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p80^{cdc25} mitotic inducer is the tyrosine phosphatase that activates p34^{cdc2} kinase in fission yeast

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We have investigated the mechanism by which fission yeast p80^{cdc25} induces mitosis. The *in vivo* active domain was localized to the C-terminal 23 kDa of p80^{cdc25}. This domain produced as a bacterial fusion protein (GST-cdc25) caused tyrosyl dephosphorylation and activation of immunoprecipitated p34^{cdc2}. Furthermore, GST-cdc25 dephosphorylated both *para*-nitrophenyl-phosphate (pNPP) and casein phosphorylated on serine *in vitro*. Reaction requirements and inhibitor sensitivities were the same as those of phosphotyrosine phosphatases (PTPases). Analysis of cdc25 C-terminal domains from a variety of species revealed a conserved motif having critical residues present at the active site of PTPases. Mutation of the cdc25 Cys480 codon, corresponding to an essential cysteine in the active site of PTPases, abolished the phosphatase activity of GST-cdc25. These data indicate that cdc25 proteins define a novel subclass of eukaryotic PTPases, and strongly argue that cdc25 proteins directly dephosphorylate and activate p34^{cdc2} kinase to induce M-phase.

Key words: cdc2/cdc25/cell cycle/mitosis/protein phosphatase

Introduction

The molecular events that bring about the onset of mitosis have been the subject of intensive investigation in recent years (reviewed by Nurse, 1990). These studies have established that the initiation of mitosis is triggered by a serine/threonine protein kinase, sometimes called M-phase promoting factor (MPF). This kinase consists of two subunits: a 34 kDa catalytic subunit, p34^{cdc2}, and a 45-60 kDa regulatory subunit. The regulatory subunit is a member of the cyclin B family of proteins, which are produced during interphase and periodically degraded as cells exit M-phase. During interphase the level of cyclin B accumulates, leading to a continuously increasing intracellular concentration of p34^{cdc2}-cyclin complex. The activity of the complex, assayed *in vitro* using histone H1 as a substrate, remains low even as the amount of p34^{cdc2}-cyclin complex increases to high levels in late G_2 phase. Activation of the complex is believed to be the critical step in the initiation of mitosis. Thus, elucidation of the mechanisms regulating the inhibition and activation of the p34^{cdc2}-cyclin complex holds the key to understanding the controls governing the initiation of mitosis.

Several important observations have been made concerning the mechanism of mitotic entry in the fission yeast *Schizosaccharomyces pombe*. Firstly, activation of the p34^{cdc2}-cyclin kinase was immediately preceded by dephosphorylation of the 15th amino acid of the p34^{cdc2} subunit, a tyrosine residue, and secondly that alteration of this residue to nonphosphorylatable phenylalanine (cdc2-F15) caused mitosis to initiate early (Gould and Nurse, 1989). These observations, together with those showing that p34^{cdc2}-cyclin immunoprecipitated from G_2 phase cells was activatable by a phosphotyrosine phosphatase (PTPase), established that the M-phase function of the p34^{cdc2} kinase is negatively regulated by phosphorylation on Tyr15 (Gould *et al.*, 1990). In animal cells, in particular Swiss 3T3 cells, it has recently been shown that the activity of p34^{cdc2} kinase is suppressed both by phosphorylation on tyrosine 15 and on threonine 14 (Norbury *et al.*, 1991).

Genetic analyses of fission yeast are being exploited to identify the proteins regulating phosphorylation of p34^{cdc2}. Of particular interest are genes involved in regulating cell size at division and, by inference, the timing of p34^{cdc2}-cyclin activation. These include the *wee1*$^+$ and *mik1*$^+$ genes which appear to operate in a co-ordinate fashion to regulate mitotic entry negatively, probably in the pathway leading to tyrosine phosphorylation of p34^{cdc2} (Russell and Nurse, 1987; Featherstone and Russell, 1991; Lundgren *et al.*, 1991; Parker *et al.*, 1991). The *wee1*$^+$ gene encodes a protein kinase which phosphorylates both serine and tyrosine residues (Featherstone and Russell, 1991), although it is not known whether it is directly responsible for p34^{cdc2} phosphorylation. The *wee1*$^+$ and *mik1*$^+$ gene functions are counteracted by the *cdc25*$^+$ rate-limiting mitotic inducer, whose product (p80^{cdc25}) increases in level during the cell cycle, reaching maximum levels in M-phase (Fantes, 1979; Russell and Nurse, 1986; Moreno *et al.*, 1990). Inactivation of *cdc25* results in a late G_2 cell cycle arrest in which p34^{cdc2} is maximally phosphorylated on Tyr15 (Gould and Nurse, 1989). These observations, together with those showing that cdc25 function is not required in cdc2-F15 mutants and in strains expressing the human T cell PTPase (Gould *et al.*, 1990), suggested that p80^{cdc25} functions in the pathway leading to tyrosyl dephosphorylation of p34^{cdc2}.

The full complement of proteins that interact with p80^{cdc25} to bring about p34^{cdc2} kinsae activation prior to mitotic onset is not known. The presumption has been that p80^{cdc25} probably does not directly dephosphorylate p34^{cdc2}, since initial comparisons of *cdc25* sequences with protein sequence databases revealed no apparent homology to any other protein, including phosphatases (Russell and Nurse, 1986). Recently, however, it was shown that human cdc25 protein produced in bacteria activated a highly purified form of pre-MPF from G_2 arrested starfish oocytes containing p34^{cdc2} and cyclin as the only major components (Strausfeld *et al.*, 1991). This suggested that the p34^{cdc2} dephosphor-

ylation reaction does not occur by activation of latent protein phosphatases, although this possibility could not be eliminated. More importantly, if left unresolved the crucial question of whether cdc25 protein has an intrinsic phosphatase activity, since both human and *Drosophila* cdc25 proteins failed to promote the dephosphorylation of substrates other than native $p34^{cdc2}$–cyclin (Kumagai and Dunphy, 1991; Strausfeld *et al.*, 1991). Two explanations were considered likely: either the substrate specificity of cdc25 protein was extremely restricted, or perhaps the $p34^{cdc2}$ or cyclin molecules participated in an active capacity with cdc25 protein to produce a phosphatase activity. In this study we provide evidence that a 207 amino acid C-terminal domain of $p80^{cdc25}$, which functions as an M-phase inducer *in vivo* and promotes the tyrosyl dephosphorylation and activation of $p34^{cdc2}$ *in vitro*, also dephosphorylates a standard phosphatase substrate (pNPP) and casein *in vitro*. Mutation of a critical cysteine residue in a region bearing limited similarity to the active site of PTPases inactivated cdc25 phosphatase activity. This proves that cdc25 protein has intrinsic phosphatase activity, and strongly supports the simplest conclusion concerning the mode of $p80^{cdc25}$ function, that it directly dephosphorylates and activates the $p34^{cdc2}$ kinase to induce the onset of mitosis.

Results

M-phase induction activity of $p80^{cdc25}$ is localized to the 23 kDa C-terminal conserved domain

To focus the biochemical analysis of $p80^{cdc25}$, we first determined which regions of the protein were required for its M-phase induction activity. Earlier studies had revealed that sequence similarities among *cdc25*$^+$ homologs from the budding yeast *Saccharomyces cerevisiae*, the fruit fly *Drosophila melanogaster* and humans were limited to a C-terminal region of ~200 amino acids (Sadhu *et al.*, 1990). These genes all rescued fission yeast *cdc25*^{ts} mutations, suggesting that the conserved C-terminal domain might be sufficient for function *in vivo*. To evaluate this, truncated versions of *cdc25*$^+$, carried on multicopy plasmids, were tested for their ability to rescue a temperature sensitive *cdc25-22* mutant. These studies showed that the C-terminal 207 amino acids, encoding a polypeptide of only M_r ~23,000, were sufficient to rescue *cdc25-22* (Figure 1A). The C-terminal 207 amino acids of $p80^{cdc25}$ correspond almost exactly to the region conserved among divergent cdc25 homologs. These results indicate that the C-terminal 23 kDa of $p80^{cdc25}$ is both necessary and sufficient to induce M-phase.

The C-terminal 23 kDa domain of $p80^{cdc25}$ activates $p34^{cdc2}$ kinase in vitro

We next determined whether the 23 kDa C-terminal region of $p80^{cdc25}$ could function as an activator of $p34^{cdc2}$–cyclin in an *in vitro* assay. We constructed a bacterial expression vector designed to produce the minimal active truncation of $p80^{cdc25}$ linked to glutathione-S-transferase (Figure 1A). GST–cdc25 protein isolated from bacteria was purified to near homogeneity by passage over a glutathione–Sepharose column (Figure 1B). Three GST–cdc25 species were consistently isolated, all of which were recognized by an antibody raised against $p80^{cdc25}$ (data not shown). The species must arise either as alternative translation products or possibly through post-translational processing of the fusion protein. As a control for many experiments, protein was isolated in an identical manner from bacteria producing glutathione-S-transferase (GST) alone (Figure 1B).

To isolate inactive $p34^{cdc2}$–cyclin complex from G_2 phase, cells bearing a temperature sensitive *cdc25-22* mutation were arrested at the restrictive temperature, cell extracts were prepared and subjected to immunoprecipitation with an anti-peptide antibody that recognizes the C terminus of the $p34^{cdc2}$ protein. Washed immunoprecipitates were incubated in the presence of either GST, GST–cdc25 or a 35 kDa fragment of human T cell PTPase produced in insect *Spodoptera frugiperda* (Sf9) cells (Zander *et al.*, 1991). The immunoprecipitates were then washed and histone H1 kinase activity was measured. As shown in Figure 2A, GST–cdc25 caused a potent activation of $p34^{cdc2}$ kinase activity (6.4 ± 0.4-fold; $n = 17$), similar to maximal activation induced by T cell PTPase (5.6 ± 0.6-fold; $n = 9$). In contrast, no activation was observed with an equal concentration of GST, even after prolonged incubation (Figure 2A). Neither bacterial preparation possessed any ability to phosphorylate histone H1 alone (data not shown). Activation of histone H1 kinase activity by GST–cdc25 was dose-dependent ($EC_{50} \approx 5$ μg/ml) with effects observed as low as 0.1 μg/ml protein (Figure 2B). Activation of $p34^{cdc2}$ kinase activity was time-dependent, and maximal after ~10 min (Figure 2C). A similar level of GST–cdc25 induced activation of histone H1 kinase activity was measured in immunoprecipitates of $p34^{cdc2}$ from extracts of *cdc25*-M51 cells arrested at the restrictive temperature (6.0 ± 0.6;$n = 4$). These results strongly suggest that in fission yeast, $p80^{cdc25}$ acts directly on G_2 phase $p34^{cdc2}$–cyclin complex to induce kinase activation and entry into mitosis, consistent with earlier studies using meiotic forms of $p34^{cdc2}$–cyclin purified from starfish oocytes (Strausfeld *et al.*, 1991) or enriched from *Xenopus* oocytes using $p13^{suc1}$ beads (Kumagai and Dunphy, 1991). Moreover, they show that the catalytic domain of $p80^{cdc25}$ is contained within its C-terminal 207 amino acids.

The C-terminal 23 kDa domain of $p80^{cdc25}$ induces vanadate-sensitive tyrosine dephosphorylation of $p34^{cdc2}$

Activation of fission yeast $p34^{cdc2}$–cyclin has been proposed to be regulated by a single dephosphorylation of residue Tyr15 in the $p34^{cdc2}$ molecule (Gould and Nurse, 1989). As all tyrosine phosphatases so far characterized are strongly inhibited by vanadate, we tested whether a vanadate-sensitive PTPase was involved in the *in vitro* activation of $p34^{cdc2}$ by GST–cdc25. Immunoprecipitates of inactive $p34^{cdc2}$–cyclin from G_2-arrested *cdc25-22* cells were treated with GST–cdc25 in the presence or absence of increasing concentrations of vanadate. As shown in Figure 2D, activation of H1 kinase activity by GST–cdc25 was completely inhibited by 1 mM vanadate ($EC_{50} = 75$ μM). These results suggested that a vanadate-sensitive tyrosyl dephosphorylation reaction was required for the activation of $p34^{cdc2}$–cyclin by GST–cdc25. This is in contrast to results showing that in the presence of 2 mM vanadate *Drosophila* cdc25 produced in bacteria induced ~5-fold activation of H1 histone kinase activity of *Xenopus* oocytes $p34^{cdc2}$–cyclin bound to $p13^{suc1}$–Sepharose (Kumagai and Dunphy, 1991).

To assess directly phosphorylation changes on the p34^{cdc2} molecule upon activation by GST−cdc25, two-dimensional phosphoamino acid analysis (2D-PAA) of p34^{cdc2} was performed before and after activation in immunoprecipitates from G_2-arrested *cdc25-22* cells that were *in vivo* labelled with [^{32}P]orthophosphate. We found that in the inactive

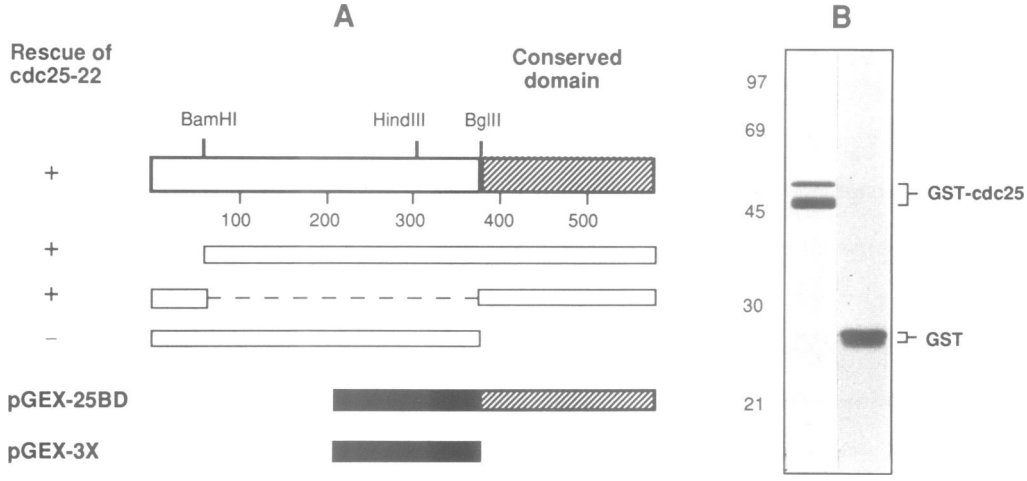

Fig. 1. Functional dissection of the *cdc25* gene and production of GST−cdc25. **A**. N-terminal and C-terminal truncations and an internal deletion of *cdc25*$^+$ were tested for their ability to rescue *cdc25-22*. Constructs having deletions upstream of the *Bgl*II site were able to rescue, whereas the C-terminal truncation was inactive. The region of the C-terminal truncation coincides with the domain conserved among cdc25 homologs (hatched). The *Bam*HI−*Bgl*II deletion also rescued a *cdc25* deletion mutation. Plasmid pGEX-25BD was constructed to produce a GST−cdc25 fusion protein (cdc25, hatched; GST, blocked in). **B**. GST−cdc25 (left lane) and GST (right) were purified by glutathione affinity chromatography, eluted with excess glutathione, and then subjected to SDS−PAGE and stained with Coomassie blue.

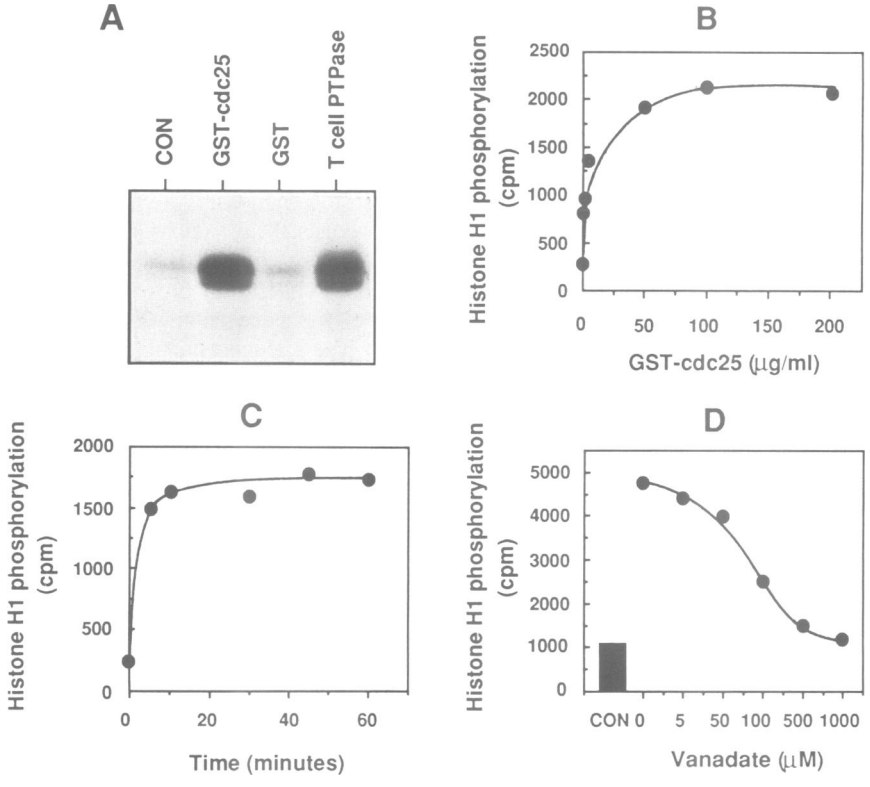

Fig. 2. Activation of p34^{cdc2} kinase by GST−cdc25. **A**. p34^{cdc2} was immunoprecipitated from extracts of late G_2 arrested *cdc25-22* cells using an antibody directed against the C terminus of p34^{cdc2}. Washed immunoprecipitates were then incubated for 1 h in phosphatase buffer at 30°C containing either no addition (CON), 100 μg/ml GST−cdc25, 100 μg/ml GST or 8 μg/ml T cell PTPase. Histone H1 kinase assays of washed immunoprecipitates were then performed. Autoradiograph of ^{32}P-labelled histone H1 separated by SDS−PAGE is shown. **B**. Effect of GST−cdc25 concentration on level of activation of p34^{cdc2} kinase. Incubations with GST−cdc25 were performed for 30 min. **C**. Time course of activation using 100 μg/ml GST−cdc25. **D**. Vanadate inhibits ability of GST−cdc25 to activate p34^{cdc2}. Immunoprecipitates of p34^{cdc2} were incubated either with no further addition (CON) or in the presence of 100 μg/ml GST−cdc25 (closed circles) and various concentrations of Na *o*-vanadate (as indicated). Immunoprecipitates were then tested for their ability to phosphorylate histone H1 as described in Materials and methods.

state, p34^{cdc2} contained only phosphotyrosine and phosphothreonine (Figure 3). This is in agreement with previous observations that fission yeast p34^{cdc2} is phosphorylated at only two sites, Tyr15 and Thr161 in late G_2 (Gould and Nurse, 1989; K.Gould, C.Norbury and P.Nurse, personal communication). p34^{cdc2} remained phosphorylated after treatment with GST−cdc25 (Figure 3), showing that GST−cdc25 cannot induce complete dephosphorylation of p34^{cdc2}. However, 2D-PAA revealed that GST−cdc25 caused almost complete dephosphorylation of p34^{cdc2} on tyrosine, while the level of phosphothreonine was unchanged (Figure 3). GST protein, on the other hand, had no effect on the level of phosphotyrosine or phosphothreonine relative to control. Tyrosyl dephosphorylation by GST−cdc25 was completely inhibited by vanadate (data not shown). These results show that GST−cdc25 activates p34^{cdc2}−cyclin by a mechanism involving tyrosine dephosphorylation of the p34^{cdc2} subunit.

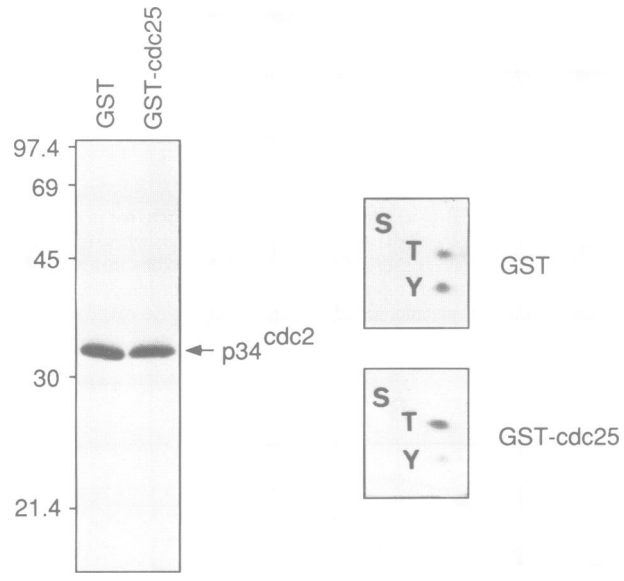

Fig. 3. Tyrosine dephosphorylation of p34^{cdc2} by GST−cdc25. p34^{cdc2} was immunoprecipitated from cdc25-22 cells grown at the restrictive temperature in the presence of [^{32}P]P_i and then treated with either GST or GST−cdc25 proteins (both at 100 μg/ml) for 30 min. p34^{cdc2} was then denatured and reprecipitated with a second anti-p34^{cdc2} antibody (4711) and analysed by SDS−PAGE (**left panel**). The autoradiograph of the fixed and dried gel was exposed to film for 24 h. The phosphoproteins corresponding to p34^{cdc2} (left panel) were excised, hydrolyzed with HCl and subjected to two-dimensional phosphoamino acid TLC as described in Materials and methods (**right panel**). S, T and Y correspond to the positions of phosphoserine, phosphothreonine and phosphotyrosine markers respectively. These data show that GST−cdc25 specifically induces tyrosyl dephosphorylation of p34^{cdc2}. Two-dimensional phosphoamino acid analysis of untreated p34^{cdc2}, or that treated with GST−cdc25 in the presence of 1 mM vanadate, appeared identical to that of the GST control. The autoradiograph was exposed for 6 days.

GST−cdc25 dephosphorylates pNPP and casein

Having established that GST−cdc25 activates p34^{cdc2}−cyclin by a mechanism involving a vanadate-sensitive tyrosyl dephosphorylation, we next turned our attention to the critical question of these studies: is p80^{cdc25} directly responsible for the tyrosyl dephosphorylation of p34^{cdc2}? In previous studies using human cdc25 protein (p54^{CDC25}) produced in bacteria, no significant dephosphorylation activity of cdc25 protein was detected using several phosphatase substrates, despite the fact that p54^{CDC25} efficiently activated p34^{cdc2}−cyclin *in vitro* via a dephosphorylation event (Strausfeld *et al.*, 1991). Similar results were obtained using *Drosophila* cdc25 (Kumagai and Dunphy, 1991). However, it was recently noticed that the conserved domain of p80^{cdc25} has a short region of sequence similarity [FLXK-(12)-IVXHCXXXXXR; the HC motif] to the active site of all previously characterized PTPases (Strausfeld *et al.*, 1991). Interestingly, cdc25 was found to have the greatest homology to the recently described vaccinia virus VH1 phosphatase, a novel enzyme shown to dephosphorylate both serine and tyrosine residues *in vitro* (Guan *et al.*, 1991; Moreno and Nurse, 1991). We have recently reported the sequence of partial cDNA clones of *cdc25* structural homologs from mouse, the clawed toad *Xenopus laevis*, the plant *Arabidopsis thaliana*, and the slime mold *Dictyostelium discoideum* (Millar *et al.*, 1991). These were cloned by PCR using a pair of highly degenerate oligonucleotide primers corresponding to two conserved regions which span the HC motif. Sequence analysis of these clones, together with previously published sequences of *cdc25* genes from fission and budding yeasts, *Drosophila* and humans, revealed that the sequence [(I/L/V)(V/I) XHCEXSXXR] was present in all the cdc25 homologs (Figure 4). An exception was one of the mouse cdc25 clones, which had a leucine in place of the histidine. Moreover, comparison of these sequences to the VH1 Tyr/Ser protein phosphatase, revealed more extensive sequence similarities both upstream and downstream of the 'HC' motif (Figure 4). In this region there is ~20% homology (identities and conservative substitutions) between VH1 phosphatase and any cdc25 protein, suggesting a structural relationship between the two phosphatases.

Reasoning that the N-terminal domain of cdc25 proteins could potentially serve to restrict substrate specificity or

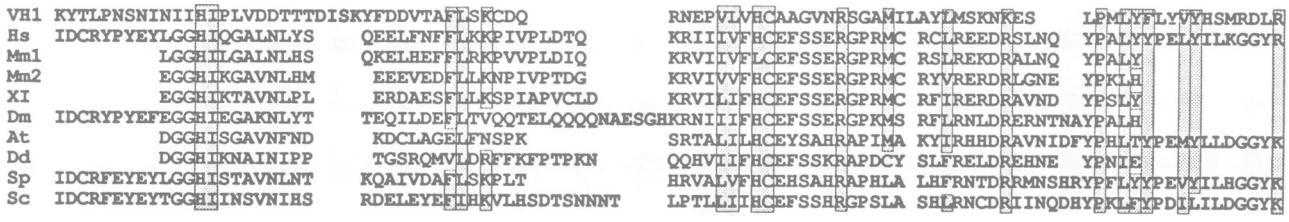

Fig. 4. Sequence homology of *cdc25*$^{+}$ gene family with VH1 Tyr/Ser phosphatase. Comparison of sequences of vaccinia virus PTPase, VH1 (Guan *et al.*, 1991) and *cdc25*$^{+}$ homologs from *Homo sapiens* (Hs), *Mus musculus* (Mm1 and Mm2), *Xenopus laevis* (Xl), *Drosophila melanogaster* (Dm), *Arabidopsis thaliana* (At), *Dictyostelium discoideum* (Dd), *Schizosaccharomyces pombe* (Sp), and *Saccharomyces cerevisiae* (Sc). Homologies (identities and conserved substitutions) found between VH1 and a majority of the cdc25 proteins are shaded.

activity of the full-length protein, we tested the ability of the GST−cdc25 protein to function in an *in vitro* phosphatase assay using PTPase substrates. No significant phosphatase activity was measured using three phosphotyrosyl substrates, including a peptide corresponding to the region of Tyr15 in p34^{cdc2}, myelin basic protein and angiotensin II (see Materials and methods). However, we found that GST−cdc25 dephosphorylated *para*-nitrophenylphosphate (pNPP) an organic compound which acts as a substrate for all classes of phosphatases (Figure 5). The dephosphorylation reaction was linear both during the time course of the assay and over a range of GST−cdc25 concentrations. Under identical conditions of dose and time, we were unable to detect any phosphatase activity associated with the GST protein alone (Figure 5). Kinetic analysis, under these conditions, revealed that GST−cdc25 dephosphorylated pNPP with a specific activity of ~110 nmol phosphate released/min/mg and with a K_m of 5.6 mM. By comparison, the specific activity of the recently reported vaccinia virus VH1 Tyr/Ser phosphatase, made as a GST−VH1 fusion protein and tested under almost identical conditions using pNPP as the substrate, was ~900 nmol/min/mg (calculated from Guan *et al.*, 1991). On a molar basis, we estimate GST−cdc25 is approximately a quarter as active as the VH1 phosphatase using pNPP.

Since of the PTPase family, cdc25 was reported to have the greatest similarity to the vaccinia VH1 Tyr/Ser phosphatase, we tested the ability of GST−cdc25 to dephosphorylate several substrates phosphorylated on threonine and serine residues (see Materials and methods). We found that GST−cdc25 failed to dephosphorylate mixed histones and glycogen phosphorylase, but remarkably was able to dephosphorylate casein that had been phosphorylated exclusively on serine residues with the catalytic subunit of cAMP-dependent protein kinase (Figure 5). This reaction was also linear both during the time course of the assay and over a range of GST−cdc25 concentrations (Figure 5). Under these conditions, GST−cdc25 dephosphorylated casein with a specific activity of ~1.3 pmol phosphate released/min/mg. Although as compared with most Thr/Ser phosphatases this activity was quite low, no activity against casein was observed for the GST protein alone, suggesting that the ability of GST−cdc25 to dephosphorylate casein is due to an authentic phosphatase activity of the cdc25 molecule (Figure 5). This conclusion is confirmed by mutational studies described below.

These results showed that GST−cdc25 harbored an intrinsic ability to cause the dephosphorylation of a limited set of substrates, including pNPP, casein and p34^{cdc2}−cyclin. This strongly argues that cdc25 product is a protein phosphatase.

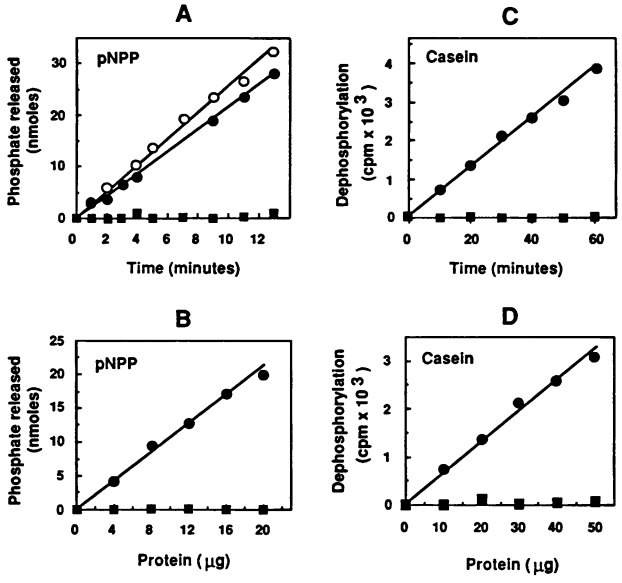

Fig. 5. Dephosphorylation of pNPP and casein by GST−cdc25. **A.** GST−cdc25 (20 μg), GST (20 μg) or T cell PTPase (0.16 μg) were incubated in the presence of 10 mM pNPP for various lengths of time (as indicated) and the release of phosphate from pNPP measured as described in Materials and methods. GST−cdc25, closed circle; GST, closed box; T cell PTPase, open circle. **B.** Various amounts of either GST−cdc25 or GST were incubated in the presence of 10 mM pNPP for 10 min and the release of phosphate from pNPP measured as described in Materials and methods. Symbols are as described in A. **C.** GST−cdc25 (50 μg) and GST (50 μg) were added to the ^{32}P-labelled casein in the appropriate phosphatase buffer at 30°C for various lengths of time (as indicated) and the release of phosphate from casein measured as described in Materials and methods. Symbols are as described in A. **D.** Various amounts of either GST−cdc25 or GST were incubated in the casein dephosphorylation assay for 10 min at 30°C and the release of phosphate measured as described in Materials and methods. Symbols are as described in A.

Reaction requirements and inhibitor sensitivities of GST−cdc25 are similar to PTPases

Our observations that the C-terminal 23 kDa of p80^{cdc25} not only dephosphorylates Tyr15 of p34^{cdc2}, but also exhibits phosphatase activity against pNPP and casein prompted us to determine whether the GST−cdc25 phosphatase exhibits functional properties similar to those of previously described tyrosine phosphatases or Ser/Thr phosphatases. We analysed the reaction requirements and inhibitor sensitivities of GST−cdc25 phosphatase, using pNPP, casein and p34^{cdc2} as substrates. In all assays the ability of GST−cdc25 to dephosphorylate pNPP and casein or activate p34^{cdc2} kinase was potently inhibited by micromolar concentrations of vanadate, a PTPase inhibitor (Table I). In contrast, GST−cdc25 phosphatase activity was unaffected by NaF, an inhibitor of Ser/Thr phosphatases, or by okadaic acid, an

Table I. Sensitivity of GST−cdc25 induced dephosphorylation to phosphatase inhibitors

Addition	GST−cdc25			T cell PTPase		
	p34^{cdc2}	pNPP	Casein	p34^{cdc2}	pNPP	Casein
o-Vanadate (500 μM)	8	0	12	0	0	n.d.
Okadaic acid (10 μM)	112	103	118	99	103	n.d.
NaF (50 mM)	n.d.	105	103	n.d.	112	n.d.
EDTA (5 mM)	100[a]	101	100[a]	100[a]	09	0

The ability of a maximum dose of either GST−cdc25 or T cell PTPase either to activate histone H1 kinse activity in immunoprecipitates of p34^{cdc2} or in parallel to dephosphorylate pNPP or casein in a standard assay was assessed in the presence of various additions as indicated. Values are described as a percentage of the activity measured without the adidtion of inhibitors. Histone H1 phosphorylation by p34^{cdc2} kinase following activation by GST−cdc25 or T cell PTPase was 2130 c.p.m. and 2342 c.p.m. respectively. Dephosphorylation of pNPP obtained with GST−cdc25 (0.412 O.D.) or T cell PTPase (0.454 O.D.) was measured at 410 nm. In the absence of other additions, GST−cdc25 released 3120 c.p.m. free phosphate from casein in a 30 min assay.
[a]All experiments involving activation of p34^{cdc2} and dephosphorylation of casein were performed in buffer containing 5 mM EDTA.
n.d., not determined.

Table II. GST−cdc25 phosphatase activity requires reducing agent, is abolished by NEM treatment and by mutation of Cys480

Addition	Substrate		
	$p34^{cdc2}$	pNPP	Casein
GST−cdc25	100	100	100
−DTT	0.4	6.8	0
+NEM	1.0	6.2	n.d.
GST−$cdc25^{S480}$	0	0	0

The ability of a maximal dose of GST−cdc25 to activate $p34^{cdc2}$ and to dephosphorylate either pNPP or casein was assessed either under standard reaction conditions or in reaction buffer lacking reducing agent or after the GST−cdc25 protein has been treated for 15 min at 4°C with 25 mM NEM and then quenched with 50 mM DTT and assayed in standard phosphatase buffer. In addition, GST−$cdc25^{S480}$ protein isolated in an identical manner was tested at the same concentration as wild type GST−cdc25 for its ability to activate $p34^{cdc2}$ and to dephosphorylate pNPP and casein. Treatment of immunoprecipitates of $p34^{cdc2}$ was for 30 min. Dephosphorylation of pNPP was performed for 13 min using 10 mM pNPP. Dephosphorylation of casein was for 30 min at 30°C. Assays for each reaction were performed exactly as described in Materials and methods. Values represent a percentage of activation obtained with wild type GST−cdc25, which was 3120 c.p.m. histone H1 phosphorylation for $p34^{cdc2}$ activation, 0.412 O.D. at 410 nm for pNPP dephosphorylation, and 3420 c.p.m free phosphate released from phosphocasein.

inhibitor of protein phosphatase type 1 and type 2A, or the presence of the calcium and magnesium ion chelating agent, EDTA (Table I). T cell PTPase exhibited the same properties against pNPP and $p34^{cdc2}$−cyclin, but was completely unable to dephosphorylate casein under any conditions (Table I). Lastly, we observed that the ability of GST−cdc25 to activate $p34^{cdc2}$ kinase was completely inhibited by 50 mM pNPP, which presumably acts as a competitive phosphatase inhibitor (data not shown). These data demonstrate that GST−cdc25 phosphatase has very similar reaction requirements to previously described PTPases and, in particular, to the T cell PTPase used as a direct comparison in this study. Furthermore, these results illustrate a striking similarity between the reaction characteristics of $p34^{cdc2}$ activation, pNPP and casein dephosphorylation by GST−cdc25, suggesting that all three reactions are performed by a similar mechanism.

Mutation of Cys480, corresponding to an essential cysteine in the active site of PTPases, abolishes the phosphatase activity of GST−cdc25

Mutation, oxidation or covalent modification of the cysteine residue in the HC motif have been shown to abolish activity of several members of the PTPase family, including the vaccinia VH1 Try/Ser phosphatase (Tonks *et al.*, 1988; Streuli *et al.*, 1990; Guan *et al.*, 1991; Guan and Dixon, 1991). This is consistent with evidence that PTPases require the presence of reducing agents for activity, presumably by maintaining the sulfhydryl moiety of the cysteine residue in a reduced state. The sequence similarity between cdc25 and the PTPases in this region prompted us to test the involvement of a sensitive sulfhydryl group in the activation and dephosphorylation of $p34^{cdc2}$ and *in vitro* phosphatase activity of GST−cdc25. We observed that like VH1 phosphatase and PTPases, GST−cdc25 demonstrated an absolute requirement for a reducing agent such as dithiothreitol to induce kinase activation of $p34^{cdc2}$ and to dephosphorylate pNPP and casein (Table II). The critical cysteine residue in the active site of PTPases is readily accessible to chemical modification by the alkylating agent *N*-ethylmaleimide (NEM), and this treatment abolishes catalytic activity of these enzymes (Tonks *et al.*, 1988). We observed that treatment of GST−cdc25 with NEM completely abolished its ability to activate $p34^{cdc2}$ and to dephosphorylate pNPP (Table II). As a control we verified that treatment of the $p34^{cdc2}$ immunocomplex with NEM did not effect subsequent activation of the kinase by either cdc25 or T cell PTPase (data not shown). Together these data strongly suggest that an active site sulfhydryl group resides in the GST−cdc25 protein, consistent with the proposal that HC motif found in cdc25 phosphatases and other PTPases are functionally equivalent active sites.

To confirm that the cysteine present in the cdc25 HC motif was essential for phosphatase activity, we performed site-directed *in vitro* mutagenesis to change cdc25 codon Cys480 to Ser480 (see Materials and methods). GST−$cdc25^{Ser480}$ was produced in bacteria in an identical manner to wild type GST−cdc25 and tested in parallel for its ability to activate $p34^{cdc2}$−cyclin and to dephosphorylate pNPP and casein. We found that the GST−$cdc25^{Ser480}$ was completely inactive as a protein phosphatase in all three assays (Table II). Together these results unequivocally demonstrate that Tyr/Ser phosphatase activity is an intrinsic property of GST−cdc25 and strongly suggests that the homology observed between cdc25, VH1 phosphatase and PTPases is of functional significance. We conclude that $p80^{cdc25}$ is the protein phosphatase that directly dephosphorylates and activates $p34^{cdc2}$−cyclin to induce the onset of mitosis.

Discussion

In this study we sought to establish conclusively if protein phosphatase activity was a fundamental feature of cdc25 function. We reasoned that this analysis would be facilitated by delineation of the regions of *S.pombe* $p80^{cdc25}$ which are essential and sufficient to promote the dephosphorylation of $p34^{cdc2}$ both *in vivo* and *in vitro*, thus allowing us to focus our efforts on the minimal active domain of $p80^{cdc25}$. With these goals in mind we showed that the C-terminal 23 kDa region of $p80^{cdc25}$, comprising ~29% of the full-length protein, was active both as a mitotic inducer *in vivo* and as an inducer of $p34^{cdc2}$ tyrosyl dephosphorylation and kinase activation *in vitro*. Notably, this region of $p80^{cdc25}$ corresponded exactly with the conserved domain of cdc25 homologs identified in earlier studies (Sadhu *et al.*, 1990).

Having defined the minimal active domain of $p80^{cdc25}$, we then sought to establish whether this domain, purified as a GST fusion protein from bacteria, would act as a phosphatase *in vitro* using typical substrates. We found that GST−cdc25 was effective at dephosphorylating pNPP, a universal substrate for all types of phosphatases, including PTPases. GST−cdc25 had a turnover rate of ~5.3 mol of pNPP/min/mol of GST−cdc25. This compares well to the activity of the VH1 Tyr/Ser phosphatase, which under similar conditions was recently shown to dephosphorylate pNPP at a rate of 20 mol of pNPP/min/mol of VH1 (calculated from Guan *et al.*, 1991). Additionally, we found that GST−cdc25 dephosphorylated casein that had been exclusively phosphorylated on Ser residues using cAMP-dependent protein kinase. These reactions were inhibited by

vanadate and required a reducing agent. Our observation that the phosphatase activity of GST−cdc25 was abolished by a single missense mutation in the C-terminal domain of cdc25 conclusively establishes that the C-terminal catalytic domain of p80^{cdc25} harbors an intrinsic phosphatase activity. Therefore there is no need to propose that cdc25 protein dephosphorylates p34^{cdc2}−cyclin either by activating latent phosphatases or by requiring the active participation of p34^{cdc2} or cyclin. Since C-terminal conserved domain of p80^{cdc25} is necessary for dephosphorylation and activation of p34^{cdc2}−cyclin *in vivo*, and is sufficient to cause the dephosphorylation and activation of immunoprecipitated p34^{cdc2}−cyclin *in vitro*, we conclude that the *in vivo* biochemical role of p80^{cdc25} is to carry out the dephosphorylation of p34^{cdc2} on Tyr15 directly.

It is remarkable that using immunoprecipitated p34^{cdc2}−cyclin, pNPP and casein as substrates, GST−cdc25 exhibits the same reaction requirements and is sensitive to the same inhibitors as previously described PTPases. This suggests that there may be a functional and structural relationship between cdc25 phosphatases and the family of PTPases. In support of this idea, a region of limited sequence homology between cdc25 homologs and PTPases has been noted (Strausfeld *et al.*, 1991; Moreno and Nurse, 1991; and this report). By far the most striking similarity occurs in the region of a conserved cysteine, the HC motif, which is believed to form a critical part of the active site of PTPases. Indeed, there is now physical evidence indicating that PTPases function by creating an unstable thiophosphate bond at this cysteine residue (Guan and Dixon, 1991). It is tempting to speculate, therefore, that this homology is of functional significance, and that the equivalent cysteine of p80^{cdc25}, Cys480, is located in the active site. That a cysteine residue is involved in catalysis by GST−cdc25 is supported by our observations that GST−cdc25, like the PTPases, has a functional requirement for reducing agents and that its activity is abolished by *N*-ethylmaleimide (NEM), a compound that covalently modifies sulfhydryl groups of exposed cysteine residues. Furthermore, we have found that a mutation changing codon Cys480 to Ser480 completely inactivates the phosphatase activity of GST−cdc25. The simplest conclusions are that Cys480 is either directly required for catalysis or for protein folding. It is important to caution that there are extensive sequence identities found among the disparate types of PTPases which are not present in cdc25 homologs. In fact, amino acid residues immediately downstream of the conserved cysteine of p80^{cdc25} (His482, Ser483) have been shown to inactivate LAR PTPase when introduced at the equivalent positions by mutagenesis (Streuli *et al.*, 1990). Thus direct biochemical proof will be required to establish conclusively that Cys480 in p80^{cdc25} plays the same functional role as the essential cysteine at the active site of PTPases.

It is interesting that GST−cdc25 is an effective phosphatase against pNPP, in view of the fact that earlier attempts using full-length *Drosophila* or human cdc25 proteins failed to detect any activity using pNPP, despite the fact that these enzymes were quite effective in dephosphorylating and activating p34^{cdc2}−cyclin (Kumagai and Dunphy, 1991; Strausfeld *et al.*, 1991). One possible explanation is that the N-terminal region of p80^{cdc25} negatively regulates the phosphatase activity of the catalytic domain. Alternatively, the substrate specificity of the C-terminal fragment of p80^{cdc25} present in the GST−cdc25 protein may be either altered or less discriminatory than full-length cdc25 proteins. Interestingly, there is a precedence for both types of phenomena for the T cell PTPase used in this study. The truncated version of T cell PTPase exhibits a dramatically different specific activity and substrate specificity from the full-length enzyme (Zander *et al.*, 1991). For example, the N-terminal truncated form of T cell PTPase displayed an ~30-fold higher activity against RCM lysozyme than did full-length T cell PTPase (Zander *et al.*, 1991). Experiments to create and test the *in vivo* and *in vitro* activity of a nested set of N-terminal truncations are under way to test these possibilities for the p80^{cdc25} phosphatase.

Our observation that the maximum activation of immunoprecipitated p34^{cdc2}−cyclin achieved by GST−cdc25 was similar in magnitude to that induced by a human T cell PTPase is consistent with a single mode of activation, namely dephosphorylation of Tyr15. However, several lines of evidence suggest that in animal cells cdc25 induces at least two post-translational modifications of the p34^{cdc2} molecule. Firstly, cdc25 protein appears to induce a vanadate-insensitive modification of *Xenopus* and starfish p34^{cdc2}, as analysed by mobility shift in SDS−PAGE, which is not due to tyrosine dephosphorylation (Kumagai and Dunphy, 1991; Strausfeld *et al.*, 1991). Secondly, in contrast to our findings in fission yeast, tyrosine dephosphorylation of mouse p34^{cdc2} *in vitro* was not sufficient to activate p34^{cdc2} histone kinase activity (Morla *et al.*, 1989; J.B.A.Millar and P.Russell, unpublished data). It has recently been demonstrated that in higher eukaryotic cells, p34^{cdc2} is doubly phosphorylated on both residues Tyr15 and Thr14 in the presumed ATP binding site of the kinase, and both are dephosphorylated on entry into mitosis (Norbury *et al.*, 1991). Since phosphorylation of Thr14 has not been detected in fission yeast, the presence of an additional inhibitory phosphate in the p34^{cdc2} molecule in higher eukaryotes that is dephosphorylated by cdc25 protein may explain these discrepancies. Although the Ser/Thr phosphatases have been classified separately from Tyr phosphatases both structurally and functionally, it has recently been reported that the vaccinia virus VH1 phosphatase is capable of dephosphorylating both Tyr and Ser residues *in vitro*, even though it bears the primary sequence hallmarks and reaction requirements of PTPases (Guan *et al.*, 1991). It is highly interesting that among PTPases, cdc25 has been reported to have the highest degree of sequence similarity to the vaccinia virus VH1 Tyr/Ser phosphatase. Our results showing that GST−cdc25 can also act as a Tyr/Ser phosphatase indicate that this degree of homology is of functional significance. This leads to the prediction that in higher eukaryotes cdc25 proteins function as a Thr/Tyr phosphatase to induce directly both dephosphorylation events on the p34^{cdc2} molecule, and thus is solely responsible for the activation of p34^{cdc2}−cyclin at mitosis. Experiments to test this prediction directly are under way.

Materials and methods

Functional dissection of cdc25 gene; production and purification of bacterially expressed truncated S.pombe cdc25

Plasmid p25SS contains a 5.3 kb genomic clone of *cdc25* and surrounding sequences cloned as an *Sst*I−*Sal*I fragment into pIRT2U, a pUC118 derivative having *ura4*$^+$ and *ars1*. Clones were transformed into a *cdc25-22* strain and tested for their ability to rescue the cell division arrest phenotype at 35.5°C. Plasmid pGX25-BD was constructed by cloning a

0.7 kb *Bgl*II–*Eco*RI fragment containing the C-terminal coding sequences of *cdc25* in pGEX-3X (Pharmacia). The *Bgl*II site is the natural one found in the *cdc25* coding sequences. It was subsequently filled in with Klenow DNA polymerase to produce an in-frame fusion with GST. The *Eco*RI site was created immediately downstream of the translation termination codon by *in vitro* mutagenesis. DH5 *Escherichia coli* transformed with pGEX25-BD were grown in L-broth at 37°C for an optical density of 0.4 and induced at 25°C for an additional 3 h in the presence of 0.4 mM IPTG. Cells were harvested and lysed at 4°C with 1 mg/ml lysozyme for 30 min in TEN buffer (0.5 mM EDTA, 10 mM DTT, 0.3 M NaCl, 50 mM Tris–HCl, pH 8.0) containing 0.2% NP-40 for 10 min. The slurry was then adjusted to 0.75 M NaCl, 6 mM $MgCl_2$, 10 mM DTT and 2 mg/ml DNAse I for an additional hour on ice, sonicated for 30 s and centrifuged for 30 min at 12 500 r.p.m. The supernatant was passed over a glutathione–Sepharose column pre-equilibrated with 10 mM DTT, 10 mM HEPES, pH 8.0, extensively washed with buffer containing 500 mM NaCl, 10 mM DTT, 10 mM HEPES, pH 8.0, and then eluted in the same buffer containing 10 mM glutathione. The eluate was dialysed against 10 mM DTT, 25 mM MOPS, pH 7.2 (unless otherwise stated), and concentrated to a final protein concentration of 1–5 mg/ml. For assays performed in the absence of reducing agent, the protein was dialysed into 25 mM MOPS, pH 7.2.

Immunoprecipitation

Immunoprecipitations were performed essentially as previously described (Moreno *et al.*, 1991). *cdc25-22* or *cdc25*-M51 strains were grown in YES medium at 25°C to early log phase (OD_{600} = 0.2–0.6) and then grown for a further 4.25 h at 35.5°C. Between 1×10^8 and 1×10^9 cells were resuspended in homogenization buffer (HB; 60 mM β-glycerophosphate, 15 mM pNPP, 15 mM EGTA, 15 mM $MgCl_2$, 1 mM DTT, 0.2 mM *o*-vanadate, 1% NP-40, 0.5 mM PMSF, 20 μg/ml leupeptin, 20 μg/ml aprotinin, 25 mM MOPS, pH 7.2) and soluble proteins extracted by vortexing with glass beads for 5 min at 4°C. The solution was clarified by centrifugation for 15 min at 100 000 *g* and antibody precipitations carried out in 1 ml by constant mixing for 3 h at 4°C. Antiserum PN24 (Gould *et al.*, 1990), raised against a peptide corresponding to the C terminus of *S.pombe* $p34^{cdc2}$, was used in all experiments. Antibodies were precipitated with protein A–Sepharose beads. Beads were washed three times with HB and three times with phosphatase buffer (PB; 5 mM EDTA, 2 mM spermidine, 2 mM DTT, 25 mM MOPS, pH 7.2) and resuspended in PB containing 0.1% BSA and various additions at 30°C as indicated in the experiments. At each stage beads were precipitated by centrifugation at 14 000 *g* for 30 s.

Histone H1 kinase assay

Immunoprecipitates were washed twice in kinase assay buffer (10 mM $MgCl_2$, 1 mM DTT, 25 mM MOPS, pH 7.2) and histone H1 assays performed essentially as previously described (Gould *et al.*, 1990). Phosphorylated histone H1 was separated and identified using 15% SDS–PAGE and autoradiography of fixed, stained and dried gels. The region of the gel corresponding to histone H1 was excised and the incorporation of radioactivity determined by Cerenkov counting in a Beckman β-counter.

Labelling with $^{32}P_i$ and two-dimensional phosphoaminoacid analysis

Exponentially growing cells (4×10^6/ml) were incubated in EMM medium in the presence of 1 mCi/ml $^{32}P_i$ for 4 h as previously described (Featherstone and Russell, 1991). After this time cells were collected and washed twice in ice-cold stop buffer (150 mM NaCl, 50 mM NaF, 1 mM NaN_3, 10 mM EDTA) and then homogenized and subjected to immunoprecipitation exactly as described above. After dephosphorylation reactions, $p34^{cdc2}$ was removed from the beads by boiling in 250 μl SDS lysis buffer (0.5% SDS, 1 mm DTT, 1 mM EDTA, 50 mM NaF, 0.2 mM Na *o*-vanadate, 1 mM PMSF, 20 μg/ml leupeptin, 20 μg/ml aprotonin, 10 mM Na_2PO_4, 25 mM MOPS, pH 7.2), and reprecipitated in RIPA buffer using an anti-$p34^{cdc2}$ antibody (Gould *et al.*, 1989). This 'reprecipitation' procedure reduced the background observed from non-specific phosphoserine containing proteins.

Phosphoproteins were extracted from fixed and dried SDS–PAGE gels after autoradiography. Gel slices containing the phosphoproteins were hydrolyzed in constant boiling HCl and 2D-PAA analysis performed as previously described (Cooper *et al.*, 1983). TLC plates were autoradiographed for 5–7 days.

Phosphatase assays

pNPP assays were performed in 200 μl of buffer containing 50 mM β-mercaptoethanol, 0.1 mg/ml BSA, 10 mM pNPP, 25 mM HEPES, pH 7.4, and incubated at 30°C for 10 min unless otherwise specified. Reactions were terminated by the addition of 800 μl 0.2 M NaOH, and the absorbance measured at 410 nm in a Hitachi U2000 spectrophotometer. Release of pNPP was calculated using a millimolar extinction coefficient of 17.8 (Tonks *et al.*, 1988).

Casein (Sigma, C4765) was phosphorylated as described by McGowan and Cohen (1988) using cAMP-dependent protein kinase purified from bovine heart according to the method of Beavo *et al.* (1974). The kinase reaction (10 mg/ml casein, 50 mM Tris–HCl, pH 7.0, 0.1 mM EGTA, 1 mM DTT, 10 mM $MgCl_2$, 0.1 mM [γ^{32}P]ATP at ~1000 c.p.m./pmol, 2 mU/ml cAMP-dependent protein kinase) was incubated for 4 h at 30°C. Unincorporated [γ-^{32}P]ATP was removed by gel filtration through a 15×1 cm^2 Sephadex G-50 Superfine column in 50 mM Tris–HCl, pH 7.4, 0.1 mM EGTA, 1 mM DTT and 0.01% NP-40. 2D-PAA confirmed that 100% of incorporated ^{32}P was in phosphoserine (data not shown). The dephosphorylation assay was performed at 30°C in a 30 μl volume containing 50 mM imidazole, pH 7.2, 5 mM EDTA, 50 mM β-mercaptoethanol and 10 μg of phosphorylated casein together with additions as described in the text. The reaction was terminated by the addition of 100 μl of 25% TCA and 100 μg BSA carrier. TCA unprecipitable ^{32}P was measured by scintillation counting. Release of free P_i was independently confirmed by TLC (data not shown).

Dephosphorylation assays were also carried out using the following substrates: bovine myelin basic protein (Sigma), angiotensin II (Calbiochem) and a synthetic peptide corresponding to the Tyr15 region of fission yeast $p34^{cdc2}$, all phosphorylated with $p60^{src}$ purified from Sf9 cells infected with recombinant baculovirus; mixed histones (Sigma) phosphorylated with cAMP-dependent protein kinase purified from bovine heart; and glycogen phosphorylase phosphorylated with phosphorylase kinase as described by Cohen *et al.* (1988). In these assays significant levels of dephosphorylation using GST–cdc25 were not detected.

SDS gel electrophoresis

Slab gel electrophoresis was performed using a 6–15% acrylamide and 0.1% SDS gel. After electrophoresis, the slabs were fixed in 40% methanol, 7.5% glacial acetic acid and dried down onto Whatman 3 mm filter paper for autoradiography with Fuji X-ray film (Fuji Photo Co. Ltd, Japan). For most purposes, dried gels were exposed to films for 1–7 days.

Mutagenesis

The oligonucleotide 5′-CTATGTTCACTATGAAAAAC-3′ was used to mutate cdc25 codon Cys480 to Ser480. Plasmid p25SS, having a 5 kb fragment containing *cdc25* in a pUC118 derivative, was used for *in vitro* mutagenesis by the method of Kunkel (1985). Two independent mutations were confirmed by DNA sequence analysis. A DNA fragment containing the mutation was subsequently cloned into pGX25-BD to produce pGX25BD-S480. Cells transformed with these two plasmids produced equal levels of GST–cdc25.

Materials

T cell PTPase (specific activity 4700 U/mg against myelin basic protein) was kindly provided by Dr Fisher and co-workers (Zander *et al.*, 1991). The GST expression vector pGEX-3X and prepacked glutathione–Sepharose columns were purchased from Pharmacia LKB Biotechnologies. [^{32}P]P_i (~285 Ci/mg phosphate) and [γ-^{32}P]ATP (4500 Ci/mmol) were purchased from ICN Radiochemicals. Histone H1, aprotonin, leupeptin, DTT, PMSF and protein A–Sepharose beads were from Boehringer Mannheim. pNPP was bought from Sigma. All other reagents were of the highest grade available.

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