. 1993; Will and Gallwitz 2001). However, Cdc16 is unable to hydrolyze GTP bound to Spg1 in an *in vitro* assay in the absence of Byr4 (Furge, Wong et al. 1998; Furge, Cheng et al. 1999). Byr4 associates directly with both Spg1 and Cdc16 and one function predicted for Byr4 is to position Cdc16 in proximity to its cognate GTPase (Furge, Cheng et al. 1999).

Spg1 has a high intrinsic rate of GDP release when compared to standard rates found for Ras or other small G-proteins studied previously (Self and Hall 1995; Furge, Cheng et al. 1999). That coupled with the fact that no GEF for Spg1 has been identified to date, it seems likely that Spg1 activity is controlled solely by its GAP. Thus, cell cycle cues are likely to control GAP activity, and Byr4 is a likely target of such regulation. In *S. cerevisiae*-Bfa1 (homolog of Byr4) is phosphorylated in a cell-cycle dependent manner and phosphorylation influence's its GAP activity *in vitro* (Hu, Wang et al. 2001; Geymonat, Spanos et al. 2003). Here I examined the role of phosphorylation in regulating Byr4 function. I find that Byr4 is a phosphoprotein and is phosphorylated on S and T residues. Byr4 is hyperphosphorylated during mitosis just prior to septation. Byr4 phosphorylation *in vivo* requires association with the SPB. Because its

phosphorylation peaks in mitosis, I examined the role of Cdc2 in Byr4 phosphorylation. I find that it is indeed a Cdc2 substrate *in vitro*. Through tryptic peptide mapping of Byr4 deletion mutants I have identified 4 Serine residues that are phosphorylated on Byr4 *in vivo*, however mutational analysis suggests that these sites are not likely to be regulatory phosphorylation sites.

Results

Byr4 is hyperphosphorylated in mitotic cells

Byr4 was predicted to be a phosphoprotein since its mobility on a SDS -PAGE gel was slower (approximately 97kD) than its predicted size of 78kD (Song, Mach et al. 1996). We generated polyclonal rabbit antibodies against the protein. These antibodies, but not pre-immune precipitated Byr4 from a protein lysate of *S. pombe* cells labeled with [^{32}P] orthophosphate, producing a single band of the expected size (Figure 15A). Phosphoamino acid analysis of ^{32}P-labeled Byr4 revealed that it is phosphorylated on both serine and threonine residues with serine being the major residue utilized for phosphorylation (Figure 15B). We then analyzed Byr4 phosphorylation status in denatured cell lysates from wild-type cells, which had been synchronized in early G2 by centrifugal elutriation (Figure 15C). We observed that the protein appeared maximally phosphorylated in late mitosis, just prior to septation. Byr4 phosphorylation was also examined in a panel of cell cycle mutants. Byr4 was immunoprecipitated from *cdc10-129*, *cdc25-22*, *nda3-km311* and *mts3-1,* that arrest in G1, G2/M, mitosis and metaphase - anaphase transition respectively, at restrictive temperature. It was also immunoprecipitated from cells arrested in S phase by treatment with hydroxyurea (HU). Immunoprecipitates were treated with λ phosphatase or mock treated with buffer alone. Although Byr4 appeared to be phosphorylated at all stages of the cell cycle (as detected by a downward mobility shift on treatment with λ phosphatase), it appeared hyperphosphorylated in mitotic mutants (Figure 15D). This is consistent with the observations from the elutriation data. The timing of Byr4 hyperphosphorylation suggests that the role of phosphorylation is most likely inhibitory to Byr4 function.

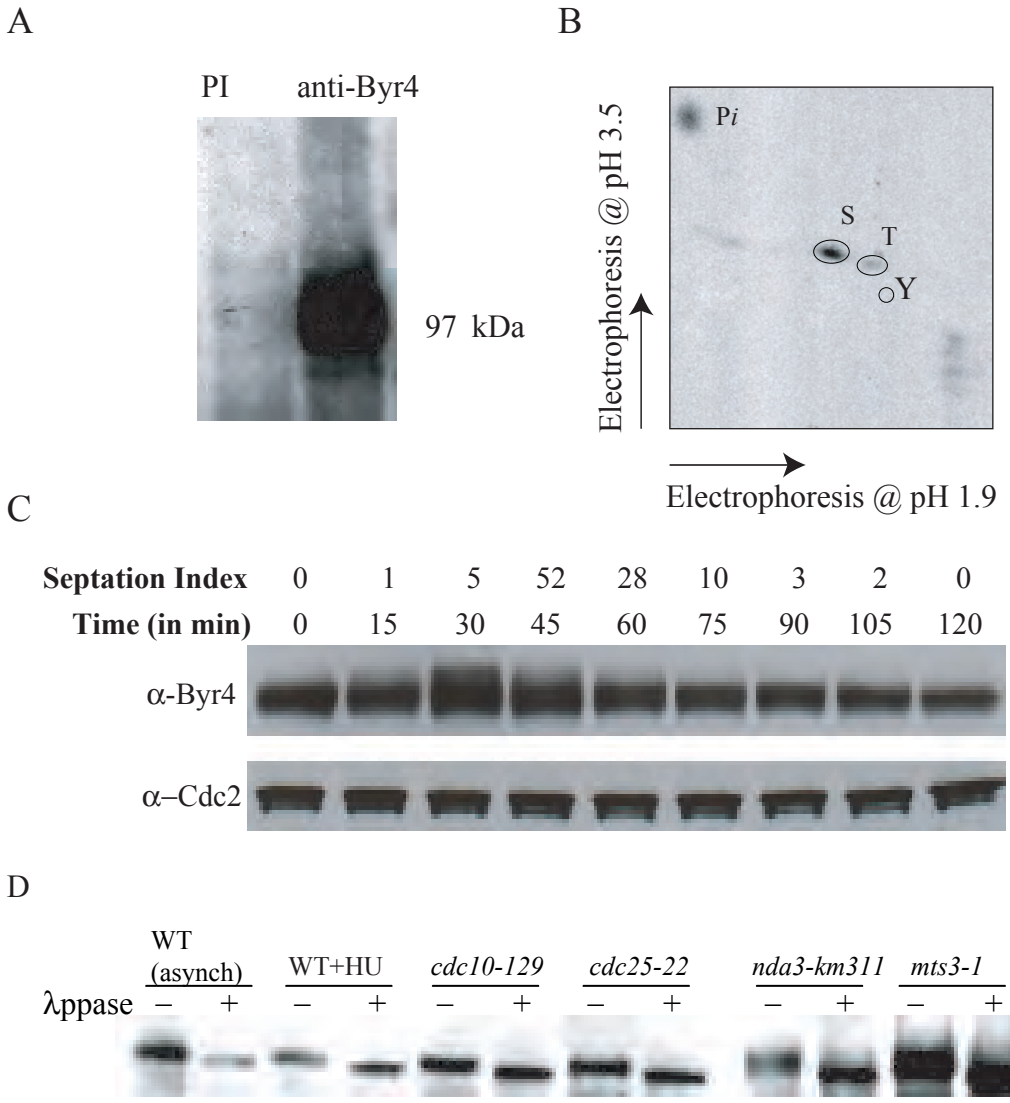


Figure 15. Byr4 is hyperphosphorylated during mitosis. A) Wild-type 246 cells were labeled with [^{32}P] Orthophosphate and lysed in SDS lysis buffer. Anti-Byr4 serum was added to one half, and pre-immune serum was added to the other half of the lysate. The immunoprecipitate were resolved by SDS-PAGE and transferred to a PVDF membrane. Labeled proteins were detected autoradiography. (B) The PVDF membrane containing Byr4 was analyzed for its phosphoamino acid content (T), (Y) and (S) stand for phosphothreonine, phosphotyrosine and phosphoserine standards.(C) Wild type cells were grown to mid-log phase and synchronized in G2 by centrifugal elutriation. Samples were taken at regular time intervals and processed Septation index as a measure of cell cycle progression.Protein lysates were prepared from each sample and run on an 3-8% Tris Acetate gel and immunoblotted for Byr4 and Cdc2 using anti-Byr4 and anti-PSTAIR antibodies. (D) Protein lysates were prepared from the indicated strains grown at restrictive temperature. Byr4 was immunoprecipitated from lysates and half of the immunoprecipitate was treated with λ ppase (+) while other half was mock-treated(-). Immunprecipitated were run on an SDS-PAGE gel and immunblotted for Byr4 using anti-Byr4 antibodies.

Byr4 phosphorylation *in vivo* requires association with the spindle pole body

To investigate whether phosphorylation of Byr4 *in vivo* required association with the spindle pole body (SPB), Byr4 was immunoprecipitated from exponentially growing *cdc16-116* cells that had been shifted to restrictive temperature for 4 hours. Previous studies had demonstrated that Byr4 localization to the SPB requires Cdc16 function (Figure 16A and (Furge, Wong et al. 1998)). Byr4 ran with a faster mobility on SDS-PAGE gels when compared to Byr4 immunoprecipated from mitotic cells (Figure 16B). The same was true for Byr4 immunoprecipitated from *sid4-SA1* mutant cells in which all the SIN components fail to localize to the SPB (Chang and Gould 2000). Furthermore, ^{32}P was incorporated into Byr4 that was immunoprecipitated from mitotically arrested *S.pombe* cell lysates but not from *cdc16-116* cell lysates (Figure 16C). These results suggest that binding to the SPB is required for Byr4 phosphorylation and/or the kinase responsible is present only at the SPB.

Byr4 is a Cdc2 substrate

Since Byr4 phosphorylation peaks just prior to septation, it was possible that it was a target of either Plo1 or Cdc2 kinases. The *S.cerevisiae* homolog of Byr4, Bfa1 is phosphorylated by polo-like kinase Cdc5 (Hu, Wang et al. 2001; Lee, Jensen et al. 2001; Geymonat, Spanos et al. 2003). To test this possibility, recombinant kinase active (KA) and kinase inactive (KD) versions of Plo1 and Cdc2/Cdc13 purified from insect cells was used to phosphorylate various N-terminal deletions of Byr4 purified from bacteria. While Byr4 served as an excellent substrate for Cdc2 *in vitro*, Plo1 kinase was unable to phosphorylate Byr4 (Figure 17A, data not shown). To address whether the *in vitro* phosphorylated sites corresponded to bonafide *in vivo* phosphorylation sites, we compared tryptic peptide maps of *in vitro* phosphorylated Byr4 with a map generated from metabolically labeled Byr4 *in vivo*. Several tryptic peptides of *in vitro* phosphorylated Byr4 comigrate with those that are phosphorylated *in vivo*, demonstrating that Cdc2 is likely the kinase phosphorylating Byr4 *in vivo* (Figure 17B). Cdc2 is a proline directed kinase that targets S/T-P or S/T-P-X-K/R sites. Byr4 has 10 such consensus sites, 5 SP and 5 TP. Mutation of 4 consensus serine Cdc2 sites to nonphosphorylatable alanine (A) residues resulted in a significant reduction of *in vitro* phosphorylation by Cdc2 (Figure 18A, left panel). However the mutant protein was capable of localizing to the spindle pole body and rescued *byr4* null cells. This was not surprising, as examination of tryptic peptide maps of *in vitro* phosphorylated

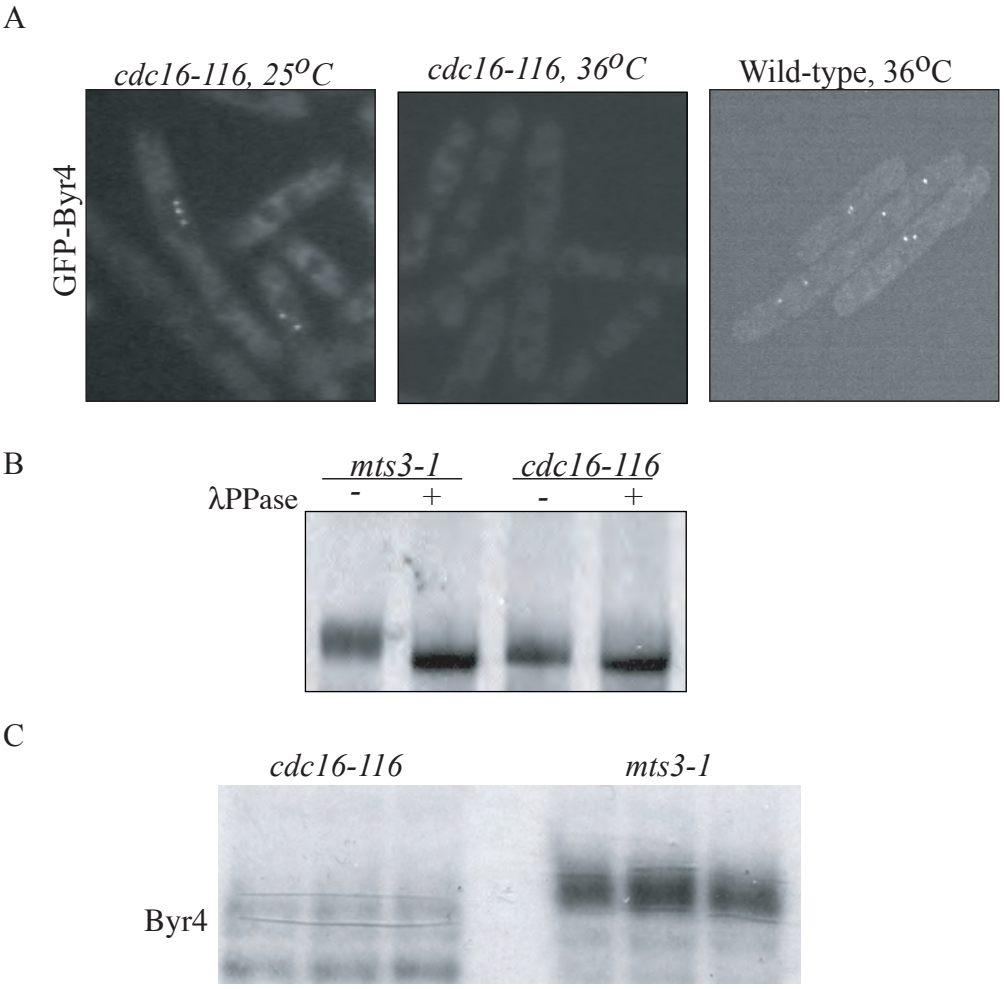


Figure 16. Byr4 phosphorylation requires association with the SPB. (A) *cdc16-116* (KGY 702) and wild type (KGY 246) cells expressing GFP-Byr4 under the control of the *nmt81* promoter were grown in the absence of thiamine at 25^{o}C for 16 hours and shifted to restrictive temperature 36^{o}C for an additional 4 hours. Images of live cells were captured. (B) Denatured cell lysates prepared from mitotic arrest *mts3-1* or *cdc16-116* mutant strains were immunoprecipitated with anti-Byr4 antibody. Immunoprecipitates were either mock treated or treated with lambda- phosphatase as described in materials and methods. (C) Autoradiogram of denatured cell lysate's prepared from ^{32}P metabolically labeled cells of indicated strains resolved by SDS-PAGE and transferred to PVDF membrane.

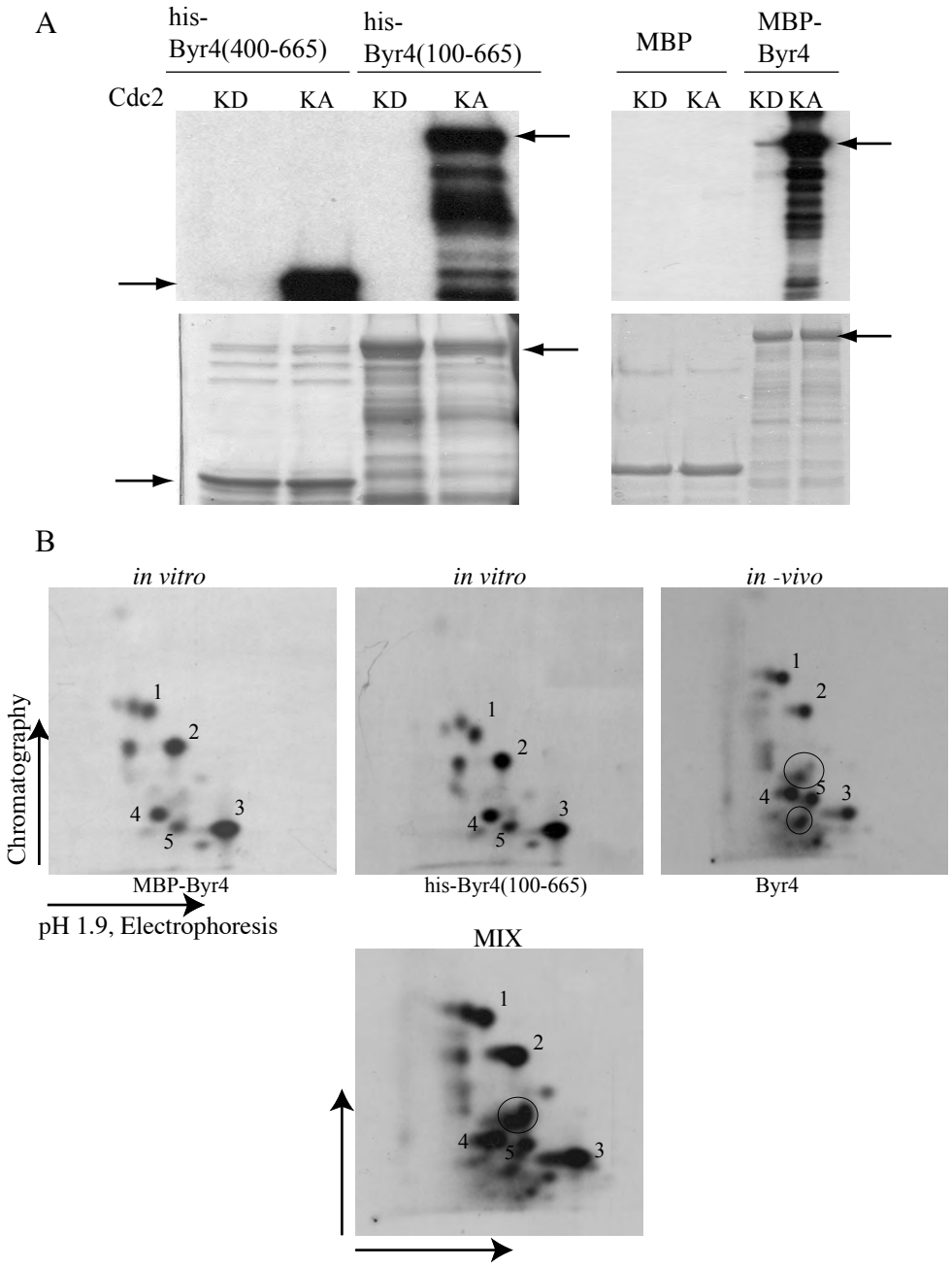


Figure 17. Byr4 is a Cdc2 substrate. A) Equal amounts of his-Byr4(100-665), his-Byr4(400-665), MBP and MBP-Byr4 was phosphorylated in vitro with baculoviral produced and purified recombinant active (KA) or kinase dead (KD) Cdk1p complex. Reactions were separated by SDS–PAGE and analyzed by coomassie blue staining (lower panel) and autoradiography(top panel). (B) The PVDF membrane containing MBP-Byr4 and Byr4(100-665) above was digested with trypsin and the peptides were separated in two-dimensions first by thin-layer electrophoresis followed by chromatography (*in vitro*), wild-type cells carrying pREP1Byr4(100-665) were labeled with [^{32}P] orthophosphate and lysed in SDS lysis buffer. Byr4 was immunoprecipitated using anti-Byr4 serum. The immunoprecipitate was resolved by SDS-PAGE and transferred to a PVDF membrane and subject to two-dimensional chromatography (*in vivo*). Bottom panel represents a mix of *in vitro* (100-665) and *in vivo* samples. Numbered spots represent those that co-migrate, circles represent *in-vivo* phophorylation events not represented within *in vitro* kinase reactions.

Byr4S4A mutant protein revealed that while 3 of the 4 major co-migrating spots were absent, 2 major spots still remained (Figure 18A, right panel). Phospho-amino acid analysis of the phosphorylated mutant protein revealed that most of the remaining phosphorylation was now concentrated on threonine residues (Figure 18B, bottom panel). Hence it is likely that although serine residues were identified as the major site of phosphorylation, the critical regulatory phosphorylations are on threonine residues.

Conclusions

Byr4 is phosphorylated at serine and threonine residues. The peak of phosphorylation occurs in mitotic cells with high Cdk activity and just prior to septation. Consistent with that observation Byr4 is phosphorylated by Cdc2 *in vitro* and several of the *in vitro* phosphorylation sites represent relevant *in vivo* phosphorylation sites. The timing of Byr4 phosphorylation suggests that Byr4 phosphorylation is most likely inhibitory in function, acting to promote septation. Byr4 phosphorylation could interfere with Spg1 or Cdc16 association, and SPB localization. However, Spg1 localizes to the SPB constitutively and Byr4 has been shown to bind Spg1 independent of GTP binding state. Therefore it is likely that phosphorylation of Byr4 influences GAP activity.

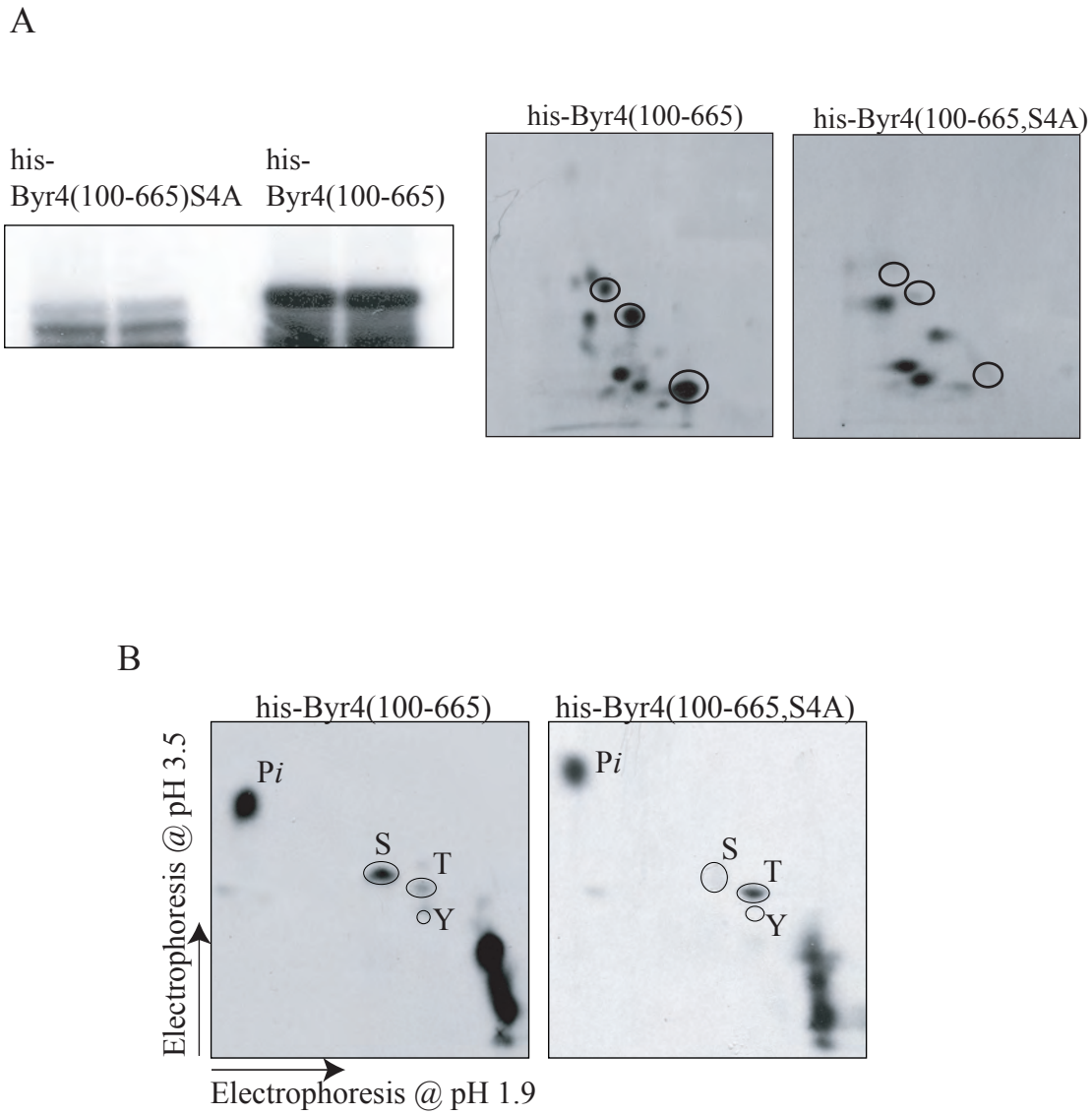


Figure 18. Byr4 is phosphorylated on atleast 3 serine residues. (A) Recombinant wild-type his-Byr4 and mutant hisByr4(100-665)S4A was phosphorylated *in vitro* with baculoviral produced and purified recombinant Cdk1 complex. Reactions were separated by SDS-PAGE and analyzed by autoradiography. The PVDF membrane containing his-Byr(100-665)WT and S4A was digested with trypsin and peptides were separated in two -dimensions (Right panel) or B) analysed for phosph-amino acid analysis after acid hydrolysis as described in material and methods.

CHAPTER VI

DISCUSSION

The ability to undergo cell division is one of the basic properties of the cell. The terminal phase of cell proliferation is cytokinesis. Cytokinesis is a complex event that involves interplay amongst several varied processes. The study of cytokinesis in simple model organisms through genetic and biochemical analysis has identified many proteins that are required for cytokinesis. Small G-proteins of the Ras and Rho family have emerged as important regulatory players in various aspects of cytokinesis. As is the emerging theme, the Septation Initiation Network (SIN) that controls the onset of cytokinesis in fission yeast, is triggered by the activation of a single GTPase, Spg1. Hence, understanding how Spg1 activation is regulated is critical to understanding the temporal regulation of cytokinesis.

We investigated the role of Cdc7, the crucial downstream effector kinase for Spg1 in the SIN. We identified several important domains that are required for Cdc7 function in the SIN. We extended our analysis to identifying functional domains within GAP protein, Byr4, to understand the molecular basis of interaction between the Byr4-Cdc16-Spg1 ternary complex. Byr4 is phosphorylated in a cell cycle dependant manner by Cdc2 and most likely serves as the critical regulatory subunit for the Byr4-Cdc16 GAP complex.

Identification of functional domains in Cdc7

Cdc7 is a protein kinase essential for cytokinesis in fission yeast. It is an effector for the ras-like GTPase Spg1, the activation of which is central to determining the timing of cytokinesis and septation in fission yeast. In the current model for SIN activation and signaling, Spg1 gets converted to its GTP-bound state, which enables association of Cdc7 to the SPB. Hence it had been suggested that the regulation of Cdc7 kinase activity is mediated by its localization to a signaling competent SPB. In this study we demonstrate that Cdc7 only associates with Spg1 during mitosis and hence its activity is apparently unaffected by association with Spg1-GTP. This suggests that the association of Cdc7 with Spg1 provides a means for Cdc7 to access its

relevant targets at the SPB and consequently regulating that association would be a key step in regulating SIN signaling.

In order to elucidate Cdc7 regulation I have identified various functional domains within Cdc7. I find that a region just adjacent to the kinase domain of Cdc7 (amino acids 250-535) acts as an effector domain and interacts with Spg1, in a nucleotide dependant manner. Cdc7 fails to localize to SPBs in germinating *spg1* null spores or *spg1-8* cells at restrictive temperature (Cerutti and Simanis 1999). While these data suggest that Spg1 is required for association of Cdc7 to the SPB I find that amino acids 250-535 within Cdc7 are capable of associating with Spg1 both *in vitro* and *in vivo* but fail to localize to the SPB. This suggests that additional residues and mechanisms exist that promote or maintain Cdc7 SPB localization. Indeed, the smallest region that could localize to the SPB (amino acids 360-870) is significantly larger than the Spg1 interacting region although it overlaps it.

In testing various fragments of Cdc7 for Spg1 and SPB interaction, I found that a GFP fusion of Cdc7 (Cdc71-900) localized efficiently to the SPB when produced at low level (Figure 7A). This result is inconsistent with a previous report suggesting that the C-terminal 162 amino acids of Cdc7 are essential for SPB targeting (Lu, Sugiura et al. 2002). It is possible that by using the strong *nmt1* promoter to drive expression at high levels, Lu et al (Lu, Sugiura et al. 2002) were unable to discriminate specific SPB staining from background. Notably the relative organization of the SPB and Spg1 binding domains, at least in primary amino acid sequence, is strikingly similar to that found in Cdc15, the *S.cerevisiae* equivalent of Cdc7 (Bardin, Boselli et al. 2003).

In the course of our studies, we found that Cdc7 self-associates through a coiled–coil domain (amino acids 360-518) that is in the middle of the Spg1 binding domain. In fact, this is also the case for *S.cerevisiae* Cdc15 (Bardin, Boselli et al. 2003). However, the residues supporting self-association are not sufficient to support binding to Spg1. Given that removal of this domain leads to reduced self- and Spg1 association in the yeast two-hybrid system, we speculate that Cdc7 dimerization is obligate for Spg1 association. Indeed, homodimerization of kinases plays a critical role in regulating their functions (Schlessinger 2000; Dan, Watanabe et al. 2001; Parrini, Lei et al. 2002). However, thus far our evidence suggests that Cdc7 dimerization is not regulated. First, a monomeric form of Cdc7 has not been observed in sedimentation analyses. Second, Spg1 itself does not self-associate and overproduction of Spg1 in a *cdc7-HA$_3$*/*cdc7-*

myc$_{13}$ diploid strain is not sufficient to disrupt Cdc7 self-association. While this region of the protein is clearly essential for Cdc7 function due to loss of both self association and Spg1 interaction, it is possible that removal of the domain interferes with yet other aspects of Cdc7 function or regulation. Interestingly this mutant accumulates at the pole when expressed from an exogenous promoter although it is also seen in several ectopic spots in the cytoplasm of the cell. Although I do not observe any changes in Cdc7 dimerization at steady state levels there may still be localized regulation of Cdc7 at the SPB. Given that Cdc7 is a protein kinase with a variety of modular domains understanding how Cdc7 is regulated through these domains to influence cytokinesis and septation in fission yeast will be a critical next step. This will require in depth structural analysis of Cdc7 in complex with Spg1. Such an analysis will help elucidate at the molecular level how Spg1 interacts with Cdc7, whether changes in Spg1 nucleotide status influence Cdc7 conformation and association with itself. Furthermore, identifying specific residues within Cdc7 that are responsible for contacting Spg1 and itself will enable the generation of specific point mutants of Cdc7 that are defective in Spg1 association but not self-association and vice versa and will be critical to investigate Cdc7 regulation.

Overproduction of Byr4 specifically displaces Cdc7 from the SPB

Byr4 is part of the two-component GAP that controls Spg1. The function of the Byr4-Cdc16 GAP complex is inhibitory as it serves to maintain Spg1 in an inactive or GDP bound state. The localization of the GAP complex to the SPBs during the cell cycle is consistent with its role in preventing inappropriate septum formation.

One interesting and as yet unexplained feature about the SIN is the asymmetric localization of the SIN components. Spg1 activation during mitosis is asymmetric, which is mirrored by the asymmetric localization of Cdc7-that occupies only the 'active' SPB. During anaphase the GAP complex Cdc16-Byr4 also localizes to only one SPB, the one without Cdc7. In fact the asymmetric localization of Cdc16-Byr4 appears to occur prior to that of Cdc7 suggesting that the complex may prevent the recruitment of Cdc7 to that SPB (Cerutti and Simanis 1999; Li, Furge et al. 2000).

Previously published observations demonstrated that overproduction of Byr4 resulted in titration of Spg1 away from the SPB and hence the inability of Cdc7 to localize to the SPB (Cerutti and Simanis 1999; Li, Furge et al. 2000). Contrary to these observations I find that

overproduction of Byr4 specifically displaces Cdc7 from the SPB. These results would be consistent with the argument that Byr4 and Cdc7 localize to the SPB mutually exclusively. We are unclear as to why there is this discrepancy between the observations. It has been shown that G-proteins can interact stoichiometrically or catalytically with their effectors and an active GTP bound G-protein can interact with multiple effectors during its lifetime. Thus termination of an interaction or complex formation must therefore require sufficient GAP concentrations to out-compete effectors (Donovan, Shannon et al. 2002).

Byr4 and Cdc7 do contain a region of sequence similarity. In our analysis of Spg1 binding domains within Byr4 we found the region of homology to be one of the Spg1 binding domains (aa475-595). However the similar region within Cdc7 was in fact responsible for Cdc7 self-association. Byr4 has 2 independent regions that bind Spg1. A modular binding domain may be an effective means of regulating Byr4 and its GAP function.

We noted that while Byr4 overproduction resulted in a SIN phenotype (due to the failure of Cdc7 to localize to the SPB), overproduction of Byr4 lacking its first 300 amino acids resulted in cells that had multiple septa. This indicates that an important regulatory domain lies within the first 300 amino acids. In fact, the first Spg1 binding domain lies within amino acids 100-300. Interestingly, we observed that removal of the second Spg1 binding domain did not affect interaction with Cdc16 or Spg1 in the yeast two-hybrid system and when overproduced was similar to full length Byr4, in that it also generated cells with a SIN phenotype. Thus, it is likely that the second Spg1 binding domain is dispensable for Byr4 function, although that remains to be tested. However, these results must be interpreted with caution as deletion of amino acids from the protein may alter its structure and therefore its function. Furthermore we haven't tested the ability of these mutant proteins to rescue a Byr4 null strain.

Byr4 association with Cdc16 is required for SPB localization

Byr4 localization to the SPB required Cdc16 function (Furge, Wong et al. 1998). The domain of Cdc16 association has been mapped to the C-terminal region of Byr4 (Furge, Cheng et al. 1999). We also find that the C-terminal 65 amino acids of Byr4 interact with Cdc16 *in vivo*. Interestingly, we find that this also is the SPB localization domain. All constructs that bear this domain can localize to the SPB; however, C-terminal deletions do not. This suggests that the association with Cdc16 is required for SPB localization of Byr4. This finding raises some

interesting questions with respect to certain observations in the literature. For example, in early mitotic cells, Byr4, but not Cdc16 is detected on the SPB and in the absence of Cdc16, Byr4 prevents GTP hydrolysis of Spg1 (Furge, Wong et al. 1998; Cerutti and Simanis 1999). However if, Byr4 association with Cdc16 is required for SPB localization then Byr4 cannot exist at the SPB in the absence of Cdc16. Furthermore, this also calls into question the *in vivo* relevance of observations from *in vitro* assays of Byr4 on Spg1 activity in the absence of Cdc16. Clearly, the two-component GAP is unique and understanding how it functions to regulate Spg1 activation at the molecular level will require structural information.

Byr4 is a phosphoprotein

Activation of GTPase, Spg1 determines the timing of cytokinesis. The nucleotide status of most G-proteins is regulated by GAPs-that inactivate G proteins by stimulating their low intrinsic GTP-hydrolysis activity and GEFs, which catalyze the exchange of bound GDP for GTP. Byr4-Cdc16 functions as a two-component GAP that maintains Spg1 in an inactive or GDP bound state. However no GEF for Spg1 GTPase has been identified. It is likely that Spg1 is controlled solely at the level of the GAP. While Cdc16 shares homology with GAPs, it has been demonstrated that Cdc16 cannot function as a GAP in the absence of Byr4 *in vitro* (Furge, Wong et al. 1998). In fact over production of Byr4 is lethal resulting in multinucleate cells, where as over production of Cdc16 has no detectable effect, suggesting that Byr4 plays a critical role in regulating the GAP (Furge, Wong et al. 1998). Various studies have implicated phosphorylation in the regulation of GAPs, although the precise mechanism is not well understood in most cases (Bernards and Settleman 2004). We demonstrate that Byr4 gets hyperphosphorylated in mitotic cells with high Cdk activity. Consistent with that it is a Cdc2 substrate *in vitro*. These data lend further support to the notion that Byr4 serves as the regulatory subunit of the GAP complex.

Byr4 phosphorylation may directly influence GAP enzymatic function, or affect GAP activity indirectly by regulating its association with Spg1 and/or Cdc16 or subcellular localization to the SPB. It must be noted that in *S.cerevisiae,* Bfa1, the Byr4 homolog, remains associated with Bub2 and Tem1 across the cell cycle; however, there is other evidence suggesting that phosphorylated Bfa1 affects Tem1 association and promotes Tem1-Cdc15 interactions (Wang, Hu et al. 2000; Lee, Jensen et al. 2001; Pereira, Tanaka et al. 2001; Ro, Song et al. 2002). Whether phosphorylation affects association with Spg1 or Cdc16 remains to be

tested. Interestingly, Spg1 resides at the SPB constitutively while GAP localization to the SPB varies across the cell cycle (Cerutti and Simanis 1999; Li, Furge et al. 2000). It is also entirely likely that SPB localization of the GAP is regulated by yet another mechanism that is independent of Byr4 phosphorylation. Additionally, as mentioned above Byr4 phosphorylation could affect the GAP activity of Cdc16. This is the case *in vitro* for Bfa1 (Geymonat, Spanos et al. 2003).

Bfa1 is a substrate of POLO-like kinase, Cdc5. Interestingly Bfa1 is de-phosphorylated by Cdc14, a phosphatase that reverses Cdk phosphorylations (Wang, Hu et al. 2000; Hu, Wang et al. 2001; Pereira, Manson et al. 2002; Park, Song et al. 2003). I demonstrated that Byr4 is in fact a Cdc2 substrate and a number of *in vitro* phosphorylation sites are bonafide *in vivo* phosphorylation sites. Our phosphorylation analysis is incomplete as after mutating all potential Cdc2 consensus SP sites, there still remains residual phosphorylation at threonine residues. A more complete mutational analysis should reveal the exact role of Byr4 phosphorylation, although as suggested above, several clues are available in the literature and can be easily tested.

Future Directions

In the last decade, efforts toward understanding the spatial and temporal co-ordination of mitotic exit with cell division has uncovered a novel signaling network, the SIN. It is likely that most of the SIN components have been identified and to a large part the roles of many components have been revealed. However, there are several critical unanswered questions. Crucial amongst them is the identity of the targets of the SIN kinases as well how these kinases are regulated to affect septum formation. It has been deduced, based on genetic epistasis analysis, that the SIN functions in a linear module. Cdc7 is the most upstream kinase in this pathway; however, to date there has been no biochemical evidence demonstrating Cdc7 phosphorylation of any of the downstream components of the SIN pathway. It has been suggested that Cdc7 kinase activity is required for mitotic hyperphosphorylation of the SIN scaffold component Cdc11, which correlates with the activation of the SIN (Krapp, Cano et al. 2003). However, this hasn't been demonstrated *in vitro* using recombinant Cdc7 or by immunoprecipitated Cdc7 from *S.pombe* cells as a source of kinase (Krapp, Cano et al. 2003).

Deconstructing protein kinase signaling pathways using conventional chemical, genetic and biochemical techniques has been difficult, largely due to the overwhelming number of

closely related protein kinases. A recent novel approach has been described that modifies a protein kinase to be specifically inhibited by a cell permeable small molecule inhibitor, that does not inhibit any wild-type kinases (Bishop, Shah et al. 1998; Bishop, Buzko et al. 2001; Kraybill, Elkin et al. 2002). The strategy involves the introduction of a functionally silent mutation within the ATP binding pocket such that it creates a larger space and renders it sensitive to a modified inhibitor (1 NM_PP1) that can only fit the modified binding pocket. Additionally a special modified ATP analog, N6-Benzyl ATP has been created and available that can only be utilized by modified kinase. I have identified a residue in Cdc7 that fulfils the criteria for the space creating mutation. Amino acid 85 is a leucine, a residue with a bulky side chain. The residue has been mutated to alanine and glycine. Genomic clones, Cdc7L85A and Cdc7L85G can rescue the temperature sensitive strain *cdc7-24* as well as the Cdc7 null strain (Figure 19). Preliminary results also suggest that the introduced mutation does render the kinase sensitive to the inhibitor at low doses but doesn't affect the wild-type kinase. Hence the stage is set to use this allele as a tool to answer critical questions about Cdc7 kinase activity. For example, at what point during the cell cycle does Cdc7 kinase activity becomes essential? Is it required during metaphase when Cdc7 occupies both poles or during anaphase when it is asymmetrically localized to one pole? What are the downstream effects of inhibiting Cdc7 kinase activity with respect to SIN component localization? The ATP-analog will serve as a powerful tool to identify substrates of Cdc7.

As mentioned in the discussion, structural information will lend great insight into how Cdc7 is regulated through its modular domains. However an additional mode of regulation could be through phosphorylation. We have been able to demonstrate that Cdc7 is also a phosphoprotein by metabolic labeling of *cdc7-myc*$_{13}$ cells with radioactive orthophosphate. Phosphoamino acid analysis revealed that Cdc7 is phosphorylated at serine residues (Figure 20). Interestingly a bacterially produced Cdc7(aa250-535) fragment that represents the domain responsible for Spg1 binding as well as self-association are excellent substrates *in vitro* for recombinant Cdc2 kinase produced from insect cells. This domain also has three consensus Cdc2 phosphorylation sites. The *S.cerevisiae* homolog of Cdc7, Cdc15, is a known phosphoprotein and remains hyper-phosphorylated in cells with high Cdk1 activity, like the APC mutant *cdc23-1* and MEN mutants' *tem1-1*, *dbf2-2* and *cdc14-1* (Jaspersen and Morgan 2000; Xu, Huang et al. 2000; Menssen, Neutzner et al. 2001). Although the phosphorylation of Cdc15 does not affect Cdc15

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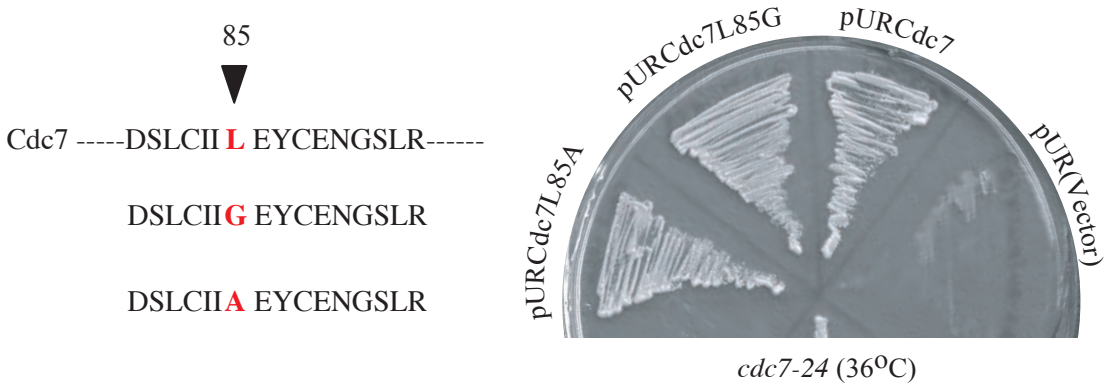

Figure 19. Cdc7 L 85 A/G mutation is viable. Partial sequence of catalytic domain showing the 'gatekeeper' position in red Leucine 85, used to introduce space creating mutations. *cdc7-24* mutant cells carrying vector alone, genomic clones of Cdc7- pURCdc7, pURCdc7L85A, pURL85G were grown on selective plates with thiamine at permissive temperature (25°C). Cells were then patched to single colonies and shifted to restrictive temperature of 36°C

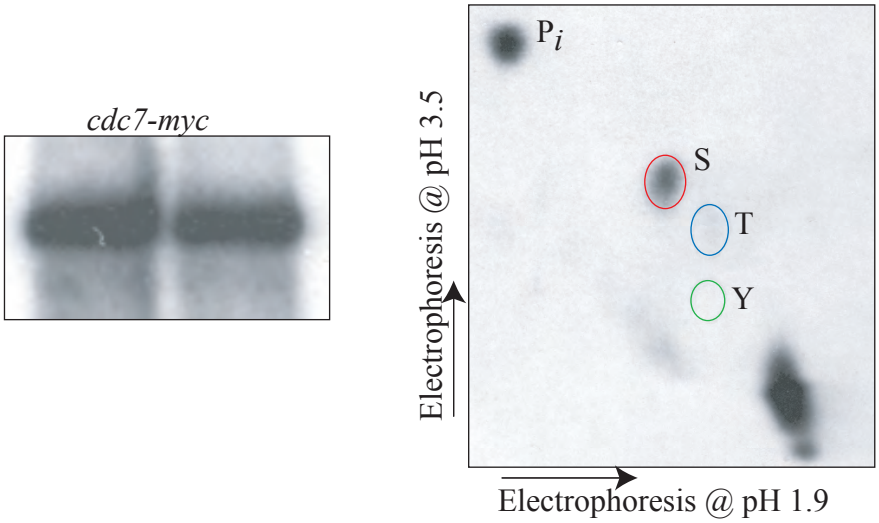

Figure 20. Cdc7 regulation by phosphorylation. *cdc7-myc$_{13}$* cells were labeled with [^{32}P] Orthophosphate and lysed in SDS lysis buffer. Anti-myc antibody was added to the lysate to immunoprecipitate Cdc7-Myc$_{13}$. The immunoprecipitate were resolved by SDS-PAGE and transferred to a PVDF membrane. Labeled proteins were detected autoradiography. Right panel, the PVDF membrane containing Cdc7-Myc$_{13}$ was analyzed for its phosphoamino acid content as described in Materials and Methods.

kinase activity a non-phosphorylatable mutant form of Cdc15 stimulates mitotic exit more than wild type suggesting that the phosphorylation of Cdc15 inhibits its function *in vivo*. It will be interesting to determine whether Cdc7 is similarly regulated by phosphorylation. This endeavor will clearly require the identification of the phosphorylated residues within Cdc7.

The pivotal regulatory point is the activation of Spg1 and its regulation by the Byr4-Cdc16 GAP complex. A two-component GAP complex is unique and solving the crystal structure of the complex will generate tremendous amount of information with respect to how it is regulated. What are the molecular roles of two independent Spg1 binding domains? How would phosphorylation events influence GAP enzymatic activity? This is of particular interest for researchers investigating GAP regulation, as although various studies have indicated phosphorylation –mediated regulatory mechanism for GAPs the precise mechanism is not understood in most cases.

Structural information will also be extremely useful in generating temperature sensitive alleles of Byr4, a resource that has been lacking and limited the genetic analysis of Byr4. Novel temperature sensitive alleles of Byr4 may uncover roles for Byr4 in checkpoint regulation of mitotic progression, similar to its *S.cerevisiae* counterpart.

Another interesting direction would be to determine the phosphatase responsible for de-phosphorylating Byr4. Byr4 needs to be returned to an active conformation as soon as SIN signaling is activated such that inappropriate septum formation is prevented. Preliminary experiments to determine if the *S.pombe* Cdc14-family phosphatase, Clp1, is responsible for Byr4 de-phosphorylation have not indicated its involvement. However this interaction may be transient and need to be examined carefully in a cell-cycle dependent manner. Nevertheless determining what the phosphatase is will be an important next step in understanding Byr4, and hence GAP complex regulation.

Given that all known SIN components localize to the SPB, it would be extremely exciting to obtain high-resolution structures of purified SPB preparations and proteomic analysis to identify additional components of the *S. pombe* SPB. Furthermore, solving the 3D structures of not only two-component complex (Byr4-Cdc16) but also, Sid1-Cdc14, Sid2-Mob1 and the GTPase Spg1 would enable us to gain more insight into how these proteins function.

CHAPTER VII

CONCLUDING REMARKS

The cell cycle field has benefited tremendously from the convergence of genetic and biochemical approaches. Clearly the identification of the Cdc2/cylin complex as the master regulator of the cell cycle and dissection of the mechanisms of Cdc2 regulation would not have been achievable without the biochemistry from model systems like frog oocytes; and data from genetically tractable organisms like budding and fission yeasts. The work done in model systems is strengthened by the knowledge that the essential biochemistry of the cell cycle remains conserved across all eukaryotes.

We have certainly entered a new era in cell cycle research. While early efforts focused on studying entry into mitosis, the last few years have seen numerous advances in our understanding of late events in the cell cycle and cytokinesis. Once again, genetic studies in fission yeast has unraveled a GTPase regulated signaling network, the SIN that serves to co-ordinate mitotic exit with onset of cytokinesis. Several components of the SIN are also conserved in higher eukaryotes, with an analogous pathway the MEN existing in *S. cerevisiae*. Given the central role of Spg1 GTPase in regulating this pathway we investigated the role of Cdc7 its effector, and Byr4 the GAP component that serves to inactivate it. While we have identified a number of functional domains within these proteins that affect SIN function, a number of questions still remain pertaining to their regulation. Discovering the physiological substrates of Cdc7 and other SIN kinases, elucidating the role of Byr4 phosphorylation will add not only to our understanding of temporal regulation of cytokinesis but also serve to lend some insights into more basic questions of GAP regulation in general.

The process of cytokinesis is highly complex and we are bound to see numerous differences between organisms. Although there are no obvious homologs of Byr4 and Cdc7 in higher eukaryotes to date, the history of cell cycle research has taught us that they often use the same processes in a manner. Furthermore, with the technologies available today such as RNAi, live cell imaging, small molecule inhibitors and high throughout genome-wide analyses, study of cytokinesis in higher eukaryotes should be rapid and rewarding. Only through the simultaneous study of this process in several organisms will we be able to reveal the similarities that exist.

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