Cdc18p can block mitosis by two independent mechanisms

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SUMMARY

The DNA replication checkpoint is required to maintain the integrity of the genome, inhibiting mitosis until S phase has been successfully completed. The checkpoint preventing premature mitosis in Schizosaccharomyces pombe relies on phosphorylation of the tyrosine-15 residue on cdc2p to prevent its activation and hence mitosis. The cdc18 gene is essential for both generating the DNA replication checkpoint and the initiation of S phase, thus providing a key role for the overall control and coordination of the cell cycle. We show that the C terminus of the protein is capable of both initiating DNA replication and the checkpoint function of cdc18p. The C terminus of cdc18p acts upstream of the DNA replication checkpoint genes rad1, rad3, rad9, rad17, hus1 and cut5 and requires the wee1p/mik1p tyrosine kinases to block mitosis. The N terminus of cdc18p can also block mitosis but does so in the absence of the DNA replication checkpoint genes and the wee1p/mik1p kinases therefore acting downstream of these genes. Because the N terminus of cdc18p associates with cdc2p in vivo, we suggest that by binding the cdc2p/cdc13p mitotic kinase directly, it exerts an effect independently of the normal checkpoint control, probably in an unphysiological manner.

Key words: cdc18p, Checkpoint, DNA replication

INTRODUCTION

The maintenance of genome integrity is important for the survival of all eukaryotic cells. This is achieved by a regulatory system involving checkpoints which monitor the completion of cell cycle events and prevent cell division until these events have been completed successfully (Hartwell and Weinert, 1989). Checkpoints operate in fission yeast to block the onset of mitosis if DNA replication is not complete or DNA damage is not repaired. Many proteins have been identified which operate in both checkpoint controls, including those encoded by rad1, rad3, rad9, rad17, rad26 and hus1 (Al-Khodairy and Carr, 1992; Enoch et al., 1992; Rowley and Young, 1992). Rad 1 is predicted to have 3’–5’ exonuclease activity and is homologous to RAD17 in Saccharomyces cerevisiae (Lydall and Weinert, 1995), rad 3 is a member of the PI-3 kinase family and is related to MEC1 in budding yeast and ATM in humans (Enoch and Norbury, 1995; Savitsky et al., 1995), rad17 is RF-C related and homologous to S. cerevisiae RAD24 (Griffiths et al., 1995). A human homologue of rad9, has been identified (Lieberman et al., 1996), as has DDC1, a S. cerevisiae homologue (Longhese et al., 1997). Human and mouse genes have recently been identified with sequence similarity to hus1, but rad26 still has no known homologue (Kostrub et al., 1998). To prevent mitosis, both pathways act to maintain cdc2p in its inactive tyrosine-15 phosphorylated form (Enoch et al., 1991; Rhind et al., 1997). The phosphorylation state of tyrosine-15 is influenced by the kinases, wee1p and mik1p, and the phosphatase, cdc25p, and their deregulation bypasses the checkpoint controls (Russell and Nurse, 1986, 1987; Gould et al., 1990; Lundgren et al., 1991).

An important aspect of the DNA replication checkpoint control is how the cell monitors whether replication has been completed. Several proteins have been identified which are both required for DNA replication and are also necessary to generate the signal that replication is in progress and so mitotic onset should be blocked. These include a number of gene products acting at the onset of DNA replication including cdc18, cdc1, the ORC related orp1, cut5 and pol1 all of which are necessary to restrain mitosis until replication is complete (Kelly et al., 1993; Saka and Yanagida, 1993; Hofmann and Beach, 1994; Saka et al., 1994; D’Urso et al., 1995; Grillert and Nurse, 1996). Deletion of these genes leads to an aberrant mitosis and cytokinesis as cells undergo mitosis in the absence of DNA replication.

One of these genes, cdc18, is crucial for regulating the onset of DNA replication as overexpression of cdc18 leads to repeated rounds of replication (Nishitani and Nurse, 1995). cdc18 encodes a 65 kDa protein of 577 amino acids with a putative NTP binding motif and six cdc2p consensus phosphorylation sites, five of which are located in the N terminus of the protein (Kelly et al., 1993). It has been proposed that phosphorylation of these sites promotes cdc18p proteolysis (Jallepalli et al., 1997). This could provide part of the explanation as to why deletion of cdc13 or high levels of the cdc2p/cdc13p mitotic kinase inhibitor rum1p can induce repeated rounds of DNA replication (Hayles et al., 1994; Moreno and Nurse, 1994; Correa-Bordes and Nurse, 1995), because both of these circumstances would reduce the cdc2p/cdc13p kinase activity. This would lead to cdc18p stabilisation, and the subsequent increase in cdc18p levels could...
bring about DNA replication. High levels of cdc18p have also been shown to block onset of mitosis (Nishitani and Nurse, 1995). A possible mechanism for this mitotic block is the activation of the checkpoint genes by high expression of cdc18p which prevents the onset of mitosis when DNA replication is ongoing. Another possibility is the direct inhibition of the cdc2p mitotic kinase because when cdc18 is expressed to a high level, cdc18p is found to be associated with cdc2p (Brown et al., 1997; Lopez-Girona et al., 1998). These observations have led to the hypothesis that the DNA replication checkpoint control may act by cdc18p, which is present at S phase, binding to and inhibiting the cdc2p mitotic kinase. When DNA replication is completed, cdc18p levels drop and the direct inhibition of the cdc2p mitotic kinase ceases allowing mitosis to proceed. Overexpression of cdc18p would therefore mimic activation of the checkpoint control to block mitosis. There has also been a suggestion that high levels of cdc18p may promote DNA replication indirectly by cdc18p inhibiting the cdc2p protein kinase, DNA replication would then be induced by an unknown mechanism (Lopez-Girona et al., 1998).

To investigate these possibilities further and to clarify the role of cdc18p in the checkpoint control, we have constructed different deletion mutants of cdc18 to focus on the role cdc2p may play in cdc18p function. We have expressed them in various checkpoint mutants. Our studies lead to the conclusion that activation of the checkpoint is unlikely to involve cdc18p acting directly on cdc2p, and that the promotion of DNA replication by high levels of cdc18p does not require inhibition of the cdc2p protein kinase.

**MATERIALS AND METHODS**

*Schizosaccharomyces pombe* strains and methods

All strains used were derived from the wild types 972h- and 975h+ and all media, growth conditions and general methods are as described by Moreno et al. (1991). Wild-type and the mutants rad1-1 leu1-32 ura4-D18 h+, rad3-136 leu1-32 h+, rad9-192 leu1-32 h+, rad17-1 hI leu1-32 h+ (Al-Khodairy and Carr, 1992), hus1-14 leu1-32 h+ (Enoch et al., 1992) and cdc2-3w leu1-32 ade6-M210 h+ (Fantes, 1981) were all grown at 32°C. Temperature sensitive mutants cut5-580 leu1-32 h+ (Saka and Yanagida, 1993) and mik1::ura4+ wee1-50 leu1-32 h- (Lundgren et al., 1991) were grown at the permissive temperature of 25°C, or the restrictive temperature of 36°C. Cells were transformed by electroporation or the lithium acetate procedure. The first five plasmids constructed were based on Rep3X-cdc18 (Nishitani and Nurse, 1995). To construct plasmid 1-141, a T AG stop promoter was induced, as described by Nishitani and Nurse (1995). Western blotting was carried out as described by Hayles et al. (1994). The antibodies used were anti-cdc18p (1:2,500 dilution), anti-C terminus cdc18p (1:2,500 dilution), anti-run1p (1:500 dilution), anti-cdc2p PSTAIR antibody (1:5,000 dilution), anti-1A2CA5 monoclonal antibody (1:2,000 dilution) and anti-α-tubulin monoclonal antibody (1:10,000 dilution) (Sigma). Proteins were detected using horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibody and an ECL kit (Amersham).

**Suc1p bead precipitation**

Extracts were prepared as described above. 10 µl of suc1p beads or BSA control beads were added to the extracts and rotated at 4°C for 1 hour. The precipitated complexes were washed 3 times in HB buffer and were boiled in 5X sample buffer for 5 minutes at 100°C. Western blots were performed as described above, and probed with anti-α-tubulin monoclonal antibody and anti-cdc2p PSTAIR antibody.

**Histone kinase assays**

Extracts were prepared from 2X10^8 cells and were broken as described above. 1 µl of rabbit polyclonal anti-cdc13p antibody SP4 was added to 400 µg of protein and incubated on ice for 1 hour. The immunocomplexes were precipitated with Protein A-Sepharose beads (Pharmacia) for 30 minutes at 4°C before being washed 3 times in HB buffer. The beads were then resuspended in 20 µl of reaction buffer containing 1 mg/ml H1 histone, 200 µM ATP and 40 µCi/ml [γ-32P]ATP and were incubated at 30°C for 20 minutes. The reactions were stopped by the addition of 5 µl of 5X sample buffer before boiling for 3 minutes at 100°C. Samples were run on a 12% SDS-polyacrylamide gel. Phosphorylated histone H1 was detected by autoradiography.

**RESULTS**

The C terminus of cdc18p is sufficient for cdc18p induced re-replication.

cdc18p can induce DNA re-replication and block the onset of...
mitosis when expressed to high levels. To investigate whether specific domains of cdc18p are essential for these functions, a series of constructs encoding mutated versions of cdc18p were generated (Fig. 1). 1-141p consists of the first 141 amino acids and includes five out of the six cdc2p consensus phosphorylation sites (S/TPXK/R). 150-577p and 150-577 (T374A)p correspond to the C terminus of cdc18p which includes the NTP binding motif. The difference between these two constructs is that the sixth cdc2p consensus phosphorylation site is mutated in 150-577 (T374A)p, leaving no consensus site for phosphorylation by cdc2p. The final construct, 1-577 (NTP)p, encodes the complete cdc18p with the NTP binding motif (GXXGXGKT) mutated (amino acid residues 204G and 205K are changed to AA). The five constructs were transformed into the temperature sensitive cdc18-K46 mutant and were expressed at the restrictive temperature of 36°C. The cells were then able to undergo at most a few divisions. None of the constructs were therefore able to complement cdc18-K46.

In order to investigate what cdc18p functions are defective in each mutant, their ability to induce re-replication and block mitosis when expressed to high levels was assayed. Each construct was introduced into a plasmid under the control of the thiamine regulatable nmt promoter (Maundrell, 1990) and transformed into wild-type cells. The phenotype was examined by microscopy and FACS analysis 20 hours after induction of the promoter at 32°C. Overexpression of the wild-type cdc18p or any of the mutants caused the cells to elongate, unlike cells containing the vector alone as a control (Fig. 2A).

These results indicate that all of the cdc18p mutants are capable of blocking mitosis. Next the ability of the cdc18p mutants to induce DNA re-replication was examined. FACS analysis showed an increase in DNA content of cells overexpressing 150-577p and 150-577 (T374A)p. Over 40% of the cells had a DNA content of greater than 4C (Fig. 2B, d and e). These constructs, like the wild-type protein, were therefore able to induce re-replication. Cells overexpressing 1-141p and 1-577 (NTP)p generated only a small apparent increase in DNA content (Fig. 2B, c and f), and less than 10% of cells had a DNA content of greater than 4C. The forward scatter dot plot

![Fig. 1. cdc18 constructs.](image)

1-577 (wt)  
1-141  
150-577  
150-577 (T374A)  
1-577 (NTP)

**Fig. 1.** cdc18 constructs. Wild-type cdc18p contains a putative NTP binding motif and six cdc2p consensus phosphorylation sites. 1-141 possesses the first 141 amino acids. 150-577 consists of amino acids 150-577 as does 150-577 (T374A), however, this has an additional mutation from T to A at amino acid 374 in the sixth cdc2p phosphorylation site. 1-577 (NTP) consists of the full length protein with the NTP binding motif mutated from GK to AA at amino acids 204-205. The black circles represent cdc2p consensus phosphorylation sites, the black rectangles depict the NTP binding motif and the grey rectangle represents the mutated NTP binding motif.

**Fig. 2.** Overexpression of the C terminus of cdc18p is required and sufficient for DNA re-replication. Wild-type cells transformed with the cdc18 constructs and a vector control were grown at 32°C for 20 hours after induction of the promoter. (A) Cells were fixed and stained with DAPI. Bar 15 μm. (B) DNA content and cell length was analyzed by FACS analysis. Vector (a), 1-577 (wt)p (b), 1-141p (c), 150-577p (d), 150-577 (T374A)p (e) and 1-577 (NTP)p (f). (C and D) Protein levels were determined by western blotting. 25 μg of protein was loaded per lane. Blots were probed with anti-cdc18p C terminus antibody (C top panel), anti-cdc18p antibody (D, top panel) and anti-α-tubulin antibody as a loading control (C and D, bottom panels).
showed that cell size increased similarly to the other constructs. The increase in DNA content observed is therefore due to either an increase in background staining or mitochondrial DNA (Sazer and Sherwood, 1990) in the subset of cells expressing the constructs (approximately 60%, see Fig. 4A, a).

Western blots were performed to verify that the cdc18 mutant proteins were expressed in all cases. Antibodies against the C terminus of cdc18p indicated that 1-577 (wt)p, 150-577p, 150-577 (T374A)p and 1-577 (NTP)p were all expressed to equal levels (Fig. 2C, lanes 1-4). To detect the N terminus of cdc18p (1-141p), antibodies against the entire protein were used. No band was seen in the vector alone control because endogenous cdc18p is expressed at a much lower level compared to that induced by the nmt promoter 20 hours after derepression (Fig. 2D, lanes 1 and 2). These results indicate that all of the mutants were able to maintain a block over mitosis, while those expressing the C terminus of the protein, 150-577p and 150-577 (T374A)p, could also efficiently promote DNA re-replication.

It has been suggested that DNA re-replication induced by high levels of cdc18p occurs indirectly due to the inhibition of the cdc2p/cdc13p mitotic kinase. There are two possibilities for how this inhibition could be achieved. Either cdc18p could directly bind and inhibit cdc2p, or overexpression of cdc18p could saturate the proteolytic machinery that degrades both itself and rum1p (Kominami and Toda, 1997). This could subsequently lead to an accumulation of the CDK inhibitor and a decrease in the mitotic kinase activity. These possibilities were investigated in three ways. Firstly, the mitotic kinase activity was assayed in cdc13p immunoprecipitates derived from cells overexpressing 1-577 (wt)p and 150-577p. The kinase activity was followed during a 20 hour time period after induction of the promoter at 32°C (Fig. 3A). High levels of wild-type cdc18p depressed mitotic kinase activity as previously reported, but 150-577p did not depress mitotic kinase activity despite inducing DNA re-replication. This experiment was repeated but in addition we also overexpressed 1-141p (data not shown). A similar decrease in kinase activity was observed between the 11 and 20 hour timepoints when 1-577 (wt)p and 1-141p were overexpressed. 150-577p overexpression again had no effect on the mitotic kinase activity. Secondly, western blots were performed with boiled extracts derived from wild-type cells overexpressing 1-577 (wt)p, 1-141p and 150-577p 20 hours after induction of the promoter. The blots were probed with anti-rum1p and anti-β-tubulin antibodies (Fig. 3B). Levels of rum1p did accumulate when 1-577 (wt)p and 1-141p but not 150-577p were overexpressed. Thirdly, cdc2p was precipitated using either suc1p beads or BSA control beads in wild-type cells expressing HA tagged 1-141p or 150-577p 16 hours after induction of the medium strength nmt promoter at 32°C. Western blots were performed and probed with anti-HA 12CA5 and anti-cdc2p PSTAIR antibodies (Fig. 3C). A greater proportion of the C terminus of cdc18p was found in the total cell extract (lanes 1 and 4) which may reflect a difference in stability of the two constructs. However, only HA 1-141p was precipitated by the suc1p beads (lanes 2 and 5). Neither cdc18p nor cdc2p were precipitated with the BSA control beads (lanes 3 and 6). This indicates that only the N terminus of cdc18p is able to interact with cdc2p. The fact that the C terminus of cdc18p does not interact with cdc2p provides further support.

Fig. 3. cdc18p induced re-replication does not require depression of the cdc2p/cdc13p mitotic kinase. (A) Histone kinase assays were performed on cdc13p immunoprecipitates from wild-type cell extracts overexpressing 1-577 (wt)p and 150-577p. (B) Protein levels were determined by western blots performed from boiled extracts of wild-type cells overexpressing 1-577 (wt)p, 1-141p and 150-577p 20 hours after induction of the promoter. Blots were probed with antibody to rum1p (top panel) and anti-β-tubulin antibody as a loading control (bottom panel). (C) Western blots were performed on suc1p or BSA control bead precipitates from wild-type cells overexpressing HA 1-141p and HA 150-577p from the Rep41 promoter for 16 hours. 5 μg of total extract and precipitates from 100 μg of extract were run. Blots were probed with anti-HA 12CA5 antibody (top panel) and anti-cdc2p PSTAIR antibody (bottom panel).
that re-replication can occur without cdc18p acting directly on cdc2p to inhibit its activity. As a consequence of these three experiments we conclude that cdc18p induced re-replication is not simply due to a direct inhibition of the cdc2p/cdc13p mitotic kinase.

Requirement for checkpoint genes for the mitotic block

It is possible that overexpression of cdc18p mimics activation of the DNA replication checkpoint and thus blocks onset of mitosis. This was examined by expressing the cdc18 mutants (Fig. 1) in strains defective in the checkpoint control. Five mutants were used, rad1-1, rad3-136, rad9-192, rad17-h21 and hus1-14, all of which are unable to send the signal preventing mitosis when blocked with hydroxyurea (HU) in S phase (Al-Khodairy and Carr, 1992; Enoch et al., 1992; Rowley et al., 1992). Two questions were addressed with these experiments; what is the position of cdc18p in the checkpoint pathway with respect to the other genes, and do the functional domains of cdc18p differ in their ability to activate the checkpoint. Cells were scored for elongation, indicating growth without division and therefore a block over mitosis, if more than 1.5 times the size of a wild-type cell 20 hours after derepression at 32°C. 60% of transformed cells in an exponentially growing population would typically contain a plasmid, and consistently, around 60% of wild-type cells transformed with any of the cdc18 constructs were found to be elongated (Fig. 4A, a). In checkpoint mutant backgrounds, cells expressing 1-577 (wt)p, 1-141p and 1-577 (NTP)p were all capable of blocking mitosis. Elongation occurred to the same level as in wild-type cells. However, in all the checkpoint mutants, expression of 150-577p and 150-577 (T374A)p did not induce elongation, indicating that these constructs were unable to send the checkpoint signal to prevent mitosis (Fig. 4A, b-f). Microscopic examination of cells overexpressing 150-577p and 150-577 (T374A)p in a rad1-1 strain confirmed their inability to send the checkpoint signal as the cells were much smaller and displayed a cut phenotype indicative of aberrant mitoses (Fig. 4B, d and e). Western blot analysis shows that all of the constructs were expressed (Fig. 4C and D) and therefore that the inability of 150-577p and 150-577 (T374A)p to block mitosis was not due to lack of protein expression. In fact, the protein levels of 150-577p and 150-577 (T374A)p in rad1-1 cells appear to be about 2 or 3 times higher than those of 1-577 (wt)p and 1-577 (NTP)p (Fig. 4C, lanes 1-4).

We conclude that high levels of wild-type cdc18p and a construct containing the N terminus can block mitosis even when the checkpoint control is defective. This strongly suggests that cdc18p overexpression does not mimic activation of the normal checkpoint control. It seems more likely that as the N terminus can bind cdc2p (Fig. 3C) it inhibits the mitotic kinase and blocks mitosis in an unspecific manner. However, when the N terminus is deleted as in the 150-577p and 150-577 (T374A)p constructs, the block over mitosis is dependent
upon an intact checkpoint control. In these cases the high levels of the protein do appear to induce activation of the checkpoint. Thus the C terminus of cdc18p appears to act upstream of the rad and hus genes examined, whilst the N terminus acts in a rad/hus checkpoint independent manner.

The NTP mutant 1-577 (NTP)p is also able to block mitosis when overexpressed but does not induce DNA re-replication (Fig. 2B, f). To test whether the N terminus region is required for the NTP mutant to block mitosis, a NTP 150-577 mutant was constructed. When over-expressed in wild-type cells no re-replication was induced and there was no block over mitosis (data not shown). Therefore, an intact NTP site is required both to induce re-replication and bring about a checkpoint dependent block over mitosis.

Requirement for cut5, and cell cycle core machinery

Another gene, cut5, has been implicated in the checkpoint control which is essential for DNA replication (Saka and Yanagida, 1993; Saka et al., 1994). To investigate the effects of the various cdc18p constructs in cut5 mutant cells, they were introduced into the temperature sensitive cut5-580 mutant which blocks DNA replication and induces mitosis at the restrictive temperature. cut5-580 cells were transformed with the cdc18 constructs and grown in the absence of thiamine to allow protein expression for 20 hours at 25°C, before the culture was split and half were shifted to the restrictive temperature of 36°C. They were allowed to grow for a further 3 generations (12 hours at 25°C and 7 hours plus 1 hour for temperature shift recovery at 36°C) before 300 cells were scored for elongation for each point.

The DNA replication checkpoint is dependent on the tyrosine-15 phosphorylation, and hence inhibition, of cdc2p to prevent mitosis (Enoch et al., 1991). The checkpoint could therefore activate the wee1p/mik1p tyrosine kinases or inhibit the cdc25p phosphatase to ultimately inhibit cdc2p. To investigate these possibilities we overexpressed the cdc18p constructs in two strains. The first was a cdc2 mutant, cdc2-3w, which allows cells to enter mitosis without cdc25p (Enoch and Nurse, 1990). If the

![Fig. 5.](image1)

![Fig. 6.](image2)
Cdc18p can block mitosis by two mechanisms

We show that a series of cdc18p mutants can prevent mitosis and would continue dividing in a cdc2-3w strain. The cells were grown at 32°C for 20 hours after induction of the promoter and were scored for elongation. All of the constructs produced elongated cells when overexpressed in the cdc2-3w strain. As cdc2-3w cdc25Δ cells are approximately double the length of wild-type cells we measured a sample of these and compared them with cdc2-3w cells that had been overexpressing 1-577 (wt)p for 20 hours. We found that cdc2-3w cells overexpressing 1-577 ( wt)p were on average twice the length of cdc2-3w cdc25Δ cells. This indicates that cdc2-3w cells overexpressing the cdc18 constructs are blocking mitosis and do not merely have a longer generation time. This therefore suggests that the checkpoint does not act solely through cdc25p (Fig. 6A). The second strain used was wee1-50 mik1Δ (Lundgren et al., 1991). At the restrictive temperature for wee1-50 (36°C) the cells do not possess a kinase capable of phosphorylating tyrosine-15 and consequently inhibiting cdc2p. The cultures were grown at the permissive temperature for 20 hours before being split and half shifted to 36°C. At 25°C all of the constructs were able to prevent mitosis thus causing cell elongation (Fig. 6A, b, and B, a and c). However, at 36°C although those expressing 1-577 (wt)p (Fig. 6B b), 1-141p and 1-577 (NTP)p were elongated, nearly all of the cells expressing 150-577p (Fig. 6B, d) and 150-577 (T374A)p were very small and displayed cut nuclei indicating aberrant mitoses. These results indicate that the wee1p/mik1p kinases are required for there to be a response to the checkpoint signal activated by overexpressing the C terminus of cdc18p which leads to a block over mitosis.

**DISCUSSION**

Previous work has shown that cdc18p overexpression in fission yeast cells results in re-replication of DNA and induces a checkpoint which blocks mitosis (Nishitani and Nurse, 1995). We show that a series of cdc18p mutants can prevent mitosis but only those encoding the C terminus of cdc18p, 150-577p and 150-577 (T374A)p, are able to induce re-replication of DNA similar to that found when wild-type cdc18p is over-expressed (Fig. 2). These results indicate that the C terminus of cdc18p with an intact NTP binding motif is able to carry out the re-replication function of cdc18p. Histone kinase data and an anti-rum1p western blot (Fig. 3A and B) shows that this re-replication can occur in the presence of some cdc2p kinase activity providing further proof that cdc18p induced re-replication does not occur solely as a result of highly reduced cdc2p/cdc13p kinase activity. This result supports previous work stating that cdc18p re-replication does not require a decrease in kinase levels; this work showed that a cdc18 construct mutated in the first five CDK phosphorylation sites was able to re-replicate even when co-expressed with cdc13p to increase cdc2p/cdc13p kinase activity (Jallepalli et al., 1997). Overexpression of the N terminus of cdc18p (1-141p) and the cdc18 NTPp mutant both caused a small increase in DNA which could be due to either increased background staining caused by cell elongation or mitochondrial DNA (Sazer and Sherwood, 1990).

We also showed that the C-terminal constructs of cdc18p, 150-577p and 150-577 (T374A)p, were unable to maintain the block over mitosis in DNA replication checkpoint deficient strains (Fig. 4). These results indicate that the C terminus of the protein acts upstream of the checkpoint genes examined and requires the rad and hus genes to send the checkpoint signal to block mitosis. In contrast constructs containing the N terminus of cdc18p (1-577 (wt)p, 1-141p and 1-577 (NTP)p) were able to prevent mitosis in the DNA replication checkpoint deficient strains. Similarly the mitotic block induced by high levels of the C terminus of cdc18p requires the cut5p protein while constructs containing the N terminus of cdc18p can block mitosis in the absence of cut5p function (Fig. 5).

These results suggest that high levels of cdc18p can block mitosis by two mechanisms. In the first mechanism the N terminus region containing five of the cdc2p consensus phosphorylation sites blocks the cdc2p mitotic kinase directly (Fig. 3C) and blocks mitosis independently of the DNA replication checkpoint control. We suggest that this is an unphysiological mechanism and does not provide any insight into the function of the checkpoint. In the second mechanism the block over mitosis is brought about by the C terminus which does operate through the DNA replication checkpoint control and requires the checkpoint rad/ hus genes. The C terminus region requires an intact NTP site to send the checkpoint signal, and the same site needs to be intact to induce DNA replication. Thus it is likely that the C terminus block over mitosis is brought about by the induction of DNA replication which then activates the normal checkpoint control. The result suggests that cdc18p acts at the beginning of the pathway to induce the DNA replication checkpoint and not at the end of the pathway by binding directly to cdc2p. However, we have not shown how the checkpoint signal is generated; it could be a replication complex on the DNA or the presence of replication intermediates once replication has been initiated. The 150-577 (NTP)p mutant expressed in wild-type cells could not induce DNA replication or activate the checkpoint signal. As these defects may have been due to the failure to form a complex or fire origins, it was impossible to separate the checkpoint function from that of replication and hence gain further insight into how the signal is initiated.

We have shown that when constructs 150-577p or 150-577 (T374A)p are overexpressed in the absence of wee1p/mik1p

![Fig. 7. Model for the Role of cdc18p in the DNA replication checkpoint. The C terminus of cdc18p acts upstream of the rad and hus genes in the DNA replication checkpoint. The pathway requires the function of the wee1p and mik1p tyrosine kinases to inhibit the cdc2p/cdc13p kinase and hence mitosis. The N terminus of cdc18p can prevent mitosis independently of the rad and hus checkpoint genes, probably via a direct interaction with cdc2p.](image-url)
function, cells enter mitosis prematurely resulting in small cells displaying cut phenotypes (Fig. 6A, b, and B, d). This suggests that the checkpoint activated by high cdc18p levels preserves cdc2p in its tyrosine-15 phosphorylated state which has reduced kinase activity. When the same constructs are overexpressed in a cdc2-3w strain which acts independently of cdc25p, the cells are able to maintain a block over mitosis indicating that the checkpoint does not act to inhibit cdc25p (Fig. 6A). This result is inconsistent with that found when HU is added to cdc2-3w cells as these continue to divide when DNA replication is blocked (Enoch and Nurse, 1990). This can be explained if the inhibitory signal sent to indicate ongoing replication is stronger than that found when replication forks are halted by the addition of HU.

The constructs containing the N terminus of cdc18p, 1-577 (wt)p, 1-141p and 1-577 (NTP)p, do not require either wee1p/mik1p or cdc2-3w to block mitosis, indicating that the inhibition in this case is not dependent on tyrosine-15 phosphorylation. These results are consistent with our conclusions that the N terminus of cdc18p prevents mitosis by binding directly to cdc2p.

The model in Fig. 7 summarises our conclusions. The C terminus of cdc18p is crucial for both the replication function of cdc18p and for the DNA replication checkpoint that acts through cut5 as well as the rad and hvs genes. cdc18p also requires an intact NTP binding motif for both of these activities. The checkpoint pathway induced by high levels of the C terminus of cdc18p exerts its effect through a pathway that requires the activity of the wee1p and mik1p tyrosine kinases, resulting in an inactive tyrosine-15 phosphorylated cdc2p/cdc13p complex.

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