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Phosphorylation of dis2 protein phosphatase at the C-terminal cdc2 consensus and its potential role in cell cycle regulation

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We show that the fission yeast dis2 protein phosphatase, which is highly similar to mammalian type 1 phosphatase, is a phosphoprotein containing phosphoserine (phospho-S) and threonine (phospho-T). It has several phosphorylation sites, two of which locate in the C-terminus. Phospho-T was abolished in the alanine substitution mutant at the C-terminal T316, which is conserved as a residue in the cdc2 consensus, TPPR, in a number of type 1-like phosphatases. In G_2-arrested *cdc2-L7* cells, the degree of T316 phosphorylation was reduced, whereas it was enhanced in metaphase-arrested *nuc2-663* mutant cells. Phospho-T was produced in dis2 by fission yeast cdc2 kinase, but not in the substitution mutant A316, indicating that the T316 residue was the site for cdc2 kinase *in vitro*. Phosphatase activity of wild type dis2 was reduced by incubation with cdc2 kinase, but that of mutant dis2-A316 was not. Phosphorylation of T316 hence has a potential significance in cell cycle control in conjunction with cdc2 kinase activation and inactivation. Overexpression phenotypes of wild type *dis2*$^+$, *sds21*$^+$ and mutant *dis2-A316*, *sds21-TPPR* genes were consistent with negative regulation of dis2 by phosphorylation. This type of regulation would explain why cells harboring the *dis2-11* mutation enter mitosis but fail to exit from it.

Key words: cdc2 kinase/cell cycle control/mitosis/protein phosphatase

Introduction

Activated cdc2−cyclin B kinase is generally agreed to be the key enzyme which promotes the G_2−M transition in the cell division cycle (reviewed in Nurse, 1990). This implies that changes in the phosphorylation state of key residues in a set of proteins alter their activity such that they switch from interphase activity to mitotic activity. It is not known how many proteins have to undergo this kind of regulation, nor to what degree their phosphorylation state changes. The protein phosphatase responsible for the dephosphorylation of that residue must also be regulated. When cdc2−cyclin B kinase is inactivated in mid-mitosis, the phosphatase(s) might be reactivated or the mitotic state would persist.

There are indications that phosphatase-deficient mutants are apt to become locked in mitosis (Doonan and Morris, 1989; Ohkura *et al.*, 1989; Axton *et al.*, 1990; Meyer-Jaekel *et al.*, 1993). In fission yeast there are two phosphatases similar to type 1 (PP1; Cohen, 1989), namely dis2 and sds21 (Ohkura *et al.*, 1989). The *dis2*$^+$ gene was initially identified by a mutation, *dis2-11*, which exhibited a failure to exit from mitosis at the restrictive temperature (Ohkura *et al.*, 1989), similar to *Aspergillus* and *Drosophila* mutants (Doonan and Morris, 1989; Axton *et al.*, 1990). The semi-dominant cold-sensitive (cs) mutant *dis2-11* is deficient in the PP1 activity so that protein serine/threonine phosphatases were thought to play an important role in completing mitosis.

Two types of multicopy suppressor genes, *sds21*$^+$ and *sds22*$^+$, were obtained for the *dis2-11* mutant. The *sds22*$^+$ gene is essential for viability. Its deletion and temperature-sensitive (ts) mutants displayed a metaphase arrest phenotype, indicating that *sds22*$^+$ is required for the progression from metaphase to anaphase (Ohkura and Yanagida, 1991; Stone *et al.*, 1993). The sds22 protein, identified by immunochemical methods, is found to be directly bound to dis2 and sds21 phosphatases in cell extracts, and altered the substrate specificity of dis2 phosphatase when histone H1 and phosphorylase were used as the substrates. The sds22 protein serves as a mitosis-specific regulator for dis2 and sds21 phosphatases (Stone *et al.*, 1993). The amino acid sequence of sds21 is 79% identical to dis2 phosphatase (Ohkura *et al.*, 1989). Amino acids differing between the two phosphatases are present in the N- and C-terminal regions. The *dis2*$^+$ and *sds21*$^+$ genes share an essential function as double gene disruption is lethal, although single disruption mutants are viable (Ohkura *et al.*, 1989). Results of immunoblotting and phosphatase activity assay show that the dis2 phosphatase is the major type 1-like phosphatase so far as the amount and activity present in cell extracts (Kinoshita *et al.*, 1990).

We addressed the question of whether dis2 protein phosphatase is regulated during the cell cycle. The phosphatase activity may be altered and proteins that are bound to dis2 possibly change. The dis2 protein could be modified, for example by phosphorylation, and the amount could oscillate during the cell cycle. In the wild type synchronous culture, however, the level of dis2 remained constant (Kinoshita *et al.*, 1990). There is an indication that dis2 phosphatase activity is regulated; the sds22-dependent H1 phosphatase activity decreased in metaphase (Stone *et al.*, 1993). The dis2 phosphatase bound to sds22, which plays an essential role in the progression from metaphase to anaphase, appears to have an elevated level of activity in interphase. As the cdc2 kinase activity is sharply reduced in the exit from metaphase (e.g., Murray and Hunt, 1993), it was of interest to examine whether the activity of dis2 phosphatase is regulated in relation to the activation and inactivation of cdc2 kinase.

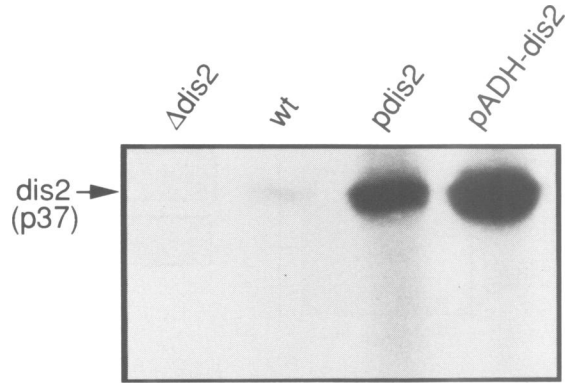

Fig. 1. Phosphorylation of dis2. Fission yeast strains were ^{32}P-labelled and cell extracts were prepared. The extracts were immunoprecipitated using affinity-purified anti-dis2 antibodies (D2F) and immunoprecipitates were run using SDS−PAGE and autoradiographed. Experimental procedures are described in Materials and methods. Δdis2, *dis2* deletion mutant (Ohkura *et al.*, 1989); wt, wild type; pdis2, wild type cells carrying plasmid with the *dis2*$^+$ gene; pADHdis2, wild type cells carrying plasmid with the *dis2*$^+$ gene ligated with the *ADH* promoter (Russell and Hall, 1983).

Here we report evidence that dis2 phosphatase is a phosphoprotein. One phosphorylation site locates nearby the C-terminus (the 316th threonine, T316) in the conserved stretch of cdc2 kinase consensus. Fission yeast cdc2 kinase purified from mitotically arrested *nda3-311* cells phosphorylated dis2 *in vitro* at the T316 site and inhibited the phosphatase activity.

Results

Dis2 phosphatase is a phosphoprotein

We examined whether dis2 phosphatase is a phosphoprotein by *in vivo* phospholabelling. Specific antibodies (D2F) against dis2, which do not cross-react with sds21 protein phosphatase, were employed for immunoprecipitation of ^{32}P-labelled cells (Figure 1); cell extracts used were wild type (wt), *dis2* deletion mutant (Δdis2), wild type carrying multicopy plasmid with the *dis2*$^+$ gene (pdis2) and wild type carrying plasmid with the *dis2*$^+$ gene downstream of the *ADH* (alcohol dehydrogenase) promoter (Russell and Hall, 1983). Immunoprecipitates, prepared as described in Materials and methods, underwent SDS−PAGE and were autoradiographed. The phosphorylated protein band at 37 kDa was obtained, the intensity of which was strikingly increased by raising the expression of the *dis2*$^+$ gene. No phosphorylated band was obtained from the *dis2* deletion mutant (Ohkura *et al.*, 1989).

The C-terminal T316 residue is a phosphorylation site

In order to identify the phosphorylation site(s) of dis2, we constructed various plasmids carrying substitution mutations which caused the change from serine or threonine to alanine or truncation mutation (some of these are shown in Figure 2A). The wild type and mutant *dis2* genes were placed downstream of the *nmt1* promoter (Maundrell, 1990) and resulting plasmids were introduced into the *dis2* deletion mutant. Transformant cells were cultured in the absence of thiamine for phospholabelling, as described in Materials and methods. Changes in the

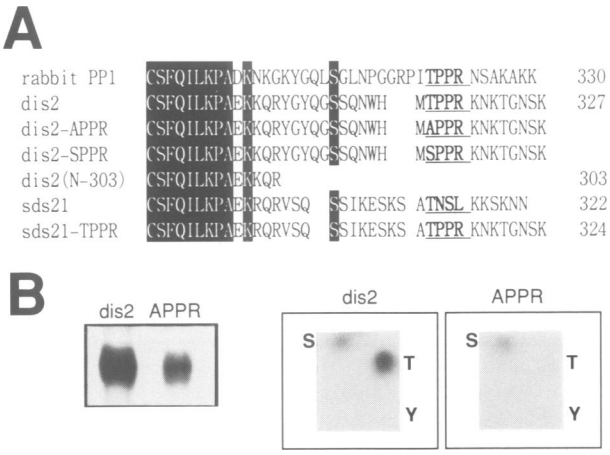

Fig. 2. Phosphorylation of the C-terminal T316 residue *in vivo*. (**A**) Substitution mutants of dis2 and sds21 made in the present study are shown with the C-terminal amino acid sequences. Rabbit PP1 sequence is shown for comparison. dis2 and sds21, wild type sequences. dis2-APPR and dis2-SPPR are the single substitution mutants at the 316th amino acid position. dis2(N-303) mutant deletes the C-terminal sequence 304–327. The end of sds21-TPPR is substituted by the dis2 C-terminal sequence. (**B**) (left) Wild type cells carrying a plasmid with the wild type gene *dis2*$^+$ (designated dis2) or the mutant gene dis2-APPR (APPR) were ^{32}P-labelled and extracts were immunoprecipitated by anti-D2F antibodies, which recognized only dis2 (Stone *et al.*, 1993), followed by SDS−PAGE and autoradiography. Labelling of the dis2 band in the mutant (APPR) was less than that in wild type (dis2). In this and following experiments, the amounts of dis2 and dis2-APPR detected by immunoblot were approximately the same. (right) Phosphoamino acid analysis (Boyle *et al.*, 1991) of labelled dis2 and APPR. Both phospho-S and -T were detected in the wild type (dis2), whereas only phospho-S was present in the mutant APPR. No phospho-Y was detected.

phosphorylation patterns of wild type and mutant dis2 proteins were studied by phosphoamino acid and peptide map analyses after immunoprecipitation.

The following evidence suggested that the C-terminal T316 residue was a phosphorylation site. This residue is a potential phosphorylation site (consensus, T/SPXK/R; Hanks and Quinn, 1991) for cdc2 kinase (Figure 2A). Wild type dis2 and dis2-A316 mutant cells were ^{32}P-labelled. Cell extracts were prepared and immunoprecipitated by D2F antibodies, followed by SDS−PAGE. The degree of phosphorylation was reduced (~50%) in the mutant dis2 protein (APPR in Figure 2B) compared with the wild type protein (dis2). Phosphoamino acid analysis of the immunoprecipitates (right panel in Figure 2B) revealed a striking difference between the wild type and mutant: the wild type dis2 showed both phosphoserine (phospho-S) and phosphothreonine (phospho-T), but the mutant A316 protein (APPR) displayed only phospho-S, even with prolonged exposure (Figure 2B, right panel).

Phosphorylation of sds21 was also examined by the same experimental procedures except for the use of antibodies capable of precipitating sds21 (Stone *et al.*, 1993). In contrast to the result with dis2, sds21 was hardly phosphorylated (data not shown). Even after prolonged exposure for autoradiography, phosphorylation of sds21 was negligible. Note that the C-terminal cdc2 consensus sequence is lacking in the sds21 amino acid sequence (Figure 2A).

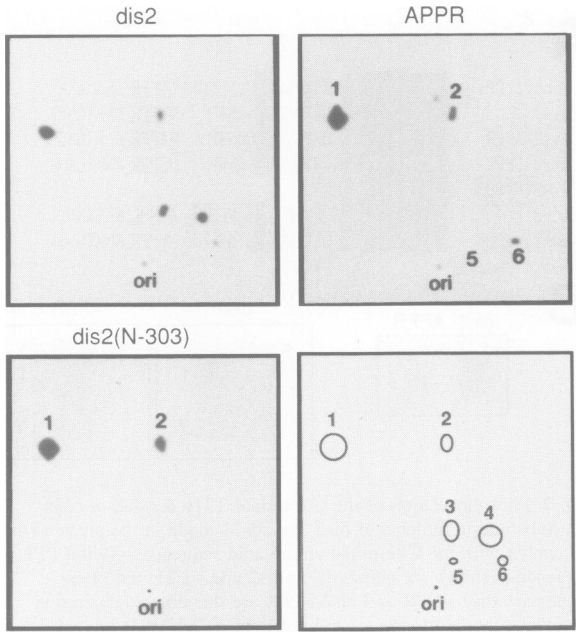

Fig. 3. Phosphopeptide mapping of ^{32}P-labelled wild type, substitution and deletion mutant dis2 proteins. Immunoprecipitates of ^{32}P-labelled dis2 proteins were digested with trypsin, and resulting peptides were separated by 2-D thin-layer chromatography (Boyle *et al.*, 1991; Kusubata *et al.*, 1992). The first dimension of electrophoresis was done at 1 kV for 40 min in 50 *n*-butanol:25 acetic acid:900 H_2O:25 pyridine at pH 4.72 [the anode, left] . The second dimension was ascending chromatography in 75 *n*-butanol:15 acetic acid:60 H_2O:50 pyridine. The peptide spots are numbered from 1 to 6. Spots 3 and 4 were missing in the mutant (APPR), while spots 3, 4, 5 and 6 were lost in the deletion mutant dis2(N-303). ori indicates the origin.

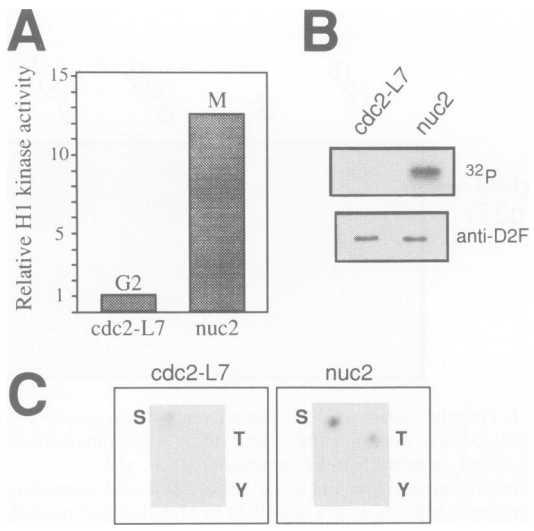

Fig. 4. Phosphorylation of dis2 in cell cycle mutant cells. Cell cycle mutants (*cdc2-L7* and *nuc2*) were arrested at 36°C for 2 h and then ^{32}P-labelled for the following 2 h at 36°C. (**A**) Histone H1 kinase activity of mutant extracts made after 4 h at 36°C. (**B**) Labelled extracts were immunoprecipitated by anti-D2F antibodies and resulting dis2 and dis2APPR were autoradiographed by the procedures described in Materials and methods. The dis2 protein is relatively more phosphorylated in *nuc2* than in *cdc2* mutant extracts (^{32}P). The level of dis2 as a control was determined by immunoblotting using anti-D2F and found to be approximately the same (anti-D2F). (**C**) ^{32}P-labelled dis2 immunoprecipitates derived from *cdc2* and *nuc2* mutant extracts were hydrolysed and phosphoamino acids were analysed. Both phospho-S and phospho-T are detected in *nuc2* mutants, whereas only phospho-S in *cdc2* mutants.

Tryptic peptide mapping of the C-terminal phosphorylation site

Peptide mapping of the immunoprecipitates was as previously described (Boyle *et al.*, 1991; Luo *et al.*, 1991). In these experiments, the amount of dis2 in the immunoprecipitates was the same (data not shown). As shown in Figure 3, a tryptic peptide map of *in vivo* ^{32}P-labelled wild type dis2 protein (dis2) displayed several phosphopeptides (numbered 1–6), two (3 and 4) of which were missing in mutant A316 cells (APPR). The C-deletion mutant dis2(N-303), which deleted the C-terminal 25 amino acids, was constructed. Immunoprecipitates of the C-deletion mutant consistently lacked spots 3 and 4 as well as spots 5 and 6. These results strongly suggested that spots 3 and 4 contained the phosphorylated T316 residue. Consistently, phosphoamino acid analysis indicated that spot 4 contained phospho-T, but not phospho-S. The radioactivity obtained from spot 3 was not sufficient for phosphoamino acid analysis.

Spots 1 and 2, containing only phospho-S, were not derived from the C-terminus, since they were present in the deletion mutant dis2(N-303). Note that phospholabelling of spot 1 increased in the APPR and C-truncation mutants. This might be compensatory for these mutations. Spots 5 and 6 probably arose from the C-end as they were missing in the deletion mutant. A putative phosphorylation site for the spots is the S326 residue. We thus concluded that dis2 phosphatase has at least three phosphorylation sites, two of which are in the C-terminal region. The T phosphorylation occurred mostly at the T316 residue as it was negligible in the A316-substituted mutant cells.

Phosphorylation in mitotically arrested cells

Whether or not phosphorylation of dis2 protein varied was examined in different cell cycle mutants. To this end, two mutant cell extracts labelled with ^{32}P were prepared. *cdc2-L7* was arrested in G_2 at 36°C for 4 h (Nurse, 1990) while *nuc2-663* was blocked at metaphase (Hirano *et al.*, 1988). Phospholabelling was done after 2 h at 36°C for 2 h. In this experiment, the dis2 plasmid was not used so that the level of dis2 protein was not elevated from that of wild type. The H1 kinase activity in *nuc2* cells after 4 h at 36°C was much higher than that in *cdc2* mutant cells (Figure 4A).

We found that the phosphorylation of dis2 was intense in mitotically arrested cells (nuc2), whereas it was greatly reduced in G_2-blocked *cdc2-L7* mutant (Figure 4B). The amounts of dis2 in the immunoprecipitates from *cdc2-L7* and *nuc2* mutant extracts detected by anti-D2F were the same.

Phosphoamino acid analysis consistently gave an intense phospho-T spot of dis2 in *nuc2* mutant extracts, but a faint phospho-T spot in *cdc2* mutant cells grown at 36°C (Figure 4C). At the permissive temperature, phosphorylation of T316 in *nuc2* mutant cells was weak as in wild type cells (data not shown).

Phosphorylation of dis2 protein by cdc2 kinase in vitro

The above results suggested that T316 was the site of phosphorylation *in vivo*. As it was in the cdc2 consensus

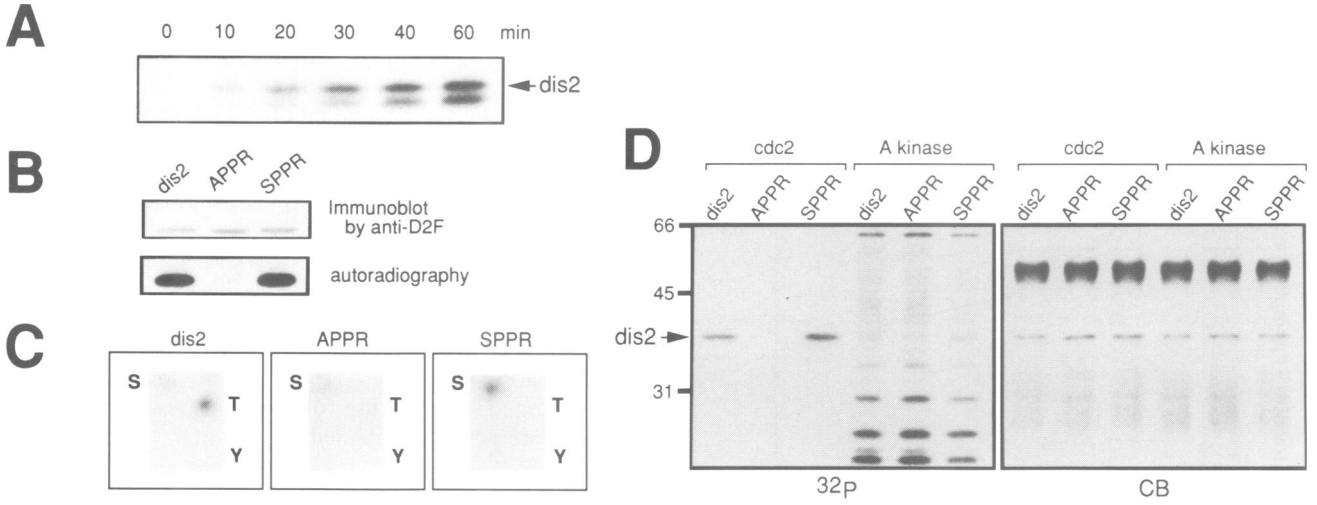

Fig. 5. Phosphorylation of dis2 by cdc2 *in vitro*. (**A**) dis2 precipitated by anti-D2F antibodies was incubated with cdc2 kinase for 0–60 min in the presence of [γ-^{32}P]ATP, followed by SDS−PAGE and autoradiography (see Materials and methods). Increased radioactive incorporation was found in the dis2 band (indicated by the arrow). The band adjacent to dis2 was probably due to proteolysis. (**B**) Precipitates of wild type dis2, mutant APPR and SPPR by anti-D2F were incubated in the presence of [γ-^{32}P]ATP and cdc2 kinase for 60 min followed by SDS−PAGE and autoradiography. The upper panel indicates immunoblot by anti-D2F and the amounts are approximately the same, while the lower panel shows the pattern of autoradiography. Incorporation of ^{32}P-radioactivity into wild type dis2 and mutant SPPR was approximately the same but that into mutant APPR was much less. (**C**) Phosphoamino acid analysis of wild type dis2, mutant APPR and SPPR. The same samples as above were hydrolysed. Mutant APPR and SPPR proteins did not contain phospho-T, suggesting that the T316 residue is the main site for phosphorylation by cdc2 kinase *in vitro*. (**D**) Specificity of dis2 phosphorylation by cdc2 kinase. Immunoprecipitates of wild type dis2 (dis2) and mutant dis2 (APPR and SPPR) were incubated with cdc2 kinase purified from mitotically-arrested *nda3–311* cells (Materials and methods) and protein kinase A catalytic subunit (Promega) in the presence of [γ-^{32}P]ATP. They were then run using SDS−PAGE, followed by Coomassie blue staining (CB) and autoradiography (^{32}P). As seen in the Coomassie blue-stained pattern, approximately equal amounts of dis2 were precipitated by antibodies (indicated by the arrow). Phosphorylation of dis2 by cdc2 was highly specific for T or S residues at the 316th position. No phosphorylation was found in the mutant APPR. Protein kinase A phosphorylated a number of proteins, the amounts of which were low in the immunoprecipitates, judging from Coomassie blue staining.

sequence, T316 might be the target of cdc2 or cdc2-like kinase. If so, T316 should be phosphorylated *in vitro* by cdc2 kinase. Hence, an *in vitro* phosphorylation experiment of dis2 by cdc2−cyclin B (cdc13) kinase was undertaken. Active cdc2 preparations were made from mitotically arrested *nda3-311* cells (Moreno *et al.*, 1989), then bound to and eluted from p13^{suc1} beads by the procedure of Kusubata *et al.* (1992) with modifications. The cdc2 preparations efficiently phosphorylated dis2 isolated from wild type extracts as an immunocomplex (Figure 5A).

Then cdc2 phosphorylation of wild type dis2, mutant A316 (APPR) and S316 (SPPR) was compared (Figure 5B). The A316 mutant protein showed only a very low level of phosphorylation, whereas wild type and mutant S316 were intensely phosphorylated, indicating that the T316 (and S316) residue was specifically phosphorylated by cdc2 *in vitro*.

Consistent results were obtained by phosphoamino acid analysis which showed intense phospho-T in wild type dis2 protein while phospho-S was obtained in the mutant S316 protein incubated with cdc2 kinase. Consistently, no phospho-T was found in the mutant APPR (Figure 5C). Weak phospho-S present in wild type and mutant APPR was possibly due to a contaminating kinase in cdc2 preparations or minor cdc2 phosphorylated sites. In contrast to the results obtained by the dis2 protein, the sds21 phosphatase was only slightly phosphorylated *in vitro* by cdc2 (data not shown).

Phosphorylation of dis2 *in vitro* by cdc2 kinase and rat brain protein kinase A was compared using three different immunocomplexes, wild type dis2, mutant A316 (APPR) and S316 (SPPR) (Figure 5D). The protein level of dis2, APPR and SPPR in the immunocomplexes (stained by Coomassie blue stain, CB) was nearly equal. Only one major band of dis2 in TPPR and SPPR was phosphorylated by cdc2 kinase, while no band was obtained in APPR (shown in the left panel, ^{32}P). However, a number of protein bands, the amount of which was low in the immunocomplex, were phosphorylated by protein kinase A. The specificity of T316 phosphorylation *in vitro* by cdc2 kinase thus was high.

dis2 phosphatase activity was reduced by T316 phosphorylation

In order to determine whether cdc2 phosphorylation of dis2 affects its activity, the S1 phosphatase activity (Kinoshita *et al.*, 1993; Stone *et al.*, 1993) of immunoprecipitates containing the wild type dis2, sds21 or mutant dis2-A316 protein was assayed before and after incubation with cdc2 kinase (Figure 6A). Peptide S1 containing the cdc2 consensus (Kinoshita *et al.*, 1993; Stone *et al.*, 1993) was ^{32}P-labelled by cdc2 and used as the substrate. The S1 phosphatase activity in mitotically arrested *sds22* mutant cells was low (Stone *et al.*, 1993).

It was found that, after incubation with cdc2 kinase for 60 min, the S1 phosphatase activity of dis2 was reduced by ~70% compared with the dis2 phosphatase activity under the same conditions without cdc2 (Figure 6A). The sds21 and mutant dis2-APPR proteins, however, retained the same level of S1 phosphatase activity after incubation with cdc2 for 60 min.

Incorporation of phosphate into dis2 was estimated by

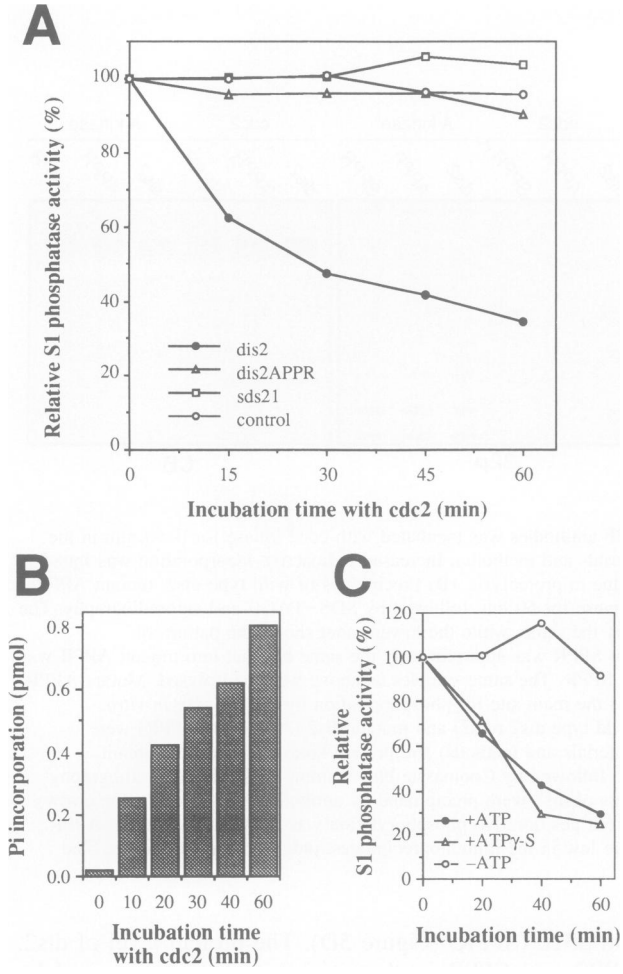

Fig. 6. Inhibition of dis2 phosphatase activity by cdc2 kinase *in vitro*. (**A**) To assay the S1 phosphatase activity of wild type dis2, sds21 and mutant dis2-A316 protein before and after incubation with cdc2 kinase, immunoprecipitation was done for extracts from the following strains using anti-D2C antibodies: *dis2* null carrying multicopy plasmid with the wild type *dis2*$^+$ gene (designated dis2); *dis2* null carrying plasmid pADH-sds21 (sds21); and *dis2* null carrying mutant plasmid pADH-dis2APPR (dis2APPR). *ADH* promoter is constitutively expressed. The oligopeptide S1 containing the cdc2 consensus was ^{32}P-labelled by fission yeast cdc2 kinase as described in Materials and methods. Then S1 phosphatase activity (Kinoshita *et al.*, 1993; Stone *et al.*, 1993) was assayed for each anti-D2C precipitate after incubation with cdc2 kinase for 0–60 min. The dis2 precipitate (derived from *dis2* null cells carrying multicopy plasmid with the *dis2*$^+$ gene) incubated without cdc2 was used as control. S1 phosphatase activity of dis2 was decreased to 30% after incubation with cdc2 for 60 min. (**B**) Incorporation of phosphate into dis2 protein by incubation with cdc2 kinase in the presence of [γ-^{32}P]ATP. The dis2 protein immunoprecipitated by anti-D2F antibody was incubated with cdc2 kinase for 0–60 min at 30°C. At each time point the radioactivity of labelled dis2 protein was estimated. Approximately 1.1 pmol (40 ng) of dis2 were taken at each time point. The amount of incorporated phosphate was estimated from the counts and the specific radioactivity. (**C**) ATPγ-S (0.1 mM) was used to replace ATP for phosphorylation of the dis2 protein by cdc2 kinase. The experimental conditions were the same as described in (A).

measuring the radioactivity of ^{32}P-labelled dis2 after incubation of the dis2 immunocomplex with cdc2 kinase in the presence of [γ-^{32}P]ATP (Figure 6B). Approximately 1.1 pmol (40 ng) of dis2 protein were used for each time point and 0.8 pmol of phosphate (estimated from the counts and the specific radioactivity) was found to be incorporated into dis2 at 60 min. The dis2 protein was thus phosphorylated with the ratio of ~0.7 mol of phosphate per mol of protein at 60 min, and therefore there is a correlation between the amount of phosphate incorporated and the inhibition of phosphatase activity. Thus cdc2 phosphorylation is inhibitory to dis2 phosphatase. The dis2 protein is the first cdc2 *in vitro* substrate derived from fission yeast.

The dis2 protein was incubated with cdc2 kinase in the presence of ATPγ-S. It was expected that the use of ATPγ-S might reduce the chance of dephosphorylation in the assay. We found that dis2 phosphatase activity was reduced by phosphorylation with ATPγ-S with the level similar to that obtained by the use of ATP; a fraction of the phosphatase activity remained (Figure 6C).

Overproduction of wild type and mutant dis2 phosphatases

To examine the potential negative regulation of dis2 phosphatase by T316 phosphorylation, we first compared the overexpression phenotype of *dis2*$^+$ and *sds21*$^+$ genes, as the *sds21*$^+$ gene did not contain the TPPR sequence. A striking phenotypic difference was found when wild type *dis2*$^+$ *and sds21*$^+$ genes (Figure 2A) were overexpressed using plasmids with the *Schizosaccharomyces pombe*-inducible promoter *nmt1* (Maundrell, 1990) in thiamine-deficient (−T) plates at 36°C (Figure 7A). When the *sds21*$^+$ gene was overexpressed, no colony was formed, while normal colonies were formed under the overexpression of *dis2*$^+$ gene downstream of the *nmt1* promoter.

The amount of dis2 and sds21 under the inducible promoter was estimated by immunoblot analysis using anti-D2C antibodies which can detect both proteins. Since the amount of dis2 detected by immunoblot analysis in the liquid cultures was greater than that of sds21 (Figure 7B), the failure of overproduced dis2 to block cell division was not due to transcription or translational inefficiencies. Overproduction occurred 10 h after the removal of thiamine. The level of cdc2 was shown to be constant by immunoblot using anti-PSTAIR antibodies.

The overproduction phenotype of *dis2*$^+$ and *sds21*$^+$ genes is shown in Figure 7C (pnmt-dis2 and pnmt-21). Cells overexpressing *sds21*$^+$ were greatly elongated with a single nucleus. By immunofluorescence microscopy using anti-tubulin, cytoplasmic microtubule arrays but not the mitotic spindle were observed (bottom panel). The DNA content of the cells overexpressing *sds21*$^+$ determined by FACS, was 2C (data not shown). Cells overexpressing dis2 (pnmt-dis2) under any conditions, however, produced normal looking cells and an increase in cell numbers at an approximately normal division rate without significant cell elongation. When the ADH promoter or multicopy plasmids were used to overexpress *sds21*$^+$, small colonies were formed (data not shown). Cells in liquid cultures were elongated but the degree of elongation was less than that of *nmt* promoter-directed overexpression.

Interestingly, when the mutant A316 (APPR) was overexpressed, colony formation was severely inhibited (Figure 7A). On the other hand, the dis2-S316 mutant behaved similarly to wild type. These results strongly suggested that the C-terminal TPPR or SPPR sequence was necessary for cells to grow when *dis2*$^+$ was overexpressed. Consist-

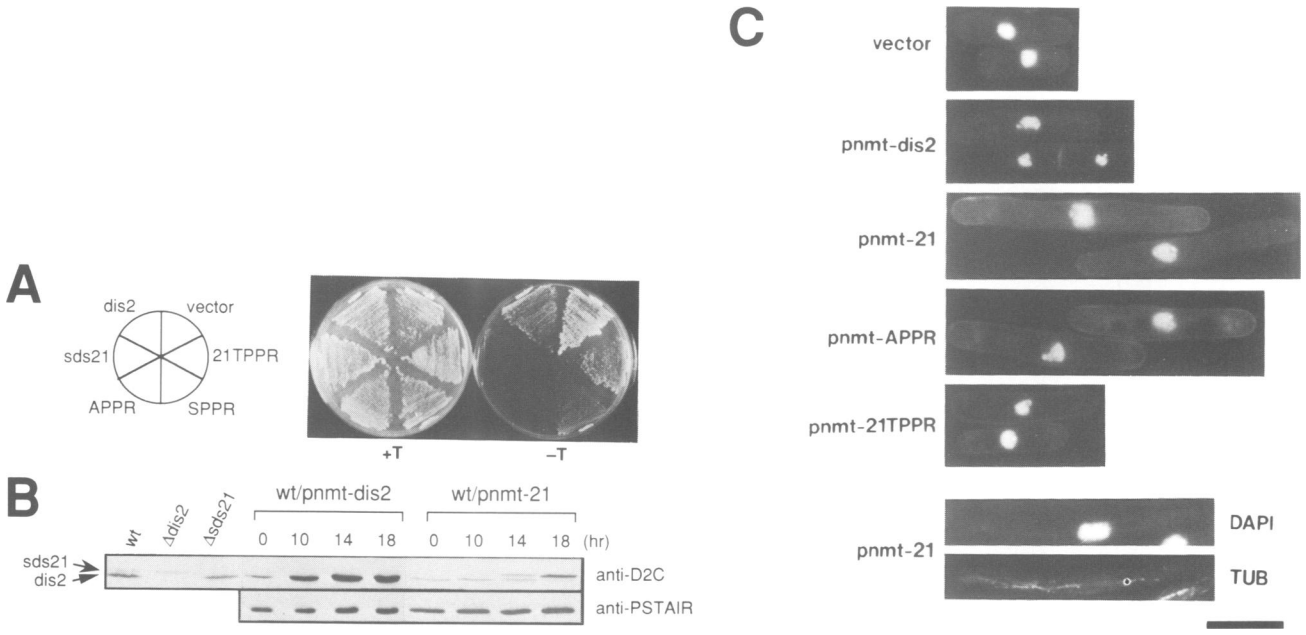

Fig. 7. Overexpression of wild type and mutant phosphatase genes. (**A**) Vector plasmid pREP1 having the *S.pombe* inducible promoter *nmt1* (Maundrell, 1990) was ligated with the initiation codons of *dis2*$^+$ (designated dis2), *sds21*$^+$ (sds21), mutant *dis2APPR* (APPR), *dis2SPPR* (SPPR) or *sds21TPPR* (21TPPR) genes. *S.pombe* transformants carrying one of these plasmids were plated on synthetic EMM2 medium containing thiamine (+T) or not (−T) at 36°C. The *nmt1* promoter was induced in −T. Colony formation of strains carrying plasmid sds21 or APPR was severely inhibited in −T, whereas colonies of dis2 and 21TPPR were normal. SPPR produced small colonies. (**B**) Immunoblot of dis2 and sds21 proteins overexpressed in wild type cells (wt) by plasmid pnmt-dis2 and pnmt-sds21. Anti-D2C antibodies recognized both dis2 and sds21 (Stone *et al.*, 1993). dis2 and sds21 proteins were identified by their respective loss in *dis2* null (Δdis2) and *sds21* null (Δsds21) (Ohkura *et al.*, 1989). The production of dis2 and sds21 increased after 10 h (Maundrell, 1990). Anti-PSTAIR antibodies against cdc2 were used as control. Note that overexpression of the *sds21*$^+$ gene caused the decreased level of dis2 protein. (**C**) Fluorescence micrographs of wild type cells transformed with the indicated plasmid, incubated in −T for 18 h and stained by DAPI are shown. Extensively elongated cells with a single nucleus were produced by pnmt-sds21 and -dis2-APPR, but cells with pnmt-sds21-TPPR were nearly normal. Cells with pnmt-sds21 stained by anti-tubulin antibodies TAT1 (Woods *et al.*, 1989) are also shown (indicated by TUB). Cytoplasmic microtubules characteristic of the interphase cells were observed. The bar indicates 10 μm.

ent with this hypothesis, the substitution mutant sds21-TPPR containing the TPPR and C-terminal amino acids of dis2 (Figure 2A) grew nearly normally when overexpressed (Figure 7A).

The overproduction phenotype of the dis2-A316 mutant is shown in Figure 7C (pnmt-APPR): cells were elongated with the single nucleus. The phenotype was similar to but less clear-cut than that of sds21 under the *nmt* promoter. Cells overproducing the sds21-TPPR mutant protein were like those of wild type, supporting the notion that the C-terminal sequence of dis2 confers the ability of cells to grow under the overproduction of dis2.

Discussion

We have shown by immunoprecipitation that the dis2 phosphatase was phosphorylated *in vivo*. The amount of label incorporated was increased by raising the level of dis2. Phosphoamino acid analysis revealed the presence of phospho-S and -T but not phospho-Y. Several phosphopeptides were detected in ^{32}P-labelled dis2 by tryptic digestion, suggesting the presence of multiple phosphorylation sites in this protein. In many of the experiments described in the present study, phospholabelling was done in cells overproducing the wild type or mutant dis2 and sds21 proteins in order to obtain higher radioactivity. The dis2 protein at the wild type level, however, was also labelled and found to contain both phospho-T and phospho-S.

The T316 residue present in the C-terminal cdc2 kinase consensus sequence was found to be one of the phosphorylation sites. Phosphopeptide mapping of wild type and mutant dis2 proteins identified peptides, the phosphorylation of which was abolished in the A316 substitution mutant. Phosphopeptides 3 and 4 appeared to contain the T316 residue. Peptide 4 contained only phospho-T. In the mutant A316, peptides 3 and 4 were not phosphorylated, but the other peptides remained phosphorylated. This result implied that the two S residues (positions 309 and 310) in the same tryptic peptide were not phosphorylated in the wild type dis2 protein. Phosphoamino acid analysis of the mutant A316 protein showed no phospho-T, suggesting that dis2 was phosphorylated at only one T residue *in vivo*. Loss of the same phosphopeptides in the C-terminal deletion mutant dis2(N-303) supported the conclusion that the T316 was *in vivo* phosphorylated.

The reason that two phosphopeptides (3 and 4) containing T316 were produced might have been incomplete tryptic digestion with the contiguous basic residues (Boyle *et al.*, 1991). Two other spots (5 and 6) were also missing in the C-deletion mutant, suggesting that they were also derived from the C-terminal position (the S326 residue).

Location of the phospho-S containing spots 1 and 2

ORGANISM	C-TERMINAL SEQUENCE	REF.
dis2	CSFQILKPAEKKQRYGYQG SSQNWHM TPPRKNKTGNSK	(1)
sds21	CSFQILKPAEKRQRVSQ SSIKESKSA TNSLKKSKNN	(1)
human PP1-α	CSFQILKPADKNKGKYGQF SGLNPGGRPITPPRNSAKAKK	(2)
rabbit PP1-α	CSFQILKPADKNKGKYGQF SGLNPGGRPITPPRNSAKAKK	(3,4)
rabbit PP1-β	CSFQILKPADKNKGKYGQL SGLNPGGRPITPPRNSAKAKK	(5)
rabbit PP1-β	CSFQILKPSEKKAKYQYGGLN SGRPV TPPRTANPPKKR	(6)
rat PP1-α	CSFQILKPADKNKGKYGQF SGLNPGGRPITPPRNSAKAKK	(7)
rat PP1-δ	CSFQILKPSEKKAKYQYGGLN SGRPV TPPRTANPPKKR	(7)
rat PP1-γ1	CSFQILKPAEKKPNATRPV TPPRGMITKQAKK	(7)
rat PP1-γ2	CSFQILKPAEKKPNATRPV TPPRVGSGLNPSIQKASNYRNNTVLYE	(7)
Dro. 96A	CSFQILKPADKRRFVYPNFG SSGRPL TPPRGANNKNKKK	(6)
Dro. 87B	CSFQILKPADKRKK	(8)
Dro. 9C	CSFQILKPSEKKAKYLYSGMN SSRPT TPQRSAPMLATNKKK	(6)
Dro. 13C	CSFQILKPVEKRKK	(9)
Aspergillus(bimG)	CSFQILKPAEKKQKYVYGAM SSGRPI TPPRKQKK	(10)
S.cere.(DIS2S1)	CSFQILKPAQKSLPRQAGGRKKK	(1)
Arabidopsis PP1	CSFQILKASEKKGNFGFGKNAGRRG TPPRKGGGKG	(11)
Arabidopsis PP1	CSFQILKPAEKKTKFMMSTKI	(11)
Arabidopsis PP1	CSFQILKPVDRRSRFF	(11)
Arabidopsis PP1-At	CSFQILKPSEKKSPFL	(12)
Trypa. PP1(4.8)	CSFIRIEPTRTLLKYFF	(13)
Trypa. PP1(5.9)	CSFVQIEPTRTLLRYFF	(13)

Fig. 8. The C-terminal cdc2 kinase consensus as a potential regulatory site. Conservation of the C-terminal cdc2 kinase consensus sequences among PP1-like phosphatases. Dro., *Drosophila*; S.cere., *S.cerevisiae*; Trypa., *Trypanosoma*. (1) Ohkura *et al.* (1989); (2) Barker *et al.* (1990); (3) Cohen (1988); (4) Cohen *et al.* (1989); (5) Berndt *et al.* (1987); (6) Dombradi *et al.* (1990); (7) Sakaki *et al.* (1990); (8) Dombradi *et al.* (1989); (9) Dombradi *et al.* (1993); (10) Doonan and Morris (1989); (11) Smith and Walker (1993); (12) Nitschke *et al.* (1992); (13) Evans and Cornelissen (1990).

and the potential role of their phosphorylation in regulating the dis2 phosphatase activity are unknown. We are currently constructing substitution and deletion mutants in order to determine the locations of unidentified phosphorylation sites. There are at least three of these sites in the dis2 protein, two of which locate in the C-terminus. In contrast to dis2, the sds21 phosphatase, although its amino acid sequence is highly similar to that of dis2, is rarely phosphorylated.

The T316 residue is in the cdc2 consensus sequence TPPR. No other cdc2 consensus (S/TPXK/R) exists in dis2. It remains to be determined whether the T316 residue is *in vivo* phosphorylated by cdc2 or a related kinase. Phosphatase responsible for dephosphorylation of T316 might be present. It was also not determined whether phosphorylation of T316 varied during the normal cell cycle. However, we showed that the T residue was highly phosphorylated in mitotically arrested *nuc2* cells, whereas it was greatly reduced in G_2-arrested *cdc2* mutant cells. To understand the *in vivo* role of T316 phosphorylation, it is essential to determine whether the degree of T316 phosphorylation peaks during normal mitosis.

We showed that the T316 residue of wild type dis2 was the major phosphorylation site *in vitro* by cdc2 kinase isolated from mitotically arrested *nda3* mutant cells. The substitution mutant S316 was nearly equally phosphorylated by cdc2 but the A316 mutant showed no phospho-T after incubation with cdc2 kinase. In comparison with protein kinase A, which barely phosphorylated the dis2

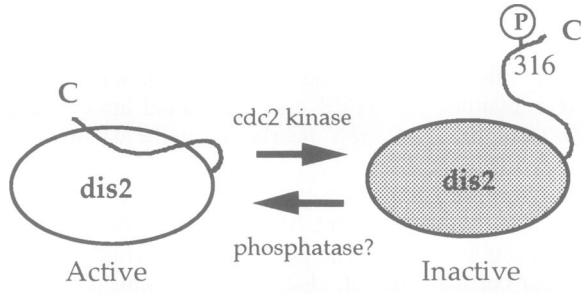

Fig. 9. Hypothetical interaction between dis2 phosphatase and cdc2 kinase. See text for explanation.

protein *in vitro*, cdc2 kinase favoured the T316 residue at least as a good *in vitro* substrate.

Evidence which supports the hypothesis that the T316 residue is a potential regulatory site by cdc2 or other kinase came from the phosphatase assay of dis2 after incubation with cdc2 kinase. The S1 phosphatase activity of dis2 (Kinoshita *et al.*, 1993; Stone *et al.*, 1993) was strikingly reduced by incubation with cdc2 kinase. However, the S1 phosphatase activity of the A316 mutant and sds21 phosphatases was not reduced by cdc2 kinase. These results strongly suggested that dis2 phosphatase was negatively regulated by phosphorylation at the T316 residue, and that cdc2 and/or other kinase which recognizes the TPPR sequence is responsible for this regulation.

The C-terminal TPPR sequence is not strictly conserved among various type 1 protein phosphatases (Figure 8). In

all the human, rabbit and rat PP1s, the TPPR sequence is conserved. However, in *Drosophila*, two of the four PP1s have the TPPR and TPPR-like TPQR sequence. In *Arabidopsis*, only one among four has it, whereas neither of the *Trypanosoma* PP1s has the TPPR. Similarly, *Saccharomyces cerevisiae* DIS2S1 does not have the TPPR, but *Aspergillus* bimG has it. The TPPR sequence thus is not essential for the phosphatase activity. This is consistent with our finding that a plasmid carrying the C-terminal deletion gene could rescue the double gene disruption mutant *dis2 sds21* (unpublished result). The TPPR sequence might be a motif for negative regulation by phosphorylation when the protein is abundant in cells or has to be inactivated within a short period of time. It might also become the signal for degradation after phosphorylation. Phosphorylation of other sites in dis2 might also cause downregulation of the activity so that such compensatory phosphorylation may enable cells to survive in the absence of phosphorylation at the T316 site. The APPR mutant expressed at wild type level did not cause severe phenotype (unpublished result).

Our results showed that the TPPR sequence conferred resistance to overproduction of dis2. The difference in overproduction phenotypes between dis2 and sds21 may be explained by the fact that dis2 phosphatase carries the TPPR sequence whereas sds21 does not. The activity of dis2 phosphatase is inhibited *in vitro* by phosphorylation of T316. However, sds21 cannot be inhibited by phosphorylation as it lacks the site for phosphorylation. Consistently, overproduction of the dis2-A316 mutation severely delayed cell division. The degree of inhibition, however, was not as strong as that of overproduced sds21. Phosphorylation at another site of dis2 may also have a negative effect on the activity.

We propose a hypothetical interaction *in vivo* between cdc2(-like) kinase and dis2 phosphatase as schematized in Figure 9. Upon entry into M phase, activated kinase counteracts dis2 phosphatase through direct phosphorylation of the T316 residue. The inhibition of dis2 phosphatase by an activated kinase would ease progression into mitosis, particularly when dis2 is abundant in cells. Upon the inactivation of cdc2 kinase in anaphase, the dis2 activity will be restored by dephosphorylation through autodephosphorylation or other phosphatase, thereby facilitating exit from mitosis and re-entry to interphase. This would explain at least partly the mutant phenotype of *dis2-11*, which can enter mitosis but cannot exit from it (Ohkura *et al.*, 1989). The mutant cells enter mitosis as the dis2 phosphatase activity is low, while they fail to exit mitosis as the dis2 phosphatase activity is not restored. The fact that H1 kinase activity is high in *dis2-11* cells at the restrictive temperature (Kinoshita *et al.*, 1991) suggests that the inactivation of cdc2 kinase might require the restoration of dis2 phosphatase activity. Conservation of the C-terminal TPPR in the termini of many PP1-like phosphatases suggests the occurrence of similar regulation in other organisms.

When sds21 or dis2-APPR phosphatase was overexpressed, cells were elongated and failed to produce the spindle. However, the H1 kinase activity was high in these cells (~3-fold increase of the activity like mitotically arrested cells; unpublished result), indicating that the activation of cdc2 kinase was not inhibited in these phosphatase-overexpressing cells. Overexpressed sds21 and dis2-APPR phosphatases might strongly dephosphorylate certain substrate proteins, phosphorylation of which may be crucial for the progression of mitotic events such as formation of the spindle.

After the submission of this paper, phosphorylation and inactivation of rabbit skeletal muscle protein phosphatase 1 by cyclin-dependent kinases were reported (Dohadwala *et al.*, 1994). Neither cdc2−cyclin A nor cdc2−cyclin B phosphorylated an active form of phosphatase 1 in which terminal threonine had been mutated to alanine. These results are consistent with our finding described in the present paper.

Materials and methods

Strains and genetical methods

The *S.pombe* strains used were previously described (Ohkura *et al.*, 1989; Kinoshita *et al.*, 1993; Stone *et al.*, 1993). The standard genetical procedures described by Gutz *et al.* (1974) were followed. The complete rich YPD (1% yeast extract, 2% bactopeptone and 2% glucose) and minimal EMM2 (Mitchison, 1970) media were employed. Transformation of *S.pombe* was done by the lithium method (Ito *et al.*, 1983).

Plasmids

A shuttle vector plasmid pDB248′ (Beach *et al.*, 1982) was used. Two *S.pombe* promoters, *ADH* (Russell and Hall, 1983) and *nmt1* (Maundrell, 1990) were ligated with the wild type $dis2^+$, $sds21^+$, mutant *dis2* and *sds21* genes. For this purpose the *Nde*I site was made for each gene at the initiation codon and used for ligation with the promoter of *ADH* and *nmt1*.

Phospholabelling of dis2 protein

The described labelling procedures (Simanis and Nurse, 1986; Shiozaki and Yanagida, 1992) were followed with modifications. EMM1 culture medium (Mitchison, 1970) was used for ^{32}P-labelling of polypeptides (50–100 μCi/ml for 3 h). For peptide mapping, each 10 mCi [^{32}P]H_3PO_4 was added to a 10 ml *S.pombe* culture (3–4×10^6 cells/ml) 12 h after the shift to thiamine-deficient medium and incubated at 33°C for 3 h. Cells were washed three times and broken with glass beads in the lysis buffer (50 mM Tris−HCl at pH 7.5, 0.4 M NaCl, 10 mM EDTA, 5 mM EGTA, 50 mM NaF, 15 mM *p*-nitrophenylphosphate, 50 mM β-glycerophosphate, 0.1 mM Na_3VO_4 and 1 mM PMSF). SDS (final concentration 1%) was added to supernatants after centrifugation at 14 000 r.p.m. (14 000 *g*) for 20 min, boiled at 100°C for 3 min and then the supernatants were diluted 10 times (SDS, final concentration 0.1%), and kept on ice for 20 min before immunoprecipitation.

Immunoprecipitation

Affinity purification of antibodies has been previously described (Stone *et al.*, 1993). Supernatants of ^{32}P-labelled extracts were incubated at 4°C for 4–6 h with anti-D2F antibodies (Stone *et al.*, 1993) bound to protein A−Sepharose in the lysis buffer containing 0.1% SDS, 0.5% NP-40 and 0.5% sodium deoxycholate. The precipitates were washed five times with the same buffer, washed once in 50 mM Tris−HCl (pH 7.5) and incubated at 4°C for 30 min in the Tris buffer containing 200 μg/ml RNase A. They were then boiled for 3 min in the sample buffer for SDS−PAGE, followed by autoradiography.

Phosphoamino acid analysis and peptide mapping

The procedures described by Boyle *et al.* (1991) and Luo *et al.* (1991) were followed. For the first dimension in peptide mapping, electrophoresis at 1 kV was done for 40 min in *n*-butanol:acetic acid:H_2O:pyridine, (50:25:900:25). The second dimension by ascending chromatography in *n*-butanol:acetic acid:H_2O:pyridine (75:15:60:50).

Phosphorylation of dis2 by cdc2 kinase in vitro.

The immunocomplex of dis2 bound to anti-D2F beads was washed three times in HB buffer [25 mM Tris−HCl (pH 7.5), 15 mM EGTA, 15 mM $MgCl_2$, 60 mM β-glycerophosphate, 15 mM *p*-nitrophenylphosphate, 2 mM Na_3VO_4, 1 mM DTT, 0.1% NP-40, 1 mM PMSF, 20 μg/ml soybean trypsin inhibitor, 20 μg/ml TPCK, 50 μg/ml leupeptin and 2 μg/ml aprotinin] containing 30 mM NaCl and incubated with cdc2 kinase in

100 µl HB buffer containing 0.1 mM ATP (50 µCi [γ-^{32}P]ATP) for 0–60 min at 30°C. The samples were washed twice in the HB buffer and boiled for 3 min in the sample buffer for SDS–PAGE. cdc2 kinase was eluted from p13^{suc1} beads as described (Kusubata *et al.*, 1992) with modification. Fission yeast β-tubulin mutant cells *nda3-311* (Hiraoka *et al.*, 1984; Moreno *et al.*, 1989) grown at 33°C were transferred to 20°C for 8 h for mitotic arrest and then disrupted in the HB buffer with glass beads (Kinoshita *et al.*, 1993). Supernatants after centrifugation at 14 000 r.p.m. (14 000 *g*) for 20 min at 4°C were incubated with p13^{suc1} beads at room temperature for 10 min, washed five times in HB at 4°C and once in 0.5 M NaCl in HB, HB and 50% ethylene glycol in HB in this order at 4°C. cdc2 kinase was eluted by 0.5 M NaCl–50% ethylene glycol in HB. The activity of H1 kinase was assayed for eluted fractions, and the peak fractions were used.

Phosphatase assay method

Immunocomplexes were obtained in 0.4 M NaCl TEG [50 mM Tris–HCl (pH 7.5), 1 mM EDTA, 1 mM 2-mercaptoethanol, 1 mM PMSF and 10% glycerol] by the method described by Kinoshita *et al.* (1993) and Stone *et al.* (1993) using anti-D2C antibodies bound to Sepharose beads. They were then suspended in 100 µl HB containing 30 mM NaCl after five washes in the same buffer and incubated in the presence of 1 mM ATP with fission yeast cdc2 kinase (described above) for 0–60 min at 30°C. cdc2-treated immunocomplexes were then washed twice in HB and then suspended in 100 µl phosphatase assay buffer (50 mM Tris–HCl at pH 7.5 containing 30 mM NaCl, 0.1 mM 2-mercaptoethanol and 0.5 mM PMSF) after three washes. The phosphatase assay (Kinoshita *et al.*, 1993; Stone *et al.*, 1993) was done using the substrate S1 (AAKAKKTPKKAKK) (Honda *et al.*, 1992) previously ^{32}P-labelled by cdc2.

Fluorescence microscopy

Staining of DNA by DAPI was previously described (Adachi and Yanagida, 1989). Immunofluorescence microscopy was done by the procedures described (Hagan and Hyams, 1988) using anti-tubulin antibodies TAT-1 (Woods *et al.*, 1989). Cells were fixed by 3.7% formaldehyde and 0.2% glutaraldehyde at 33°C for 1 h. The cells were digested with Zymolyase (0.6 mg/ml; Seikagaku Corp., Tokyo), followed by permeabilization with 1% Triton X-100.

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References

Adachi,Y. and Yanagida,M. (1989) *J. Cell Biol.*, **108**, 1195–1207.
Axton,J.M., Dombradi,V., Cohen,P.T.W. and Glover,D.M. (1990) *Cell*, **63**, 33–46.
Barker,H.M., Jones,T.A., da Cruz e Silva,E.F., Spurr,N.K., Sheer,D. and Cohen,P.T.W. (1990) *Genomics*, **7**, 159–166.
Beach,D., Piper,M. and Nurse,P. (1982) *Mol. Gen. Genet.*, **187**, 326–329.
Berndt,N., Campbell,D.G., Caudwell,F.B., Cohen,P., da Cruz e Silva,E.F., da Cruz e Silva,O.B. and Cohen,P.T.W. (1987) *FEBS Lett.*, **223**, 340–346.
Boyle,W.J., van der Geer,P. and Hunter,T. (1991) *Methods Enzymol.*, **201**, 110–149.
Cohen,P. (1989) *Annu. Rev. Biochem.*, **58**, 453–508.
Cohen,P.T.W. (1988) *FEBS Lett.*, **232**, 17–23.
Cohen,P.T.W., Schelling,D.L., da Cruz e Silva,O.B., Barker,H.M. and Cohen,P. (1989) *Biochim. Biophys. Acta*, **1008**, 125–128.
Dohadwala,M., da Cruz e Silva,E.F., Hall,F.L., Williams,R.T., Carbonard-Hall,D.A., Nairn,A.C., Greengard,P. and Berndt,N. (1994) *Proc. Natl Acad. Sci. USA*, **91**, 6408–6412.
Dombradi,V., Axton,J.M., Glover,D.M. and Cohen,P.T.W. (1989) *Eur. J. Biochem.*, **183**, 603–610.
Dombradi,V., Axton,J.M., Brewis,N.D., da Cruz e Silva,E.F., Alphey,L. and Cohen,P.T.W. (1990) *Eur. J. Biochem.*, **194**, 739–745.
Dombradi,V., Mann,D.J., Saunders,R.D.C. and Cohen,P.T.W. (1993) *Eur. J. Biochem.*, **212**, 177–183.
Doonan,J.H. and Morris,N.R. (1989) *Cell*, **57**, 987–996.
Evans,R. and Cornelissen,A.W.C.A. (1990) *Nucleic Acids Res.*, **18**, 5089–5095.
Gutz,H., Heslot,H., Leupold,U. and Lopreno,N. (1974) In King,R.C. (ed.), *Handbook of genetics 1*. Plenum Press, New York, pp. 395–446.
Hagan,I. and Hyams,J.S. (1988) *J. Cell Sci.*, **89**, 343–357.
Hanks,S.K. and Quinn,A.M. (1991) *Methods Enzymol.*, **200**, 38–81.
Hirano,T., Kinoshita,N., Morikawa,K. and Yanagida,M. (1988) *J. Cell Biol.*, **106**, 1171–1183.
Hiraoka,Y., Toda,T. and Yanagida,M. (1984) *Cell*, **39**, 349–358.
Honda,R., Ohba,Y. and Yasuda,H. (1992) *Biochem. Biophys. Res. Commun.*, **186**, 1333–1338.
Ito,H., Fukuda,Y., Murata,K. and Kimura,A. (1983) *J. Bacteriol.*, **153**, 163–168.
Kinoshita,N., Ohkura,H. and Yanagida,M. (1990) *Cell*, **63**, 405–415.
Kinoshita,N. *et al.* (1991) *Cold Spring Harbor Symp. Quant. Biol.*, **56**, 621–628.
Kinoshita,N., Yamano,H., Niwa,H., Yoshida,T. and Yanagida,M. (1993) *Genes Dev.*, **7**, 1059–1071.
Kusubata,M. *et al.* (1992) *J. Biol. Chem.*, **267**, 20937–20942.
Luo,K., Hurley,T.R. and Sefton,B.M. (1991) *Methods Enzymol.*, **201**, 149–152.
Maundrell,K. (1990) *J. Biol. Chem.*, **265**, 10857–10864.
Mayer-Jaekel,R., Ohkura,H., Gomes,R., Sunkel,C.E., Baumgartner,S., Hemmings,B.A. and Glover,D.M. (1993) *Cell*, **72**, 621–633.
Mitchison,J.M. (1970) *Methods Cell Physiol.*, **4**, 131–165.
Moreno,S., Hayles,J. and Nurse,P. (1989) *Cell*, **58**, 361–372.
Murray,A. and Hunt,T. (1993) *The Cell Cycle: An Introduction.* W.H.Freeman and Company, New York.
Nitschke,K., Fleig,U., Schell,J. and Palme,K. (1992) *EMBO J.*, **11**, 1327–1333.
Nurse,P. (1990) *Nature*, **344**, 503–508.
Ohkura,H. and Yanagida,M. (1991) *Cell*, **64**, 149–157.
Ohkura,H., Kinoshita,N., Miyatani,S., Toda,T. and Yanagida,M. (1989) *Cell*, **57**, 997–1007.
Russell,P. and Hall,B.D. (1983) *J. Biol. Chem.*, **258**, 143–149.
Sakaki,K., Shima,H., Kitagawa,Y., Irino,S., Sugimura,T. and Nagao,M. (1990) *Japan J. Cancer Res.*, **81**, 1272–1280.
Shiozaki,K. and Yanagida,M. (1992) *J. Cell Biol.*, **119**, 1023–1036.
Simanis,V. and Nurse,P. (1986) *Cell*, **45**, 261–268.
Smith,R.D. and Walker,J.C. (1993) *Plant Mol. Biol.*, **21**, 307–316.
Stone,E.M., Yamano,H., Kinoshita,N. and Yanagida,M. (1993) *Curr. Biol.*, **3**, 13–26.
Woods,A., Sherwin,T., Sasse,R., McRae,T.H., Baines,A.J. and Gull,K. (1989) *J. Cell Sci.*, **93**, 491–500.

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