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cdc25^{+} Encodes a Protein Phosphatase that Dephosphorylates p34^{cdc2}

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To determine how the human cdc25 gene product acts to regulate p34^{cdc2} at the G2 to M transition, we have overproduced the full-length protein (cdc25Hs) as well as several deletion mutants in bacteria as glutathione-S-transferase fusion proteins. The wild-type cdc25Hs gene product was synthesized as an 80-kDa fusion protein (p80$^{GST\text{-}cdc25}$) and was judged to be functional by several criteria: recombinant p80$^{GST\text{-}cdc25}$ induced meiotic maturation of *Xenopus* oocytes in the presence of cycloheximide; p80$^{GST\text{-}cdc25}$ activated histone H1 kinase activity upon addition to extracts prepared from *Xenopus* oocytes; p80$^{GST\text{-}cdc25}$ activated p34^{cdc2}/cyclin B complexes (prematuration promoting factor) in immune complex kinase assays performed in vitro; p80$^{GST\text{-}cdc25}$ stimulated the tyrosine dephosphorylation of p34^{cdc2}/cyclin complexes isolated from *Xenopus* oocyte extracts as well as from overproducing insect cells; and p80$^{GST\text{-}cdc25}$ hydrolyzed *p*-nitrophenylphosphate. In addition, deletion analysis defined a functional domain residing within the carboxy-terminus of the cdc25Hs protein. Taken together, these results suggest that the cdc25Hs protein is itself a phosphatase and that it may function directly in the tyrosine dephosphorylation and activation of p34^{cdc2} at the G2 to M transition.

INTRODUCTION

The induction of DNA synthesis (S phase) and mitosis (M phase) in eukaryotic cells requires a serine/threonine protein kinase, known as p34^{cdc2} (Reed, 1980; Nurse and Bissett, 1981; Simanis and Nurse, 1986; Blow and Nurse, 1990; Furukawa *et al.*, 1990). p34^{cdc2} is the human homolog of the cdc2 gene product in the fission yeast, *Schizosaccharomyces pombe*, and of the CDC28 gene product in the budding yeast, *Saccharomyces cerevisiae* (Beach *et al.*, 1982; Lee and Nurse, 1987). p34^{cdc2} is ubiquitous in eukaryotes (for review see Nurse, 1990). Although the amount of p34^{cdc2} remains constant throughout the cell cycle, its activity oscillates dramatically (Draetta and Beach, 1988; Labbe *et al.*, 1988; Booher *et al.*, 1989; Gautier *et al.*, 1989; Moreno *et al.*, 1989). The regulation of p34^{cdc2} involves changes in both its state of phosphorylation and its association with other cell cycle regulatory proteins.

In *S. pombe*, two proteins, p13^{suc1}, the product of the suc1^{+} gene (Brizuela *et al.*, 1987) and p56^{cdc13}, the product of the cdc13^{+} gene (Moreno *et al.*, 1989), physically associate with p34^{cdc2}. Data from *S. pombe* suggest that p13^{suc1} may be involved in inactivating p34^{cdc2} and facilitating exit from mitosis (Moreno *et al.*, 1989). In *Xenopus*, however, p13^{suc1} has been shown to inhibit activation and tyrosine dephosphorylation of p34^{cdc2} (Dunphy and Newport, 1989). p56^{cdc13} is a B-type cyclin. Cyclins fall into two classes on the basis of sequence differences as well as differences in the timing of their accumulation and degradation throughout the cell cycle (Swenson *et al.*, 1986; Pines and Hunter, 1989, 1990; Westendorf *et al.*, 1989; Minshull *et al.*, 1990). It is a B-type cyclin that along with p34^{cdc2} comprises maturation promoting factor (MPF) (Dunphy *et al.*, 1988; Gautier *et al.*, 1988, 1990; Lohka *et al.*, 1988; Draetta *et al.*, 1989). Association with the cyclins is necessary for the activation of p34^{cdc2} kinase activity and for the entry of cells into mitosis (Minshull *et al.*, 1989; Murray and Kirschner, 1989). Likewise, proteolytic degradation of cyclin is required for inactivation of p34^{cdc2} kinase ac-

tivity and exit of cells from mitosis (Murray *et al.*, 1989; Roy *et al.*, 1991).

The following model for the regulation of $p34^{cdc2}$ has emerged through the work of several laboratories over the past few years. Early in the cell cycle (G1), $p34^{cdc2}$ exists in monomeric form and serine phosphorylation is detected (Draetta and Beach, 1988; Krek and Nigg, 1991). As cells progress from the G1 to the G2/M phases of the cell cycle, cyclins accumulate and bind to $p34^{cdc2}$ (Draetta *et al.*, 1989; Minshull *et al.*, 1990). Cyclin binding facilitates the phosphorylation of $p34^{cdc2}$ (Solomon *et al.*, 1990; Meijer *et al.*, 1991; Parker *et al.*, 1991). Both tyrosine (Tyr 15) and threonine (Thr 14 and presumably Thr 161) residues are phosphorylated (Draetta *et al.*, 1988; Gould and Nurse, 1989; Lewin, 1990; Krek and Nigg, 1991). Just before entry into mitosis, $p34^{cdc2}$ is dephosphorylated on Thr 14 and Tyr 15 and its kinase activity is activated (Dunphy and Newport, 1989; Gautier *et al.*, 1989; Gould and Nurse, 1989, 1990; Morla *et al.*, 1989; Krek and Nigg, 1991). In fission yeast, the substitution of phenylalanine for tyrosine (at position 15) advances cells prematurely into mitosis, indicating that the dephosphorylation of tyrosine 15 is a key step in the activation of $p34^{cdc2}$ function (Gould and Nurse, 1989).

Although the kinases and phosphatases that are responsible for the cell cycle-dependent phosphorylations/dephosphorylations of $p34^{cdc2}$ have not yet been identified, two mitotic control genes have been identified in *S. pombe* that are thought to regulate $p34^{cdc2}$ function by altering its state of phosphorylation. These genes include $cdc25^+$ and $wee1^+$ (Russell and Nurse, 1986, 1987). Genetic evidence suggests that $cdc25^+$ encodes an activator of $p34^{cdc2}$, whereas $wee1^+$ encodes a negative regulator. The products of both the $wee1^+$ and the $cdc25^+$ genes act antagonistically to regulate $p34^{cdc2}$ and thereby precisely control the timing of entry into mitosis (Russell and Nurse, 1987).

To decipher the temporal and molecular regulation of $p34^{cdc2}$ as well as to identify the kinases and phosphatases that modify $p34^{cdc2}$ throughout the cell cycle, we overproduced $p34^{cdc2}$ as well as several of its regulators using a baculoviral expression system (Parker *et al.*, 1991). We made the surprising observation that $p34^{cdc2}$ becomes phosphorylated on tyrosine 15 when coproduced with $p107^{wee1}$ in insect cells, suggesting a role for $p107^{wee1}$ in the regulation of $p34^{cdc2}$ by tyrosine phosphorylation. In support of this, genetic data in fission yeast have demonstrated that $wee1^+$ acts redundantly with $mik1^+$ in regulating the tyrosine phosphorylation of $p34^{cdc2}$ (Lundgren *et al.*, 1991).

If $wee1^+$ is involved in regulating the tyrosine phosphorylation of $p34^{cdc2}$ and if $cdc25^+$ acts in opposition to $p107^{wee1}$, then one might predict that $cdc25^+$ is involved in the tyrosine-dephosphorylation of $p34^{cdc2}$. Several lines of evidence are supportive of this model: 1) in yeast mutants containing a temperature-sensitive allele of $cdc25^+$, the tyrosine phosphorylated/inactive form of $p34^{cdc2}$ accumulates at the nonpermissive temperature. Upon shift to the permissive temperature, $p34^{cdc2}$ becomes dephosphorylated on tyrosine coincident with its activation (Gould and Nurse, 1989); 2) the phosphotyrosine-specific protein phosphatase from human T cells is able to functionally complement $cdc25^+$ in fission yeast (Gould *et al.*, 1990); 3) bacterially produced *string* protein (*Drosophila* homolog of $cdc25^+$) dephosphorylates and activates *Xenopus* $p34^{cdc2}$ in whole cell extracts as well as in $p13^{suc1}$ precipitates (Kumagai and Dunphy, 1991); and 4) bacterially produced human cdc25 protein dephosphorylates and activates $p34^{cdc2}$ purified from starfish oocytes (Strausfeld *et al.*, 1991). In each of these assay systems it can not be rigorously excluded that the cdc25 protein actually regulates the activity of a phosphatase rather than possessing intrinsic protein phosphatase activity.

To characterize the biochemical activity associated with the $cdc25^+$ protein and to elucidate the contribution made by $cdc25^+$ to the regulation of $p34^{cdc2}$, we have overproduced full length as well as deletion mutants of the cdc25 protein (human homolog) in bacteria. Recombinant cdc25Hs protein was assayed functionally by microinjection into *Xenopus* oocytes as well as in vitro in extracts prepared from *Xenopus* oocytes. cdc25Hs protein induced meiotic maturation of *Xenopus* oocytes in the presence of cycloheximide. In addition, cdc25Hs protein induced the activation of $p34^{cdc2}$ in vitro on incubation in extracts prepared from *Xenopus* oocytes. Sodium vanadate (an inhibitor of phosphotyrosine-specific protein phosphatases) ablated the effects of recombinant cdc25Hs protein. Incubation of precipitates of $p34^{cdc2}$/cyclin complexes (from *Xenopus* extracts or from insect cells infected with recombinant baculoviruses) with recombinant cdc25Hs protein induced the tyrosine dephosphorylation of $p34^{cdc2}$ in vitro. Furthermore, cdc25Hs protein hydrolyzed *p*-nitrophenylphosphate (PNPP), a commonly used substrate for assaying tyrosine-specific protein phosphatase activity. Finally, mutational analysis indicated that residues 258–435, located within the C terminus of the cdc25 protein, were critical for cdc25Hs function in these assays.

MATERIALS AND METHODS

Bacterial Expression

pBSK1 (Sadhu *et al.*, 1990) was digested with *Bam*H1 and *Xho* I. The 2-kb insert encoding the human homolog (cdc25Hs) of the *S. pombe* $cdc25^+$ gene was isolated and ligated into the *Bam*H1/*Xho* 1 site of pGC52 to generate pGC52(cdc25Hs). pGC52(cdc25Hs) was incompletely digested with Acc1 and then ligated to two partially overlapping oligonucleotides 5′ CTGGATCCATGT 3′ and 5′ AGACATGGATCC 3′ to generate pGC52(cdc25Hs)-2. This step places a *Bam*H1 site upstream of the initiation codon of cdc25Hs. pGC52(cdc25Hs)-2 was digested with *Bam*H1 and the 1.9-kb insert containing cdc25Hs was isolated and ligated into the *Bam*H1 site of pGEX-2T (Pharmacia, Piscataway, NJ) to generate pML25. JM109 cells were transformed with pGEX-2T or pML25 and protein expression was induced with

0.5 mM isopropyl β-D-thiogalactopyranoside. One-liter cultures were routinely induced at an OD_{595} of 0.4–0.8 for 3 h at 37°C and pelleted at 3800 × *g* for 15 min. The pellet was resuspended in 50 ml of phosphate-buffered saline (PBS) and the suspension was repelleted at 3200 × *g* for 15 min. Bacterial pellets were stored at −80°C.

Mutants

Mutant constructs were created from pML25 as follows. N459: pML25 was partially digested with *Pvu* II and the 6.8-kb linear form was isolated and digested to completion with *Sma* I. The 6.3-kb fragment containing the majority of the 5′ cdc25Hs coding sequence was isolated and recircularized by blunt-end ligation to create the plasmid pML25-N459. This construct removes the coding region for the C-terminal 14 amino acids of cdc25Hs and replaces them with four unrelated amino acids. N435: pML25 was partially digested with *Pst* I and the linear form was isolated and cut to completion with *Nsi* I. The 6.2-kb product was isolated and recircularized creating the plasmid pML25-N435, which codes for the cdc25 protein with the C-terminal 38 amino acids deleted and replaced with 11 unrelated amino acids. N300: pML25 was digested to completion with *Bgl* II and *Sma* I, and the 5.9-kb backbone was isolated and a forced ligation was set up to recircularize the plasmid. The resulting plasmid pML25-N300 codes for the N-terminal 300 amino acids of cdc25 protein with an additional 4–7 unrelated amino acids at the C terminus. N258: pML25 was digested to completion with *Dra* I and the 926-bp fragment was isolated and digested with *Bam*HI. The resulting 787-bp fragment was ligated as a *Bam*H1/*Sma* I fragment into pGEX-2T to generate pML25-N258, which codes for the N-terminal 258 amino acids of cdc25 protein with an additional five unrelated amino acids at the C terminus. C215: pML25 was digested to completion with *Dra* I and the 1062-bp *Dra* I–*Dra* I fragment containing the coding region for the C-terminal 215 amino acids of the cdc25^{+} protein was isolated and ligated into *Sma* I-linearized pGEX-2T to create pML25-C215. C173: pML25 was digested to completion with *Bgl* II and *Sma* I and the 964-bp fragment was isolated and ligated into *Bam*H1/*Sma* I-linearized pGEX-1 to create pML25-C173. This plasmid codes for the C-terminal 173 amino acids of cdc25^{+} protein. Plasmids containing mutant constructs were transformed into JM109 cells and induced as described for the wild-type cdc25 protein. The predicted molecular weights for the mutant fusion proteins are as follows: N459, 79 kDa; N435, 77 kDa; N300, 61 kDa; N258, 57 kDa; C215, 51 kDa; and C173, 47 kDa.

Protease Inhibitors

Protease inhibitors were added to lysis, wash and reaction buffers as indicated in the text at the following concentrations: phenylmethylsulfonyl fluoride (PMSF) 2 mM, aprotinin (AP) 0.15 U/ml, leupeptin (LP) 20 μM, pepstatin (PP) 20 μM.

Preparation of Glutathione Beads

Glutathione agarose beads (Sigma Chemical, St. Louis, MO) were hydrated in PBS and then washed once in NETN (20 mM tris[hydroxymethyl]aminomethane [Tris] pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% vol/vol NP40, PMSF, AP, and LP), once in NETN containing 0.5% bovine serum albumin (BSA) (wt/vol), followed by three washes in NETN (Kaelin *et al.*, 1991).

Purification of Glutathione S-transferase (GST) Fusion Proteins

Method 1 (Column Chromatography). Frozen bacterial pellets from 1-l cultures were resuspended in 80–100 ml of lysis buffer (either PBS with 1% vol/vol Triton X-100 and PMSF, AP, LP, PP for microinjection experiments or NETN for all other experiments) and lysozyme was added to 0.5 mg/ml. After incubation on ice for 30 min, pellets were sonicated three times for 10 s each with a probe tip sonicator (model 185, Branson Sonic Power Co., Danbury, CT) at the microtip limit. Lysates were clarified by centrifugation at 27 000 × *g* for 1 h at 4°C, transferred to a fresh tube and re-centrifuged at 27 000 × *g* for 15 min at 4°C. Clarified supernatants were recycled over a 1-ml column of glutathione agarose for 16 h at 4°C. Columns were washed extensively with lysis buffer and then elution buffer (either 20 mM Tris, pH 8.0, 1 mM dithiothreitol [DTT] with PMSF, AP, LP, and PP for microinjection experiments or phosphatase buffer [25 mM imidazole, pH 7.2, 1 mM EDTA, 0.1% vol/vol 2-mercaptoethanol with PMSF, AP, LP, and PP]). Bound proteins were then eluted with elution buffer containing 10 mM reduced glutathione.

Method 2. GST fusion proteins were purified essentially as described (Kaelin *et al.*, 1991). Frozen bacterial pellets from 50-ml cultures of induced JM109 cells were suspended in 5 ml NETN. Cell suspensions were then sonicated three times for 5 s each, using a probe tip sonicator at its maximum setting. Sonicates were spun at 12 000 × *g* for 7 min. Cleared supernatants were added to glutathione beads and were incubated at 4°C for 30 min (1–5 ml of supernatant per 25 μl of packed beads). Beads were washed five times with NETN and boiled in sodium dodecyl sulfate (SDS) sample buffer. Bound proteins were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) and visualized by staining with Coomassie blue. For functional assays, the beads were washed four times with phosphatase buffer and were used directly or the recombinant proteins were eluted from the beads in phosphatase buffer containing 10 mM glutathione.

Xenopus Oocytes

Frogs were primed with 50 IU pregnant mares' serum gonadotropin in 0.1 ml 36 h before surgical removal of ovaries. *Stage VI* oocytes were manually dissected from ovarian tissue and maintained in modified OR-2 medium (25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES] pH 7.4, 73 mM NaCl, 1 mM $MgCl_2$, 0.5 mM $CaCl_2$, 1 mM KCl, 50 μg/ml gentamycin [GIBCO, Grand Island, NY], 10 mM $NaHCO_3$, and 100 μg/ml BSA) at 18°C.

Meiotic Maturation

Defolliculated oocytes were stimulated to mature with 10 μg/ml progesterone in modified OR-2 medium. For microinjection experiments, 50–100 nl of sample containing bacterially produced recombinant protein (purified by column chromatography) in 20 mM Tris, pH 8.0, 1 mM DTT, and 10 mM glutathione was injected into oocytes. cdc25 fusion protein (p80$^{GST\text{-}cdc25}$) and GST were injected at varying concentration (20–400 nM final concentration). Injected oocytes were incubated at 18°C for the duration of the experiment. When cycloheximide was used, oocytes were incubated in medium supplemented with 0.5 μg/ml cycloheximide for 30 min before microinjection and were maintained in this concentration of drug for the entire experiment. Germinal vesicle breakdown (GVBD) was scored by white spot formation and was confirmed by fixation of the oocytes in 10% trichloroacetic acid followed by manual dissection of the oocytes.

Extract Preparation

Dilute Prophase Extracts. *Stage VI* oocytes were homogenized in modified MPF extraction buffer (80 mM beta glycerol phosphate, 50 mM NaF, 20 mM Na ethylene glycol-bis[β-amino ethyl ether]-*N*,*N*,*N*′,*N*′-tetraacetic acid [EGTA], 15 mM $MgCl_2$, 20 mM HEPES, pH 7.5, 1 mM DTT, PMSF, LP, AP, and PP at a concentration of 10 μl of buffer per oocyte). In some instances vanadate was added to 1 mM. The cytoplasmic protein fraction was prepared by centrifuging the lysate at 100 000 × *g* for 1 h at 4°C. The supernatant was removed and stored at −80°C.

Concentrated Prophase Extracts. Extracts were prepared as described above except that oocytes were homogenized at a concentration of 1 μl of MPF extraction buffer per oocyte.

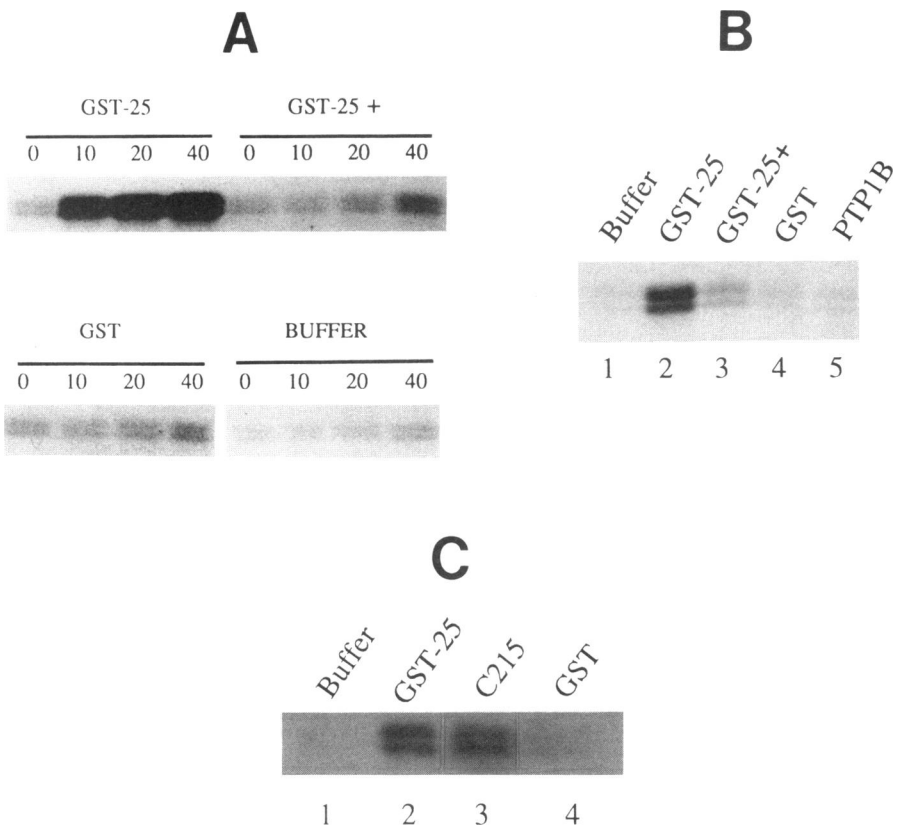

Figure 1. Activation of MPF kinase activity by cdc25Hs protein. (A) Extracts were prepared from *Xenopus* oocytes as described in MATERIALS AND METHODS. Extracts were incubated for 0–40 min with buffer only (buffer), bacterially produced glutathione-S transferase (GST), or with $p80^{GST\text{-}cdc25}$ in the absence (GST-25) or in the presence (GST-25+) of 1 mM vanadate. At the indicated times, aliquots were removed and histone H1 kinase assays were performed in vitro. Reaction products were resolved by SDS-PAGE and analyzed by autoradiography. Exposure time was 45 min with an intensifying screen at −80°C. (B) Extracts were prepared from *Xenopus* oocytes as described in MATERIALS AND METHODS. $p34^{cdc2}$ was immunoprecipitated with an antibody specific for *Xenopus* $p34^{cdc2}$. Immunoprecipitates were washed and then incubated for 40 min at room temperature with buffer only (lane 1), $p80^{GST\text{-}cdc25}$ (lane 2), $p80^{GST\text{-}cdc25}$ in the presence of 1 mM vanadate (lane 3), glutathione-S-transferase (lane 4), or PTP1B (lane 5). Immune complex kinase assays were then performed in the presence of histone H1. Exposure time was for 5 min at room temperature. (C) *Xenopus* $p34^{cdc2}$ was immunoprecipitated as described above and incubated with PTP1B for 30 min at room temperature. Precipitates were then washed and incubated with buffer only (lane 1), $p80^{GST\text{-}cdc25}$ (lane 2), C215 (lane 3), or GST only (lane 4) for 15 min at room temperature. Immune complex kinase assays were then performed with histone H1.

Activation of MPF in Extracts Prepared from Stage VI Oocytes

Reaction mixes were prepared in a final volume of 30 μl containing 15 μl of dilute prophase extract and a final concentration of 40 mM beta glycerol phosphate, 25 mM NaF, 7.5 mM $MgCl_2$, 10 mM HEPES, pH 7.5, 0.5 mM DTT, 10 mM NaEGTA, 10 mM imidizole, pH 7.2, 0.4 mM NaEDTA, 0.04% vol/vol B-mercaptoethanol, 10 mM creatine phosphate, 1 mM ATP, 0.04 mg/ml $p80^{GST\text{-}25}$, or 0.16 mg/ml GST, and when included, 1 mM sodium orthovanadate. Reaction mixes were incubated at 30°C and at the times indicated in the Figures; 5-μl aliquots were removed and diluted into an equal volume of 2 mM vanadate and placed on ice. On completion of the time course, histone H1 kinase assays were performed as follows: the 10-μl samples were added to 30 μl of kinase reaction mix to give a final concentration of 20 mM HEPES, pH 7.5, 30 mM B-mercaptoethanol, 0.1 mg/ml BSA, 10 mM $MgCl_2$, 0.1 mM ATP, 80 mM beta glycerol phosphate, PMSF, AP, LP, PP, 1 mM vanadate, 0.2 μg of protein kinase A inhibitor, 0.5 mg per ml histone HI, 1×10^7 cpm gamma-^{32}P-ATP. Reactions were incubated at 30°C for 10 min. Reactions were stopped by the addition of SDS sample buffer and were boiled at 100°C for 5 min. Samples were resolved by SDS-PAGE on 12% gels. Labeled histone H1 was excised from each lane after staining gels with Coomassie blue, and radioactivity was measured by Cherenkov counting. In experiments using mutant proteins, 25 μl of dilute prophase extracts (adjusted to 10 mM creatine phosphate and 1 mM ATP) was added directly to 25 μl of mutant cdc25 proteins (bound to glutathione beads) in phosphatase buffer (prepared as in Method 2).

Activation of MPF in Anti-$p34^{cdc2}$ (Xenopus) Precipitates

$p34^{cdc2}$ was immunoprecipitated from 100 μl of dilute prophase extracts with 10 μl of *Xenopus* $p34^{cdc2}$-specific antibody (antipeptide antiserum directed against the carboxy-terminal 12 amino acids of *Xenopus* $p34^{cdc2}$) by incubation at 4°C for 2 h with gentle rocking. Protein A Sepharose was added and the incubation was continued for an additional hour. Immunoprecipitates were washed three times in MPF extraction buffer containing 1 mM vanadate; three times in RIPA buffer (Parker *et al.*, 1991) containing PMSF, AP, LP, PP, and 1 mM vanadate; three times in lithium chloride wash buffer (0.5 M lithium chloride, 20 mM Tris, pH 8.0, PMSF, AP, LP, PP, and 1 mM vanadate); and two times with phosphatase buffer. The immunoprecipitates were then incubated with recombinant $p80^{GST\text{-}25}$ (0.18 mg/ml) or GST (1.8 mg/ml) protein in phosphatase buffer or PTP1B (final concentration of 0.013 mg/ml). When included, vanadate was present at 1 mM. Reactions were incubated at room temperature for 1 h and the reaction was stopped by washing the beads two times in kinase wash buffer (20 mM HEPES, pH 7.5, 30 mM B-mercaptoethanol, 0.1 mg/ml BSA, 10 mM $MgCl_2$, 80 mM beta glycerol phosphate, PMSF, AP, LP, PP, and 1 mM vanadate). Kinase assays were performed by the addition of 40 μl of kinase reaction mix with final concentrations as described above. The reaction products were analyzed by SDS-PAGE and autoradiography as described above. In Figure 1C, precipitates were prepared and washed as described above and then incubated with PTP1B (0.013 mg/ml) for 30 min at room temperature. Precipitates were then washed three times with phosphatase buffer and incubated

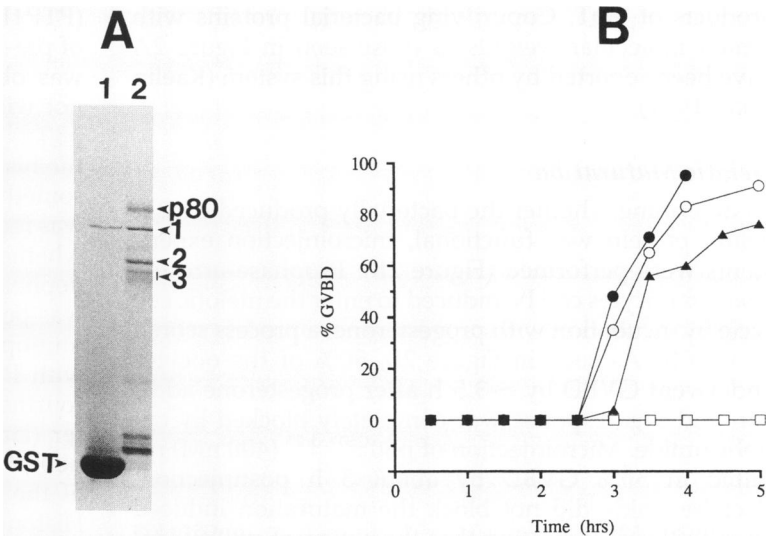

Figure 2. Bacterially produced cdc25Hs protein induces meiotic maturation. (A) Lysates prepared from bacteria expressing either the glutathione S-transferase leader sequence from pGEX-2T (lane 1) or the cdc25-fusion protein from pML25 (lane 2) were prepared and incubated with glutathione-agarose beads as described in MATERIALS AND METHODS. The beads were washed and the bound proteins were eluted by boiling in SDS-sample buffer. Proteins were resolved by SDS-PAGE on a 12% gel and visualized by Coomassie blue staining. *Species 1* and *2* are copurifying bacterial proteins and *species 3* is a breakdown product of p80$^{GST\text{-}cdc25}$. (B) *Xenopus* oocytes were incubated in progesterone in the absence (▲, n = 25) or in the presence (□, n = 17) of cycloheximide. Alternatively, oocytes were microinjected with p80$^{GST\text{-}cdc25}$ in the absence (●, n = 23) or in the presence of cycloheximide (○, n = 21) or with bacterially produced glutathione S-transferase (GST, ■, n = 22). Assay points for GST (■) remained at baseline throughout the 5-h incubation period. Values are expressed as percent of cells that had undergone GVBD at the time indicated.

with p80$^{GST\text{-}cdc25}$ (0.06 mg/ml), C215 (0.05 mg/ml), GST (0.85 mg/ml), or buffer alone for 15 min at room temperature. Histone H1 kinase assays were then performed as described above and the reaction products were analyzed by SDS-PAGE and autoradiography.

Purification of Phosphotyrosine-Containing Form of p34^{cdc2}/Cyclin Complex From Overproducing Insect Cells

p34^{cdc2} phosphorylated on tyrosine 15 was produced using a baculoviral expression system as described previously (Parker *et al.*, 1991). Ten × 10^6 Sf9 cells were seeded onto 100-mm tissue culture dishes. After attachment, cells were coinfected with recombinant viruses encoding human p34^{cdc2}, clam cyclin B, and *S. pombe* p107^{wee1}, each at multiplicity of infection of ~10. At 37 h postinfection, plates were rinsed once with methionine-free Graces media (GIBCO) followed by incubation at 26°C for 3 h in 1.2 ml Graces methionine-free media supplemented with 2 mM glutamine, 1.5% dialyzed calf serum, and 500 μCi/ml ^{35}S-Trans label (ICN Biomedicals Inc., Costa Mesa, CA). After the incubation period, cells were rinsed twice with PBS and then were lysed in 1.3 ml of cdc2 lysis buffer (50 mM Tris, pH 7.4, 0.1% vol/vol NP-40, 50 mM NaF, 10 mM NaPPi, 250 mM NaCl, PMSF, AP, LP, and 1 mM sodium vanadate) at 4°C for 15 min. Lysates were centrifuged at 10 000 × *g* for 10 min. Supernatants were collected and the p34^{cdc2}/cyclin B complex was precipitated using either a polyclonal antibody against human p34^{cdc2} or p13^{suc1} beads as described previously (Parker *et al.*, 1991).

Phosphatase Assays

p34^{cdc2} phosphorylated on tyrosine 15 was purified from Sf9 cells using either p34^{cdc2}-specific antibody coupled to protein A Sepharose or p13^{suc1} beads. The beads were washed four times with phosphatase buffer. After the final wash, 200 μl of phosphatase buffer containing bacterially produced recombinant protein at a concentration of 60 ng/μl (isolated by Method 2) was added. Tubes were incubated at 25°C and at the times indicated in the Figures; 100-μl aliquots were removed and added to 500 μl of cdc2 lysis buffer containing 2 mM vanadate. The reaction mixes were centrifuged at 1000 × *g* for 1 min and the supernatant was discarded. The pelleted beads were boiled in SDS sample buffer and proteins were resolved by SDS-PAGE and visualized by autoradiography.

PNPP Assays

Recombinant protein bound to glutathione beads was prepared by Method 2 except that beads were washed once with PNPP wash buffer (50 mM Tris, pH 7.4, 0.1% vol/vol B-mercaptoethanol, AP, PP, LP, and PMSF) rather than phosphatase buffer. Assays were performed by adding 500 μl of reaction buffer (PNPP wash buffer containing 20 mM PNPP) to the recombinant protein and incubating the mixture at room temperature with gentle rocking. At the indicated time points, the reaction was stopped by addition of 500 μl of 0.5 M NaOH. Absorbance at 410 nm was measured using a Beckman DU7 spectrophotometer (Beckman, Fullerton, CA). Units were calculated by determining the slope of the standard curve (in OD units/min) and using it in the equation (slope)/(1.75 × 10^4 mol·cm^{-1}·ml^{-1}). Specific activity is expressed as units (U = pmole PNPP hydrolyzed/min) per mg of recombinant protein.

RESULTS

Protein Production

To facilitate the production and purification of the human cdc25 gene product, a bacterial expression vector was constructed that expressed the full length cdc25^{+} gene product as a fusion protein with the *Schistosoma japonicum* GST gene. The majority of the 80-kDa cdc25 fusion protein (p80$^{GST\text{-}cdc25}$) was present in the insoluble fraction of the bacterial lysate; however, the soluble fraction (~1%) was easily purified by affinity chromatography on glutathione coupled to agarose (Figure 2A, lane 2). As seen in lane 1, GST migrated with a molecular weight of ~26 kDa. Also evident in lanes 1 and 2 is an endogenous bacterial protein of ~70 kDa (*species 1*) that copurified with GST and p80$^{GST\text{-}cdc25}$ (also see Figure 5B). Another bacterial protein of ~60 kDa (*species 2*, lane 2) copurified with p80$^{GST\text{-}cdc25}$ (see Figure 5B). *Species 3* was generated during the purification of p80$^{GST\text{-}cdc25}$, was recognized by an antibody specific for the cdc25 protein, and thus is likely to be a degradation product of p80$^{GST\text{-}cdc25}$. Smaller species of 27–30 kDa were also detected and may represent read-through

products of GST. Copurifying bacterial proteins with similar molecular weights to those seen in Figure 2A have been reported by others using this system (Kaelin *et al.*, 1991).

Meiotic Maturation

To determine whether the bacterially produced cdc25-fusion protein was functional, microinjection experiments were performed (Figure 2B). Prophase-arrested *Xenopus* oocytes can be induced to enter the meiotic cell cycle by incubation with progesterone, a process scored by GVBD. As seen in Figure 2B, 50% of the oocytes underwent GVBD by ~3.5 h after progesterone addition and this response was completely blocked by cycloheximide. Microinjection of $p80^{GST\text{-}cdc25}$ (400 nM) resulted in 50% GVBD by about 3 h postinjection. Cycloheximide did not block the maturation induced by $p80^{GST\text{-}cdc25}$ indicating that the action of $p80^{GST\text{-}cdc25}$ was independent of new protein synthesis. Bacterially expressed GST (control) did not induce GVBD under similar assay conditions.

Activation of pre-MPF in vitro

The role of cdc25 protein in the activation of pre-MPF was further investigated in extracts prepared from *stage VI* oocytes (Figure 1A). Addition of $p80^{GST\text{-}cdc25}$ (at a final concentration of ~500 nM) to extracts stimulated histone HI kinase activity (GST-25) sixfold within 20 min. In the absence of an ATP-regenerating system the activation process was delayed, requiring 60 min to reach the sixfold increase. The activation by $p80^{GST\text{-}cdc25}$ was inhibited by the protein-tyrosine phosphatase inhibitor sodium orthovanadate (GST-25+), suggesting the involvement of a tyrosine-specific protein phosphatase in the activation process. Buffer alone or bacterially expressed GST had no effect on the kinase activity of pre-MPF in the extract.

These results suggested that the bacterially produced cdc25-fusion protein was capable of activating pre-MPF in intact oocytes as well as in extracts prepared from oocytes and that the activation was dependent, at least in part, on the activity of a tyrosine-specific protein phosphatase. To look more directly at the effects of $p80^{GST\text{-}cdc25}$ on pre-MPF, immunoprecipitation experiments were performed. $p34^{cdc2}$/cyclin complexes (pre-MPF) were immunoprecipitated from oocyte extracts using an antibody specific for *Xenopus* $p34^{cdc2}$. The immunoprecipitates were stringently washed with RIPA buffer and 0.5 M LiCl and were then assayed for their ability to be activated under a variety of conditions. As seen in Figure 1B (lane 2), $p80^{GST\text{-}cdc25}$ activated the histone H1 kinase activity of pre-MPF in vitro 15-fold over controls (buffer only, lane 1) when added to the immunoprecipitates. The activation of pre-MPF by $p80^{GST\text{-}cdc25}$ was inhibited by vanadate (lane 3). Neither GST (lane 4) nor human placental phosphatase 1B (PTP1B, lane 5) activated the histone H1 kinase activity of pre-MPF. The tyrosine dephosphorylation of $p34^{cdc2}$ was observed with both $p80^{GST\text{-}cdc25}$ and PTP1B but not with GST or when vanadate was included in the $p80^{GST\text{-}cdc25}$ reaction. The failure of PTP1B to activate the histone H1 kinase activity of $p34^{cdc2}$ suggested that something in addition to tyrosine dephosphorylation was required for the activation of $p34^{cdc2}$. To determine whether $p80^{GST\text{-}cdc25}$ could activate $p34^{cdc2}$ that had been dephosphorylated by PTP1B, the following experiment was performed (Figure 1C). $p34^{cdc2}$ (pre-MPF) was immunoprecipitated from *Xenopus* extracts and incubated with PTP1B. The reaction mix was then divided into fourths. The aliquots were either incubated with buffer (lane 1), $p80^{GST\text{-}cdc25}$ (lane 2), C215, a mutant of $p80^{GST\text{-}cdc25}$ containing the C-terminal 215 amino acids of the protein (see below), (lane 3), or GST (lane 4). Histone H1 kinase assays were then performed. As shown in Figure 1C, incubation of the immunoprecipitates with either $p80^{GST\text{-}cdc25}$ or the mutant C215 (but not with either buffer or GST) resulted in the activation of $p34^{cdc2}$.

Tyrosine-Dephosphorylation of Insect Cell-Derived $p34^{cdc2}$

We have previously demonstrated that $p34^{cdc2}$ is phosphorylated on tyrosine 15 when coproduced with cyclin and the $wee1^+$ gene product in insect cells using a baculoviral expression system (Parker *et al.*, 1991). The tyrosine phosphorylated form of $p34^{cdc2}$ has a slower electrophoretic mobility than other forms of $p34^{cdc2}$ and can therefore be distinguished by SDS-PAGE (Figure 3A). We used the insect-derived $p34^{cdc2}$/cyclin complex as an independent assay for monitoring $p80^{GST\text{-}cdc25}$ function. The validity of this assay is demonstrated in Figure 3A. ^{35}S-labeled $p34^{cdc2}$/cyclin complexes were immunoprecipitated (using anti-$p34^{cdc2}$ serum) from lysates derived from cells coproducing $p34^{cdc2}$, cyclin B, and $p107^{wee1}$. As seen in Figure 3A (left), cyclin B as well as 2 forms of $p34^{cdc2}$ (differing in electrophoretic mobility) are evident. Only the slower electrophoretic form of $p34^{cdc2}$ reacted with an anti-phosphotyrosine antibody (right). Incubation of the immunoprecipitates with bacterially produced placental phosphatase 1B (PTP1B) (Chernoff *et al.*, 1990) resulted in the tyrosine dephosphorylation of $p34^{cdc2}$ as monitored by the disappearance of the slower electrophoretic form of $p34^{cdc2}$ (left, lane 3) coincident with the loss in immunoreactivity with the anti-phosphotyrosine antibody (right, lane 3).

The ability of bacterially derived $p80^{GST\text{-}cdc25}$ to cause the tyrosine dephosphorylation of $p34^{cdc2}$ was tested using this assay. As seen in Figure 3B, addition of $p80^{GST\text{-}cdc25}$ to $p13^{suc1}$ precipitates of ^{35}S-labeled $p34^{cdc2}$/cyclin complexes stimulated the tyrosine dephosphorylation of $p34^{cdc2}$, as monitored by the loss of the slower electrophoretic form of $p34^{cdc2}$ (lanes 4–6). Vanadate

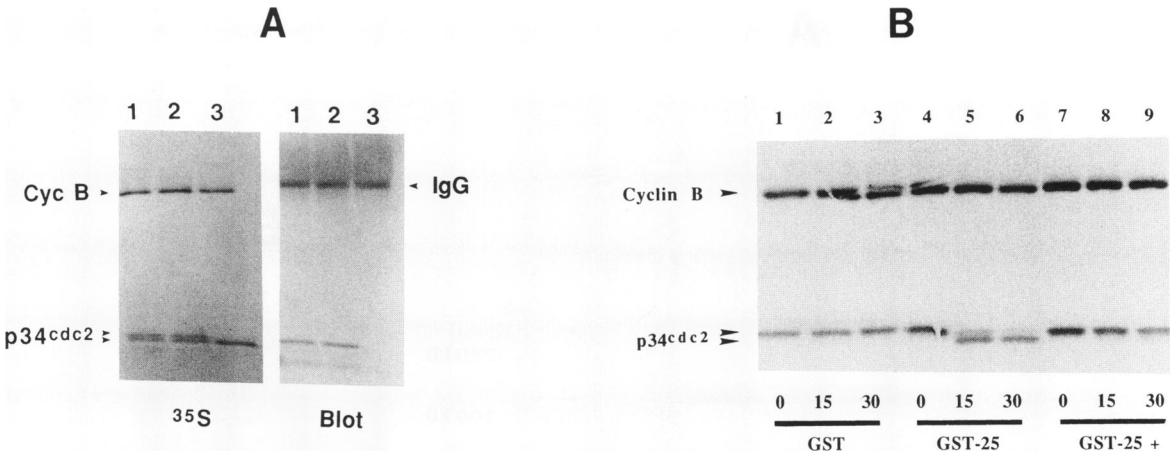

Figure 3. Tyrosine-dephosphorylation of p34^{cdc2} produced in insect cells. (A) ^{35}S-labeled p34^{cdc2}/cyclin complexes were immunoprecipitated (using anti-p34^{cdc2} serum) from insect cells coproducing p34^{cdc2}, cyclin B, and p107^{wee1} as described in MATERIALS AND METHODS. Immunoprecipitates were divided into thirds. One-third was left untreated (lane 1), one-third was incubated in phosphatase buffer alone (lane 2), and the final third was incubated with PTB1B (lane 3). Proteins were resolved by SDS-PAGE, blotted onto nitrocellulose, and probed with a monoclonal antibody directed against phosphotyrosine (right). The blot was then exposed to film and ^{35}S-labeled proteins were detected by autoradiography (left). (B) ^{35}S-labeled p34^{cdc2}/cyclin complexes were precipitated from insect cells coproducing p34^{cdc2}, cyclin B, and p107^{wee1} using p13^{suc1} beads. The precipitates were divided into thirds. One-third was incubated with GST (lanes 1–3), one-third was incubated with the p80$^{GST\text{-}cdc25}$ (lanes 4–6), and the final third was incubated with p80$^{GST\text{-}cdc25}$ and 1 mM vanadate (lanes 7–9). Aliquots were collected at 0, 15, and 30 min and analyzed by SDS-PAGE and autoradiography.

blocked the p80$^{GST\text{-}cdc25}$-dependent dephosphorylation of p34^{cdc2} (GST-25+, lanes 7–9), and GST was negative in this assay (GST, lanes 1–3). Identical results were obtained when the p34^{cdc2}/cyclin complex was immunoprecipitated with anti-p34^{cdc2} sera rather than with p13^{suc1} beads.

The C Terminus of cdc25Hs Possesses Intrinsic Phosphatase Activity

The cdc25Hs protein consists of 473 amino acids. Regions of homology with other cdc25 homologs are localized within the C terminus (residues 245–473) of cdc25Hs (Edgar and O'Farrell, 1989; Russell *et al.*, 1989; Jimenez *et al.*, 1990; Sadhu *et al.*, 1990). To localize the structural domain of the cdc25 protein required for function, a series of amino- and carboxy-terminal deletion mutants of p80$^{GST\text{-}cdc25}$ were constructed and expressed in bacteria as fusion proteins (Figure 4). The mutant proteins were tested for their ability to activate histone H1 kinase activity in extracts prepared from *Xenopus* oocytes (Figure 5A), for their ability to stimulate the tyrosine-dephosphorylation of insect-cell derived p34^{cdc2} in vitro (Figure 5B), and for their ability to hydrolyze PNPP (Figure 6).

As seen in Figure 4B, recombinant fusion proteins of the expected molecular weights were easily purified from induced bacteria using glutathione beads. As was seen in Figure 2A, a 70-kDa bacterial protein (species 1) copurified with GST as well as with each of the cdc25-fusion proteins, with the exception of C173 (lane 7). C173 was constructed in vector pGEX-1, whereas all the other mutants were constructed in vector pGEX-2T. Interestingly, pGEX-2T differs from pGEX-1 in that it encodes for a thrombin cleavage site and this may be the region involved in binding to p70. *Species* 2 (60-kDa bacterial protein) copurified with p80$^{GST\text{-}cdc25}$ as well as the deletion mutants of cdc25 that retained the carboxy-terminal domain (lanes 1–3, 6, and 7). However, copurification of *species* 2 did not correlate with cdc25 function (see below).

As shown in Figure 5, the activities of each of the mutant proteins were tested in two distinct assays: activation of *Xenopus* pre-MPF in prophase extracts (A) and tyrosine dephosphorylation of p34^{cdc2} produced in insects cells (B). As seen in Figure 5A and B, the C-terminal deletion mutants of cdc25Hs (N300 and N258) were negative in both assays, whereas mutant C215 that completely lacked the N-terminus of cdc25Hs but retained C-terminal residues 258–473 was fully functional. Deletion mutants N459 and N435 that lack the C-terminal 14 and 37 amino acids, respectively, were also fully functional in both assays. These results point to the importance of the conserved C-terminal domain of cdc25Hs for function. C173, a slightly larger deletion mutant than C215 (retains residues 301–473), was negative in both assays. Thus, sequences located between residues 258 and 301 are essential for cdc25Hs function. As mentioned above, *species* 2 (60-kDa bacterial protein) copurified with p80$^{GST\text{-}cdc25}$ in addition to each of the mutants that retained the C terminus of the cdc25 protein (lanes 1–3, 6, and 7). However, binding of p60 to p80$^{GST\text{-}cdc25}$ did not correlate with function as mutant C173 (lane 7) bound p60 yet was nonfunctional in the

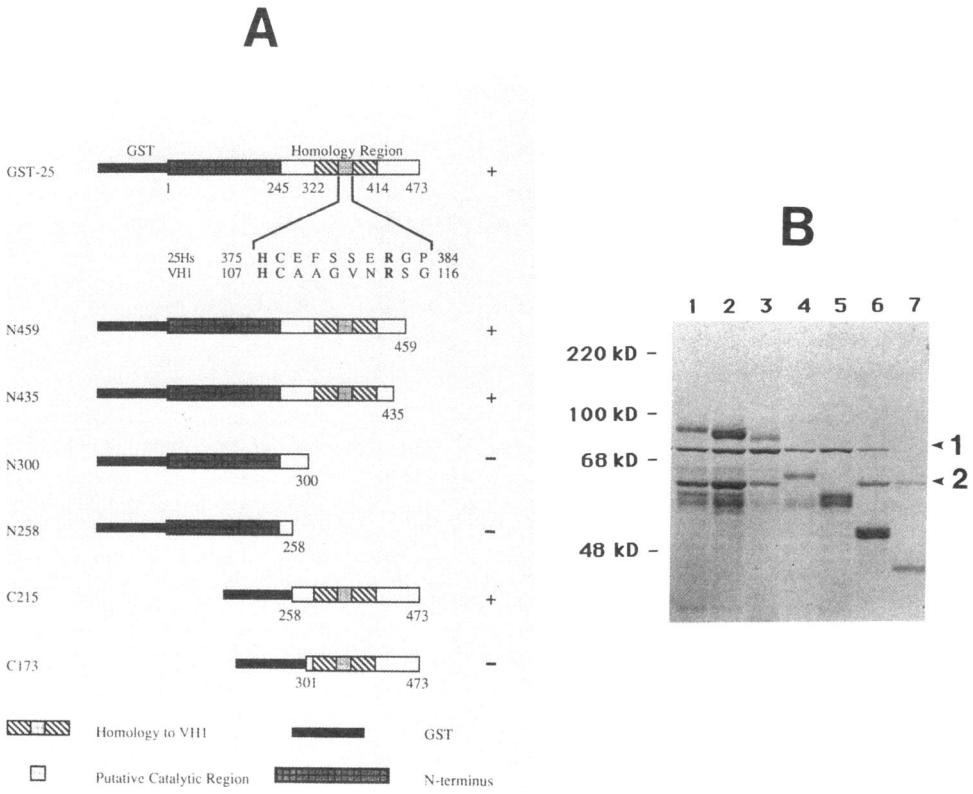

Figure 4. Expression and purification of deletion mutants of cdc25Hs protein. (A) Structure of glutathione S-transferase fusion proteins. (+) indicates that the protein was functional; (−) indicates that the mutant protein was nonfunctional. (B) Lysates prepared from bacteria expressing $p80^{GST\text{-}cdc25}$ (lane 1) or deletion mutants of cdc25Hs (lanes 2–7) were prepared and incubated with glutathione beads as described in MATERIALS AND METHODS. The beads were washed and the bound proteins were eluted by boiling in SDS-sample buffer. Proteins were resolved by SDS-PAGE on a 12% gel and were visualized by Coomassie blue staining. N459 (lane 2), N435 (lane 3), N300 (lane 4), N258 (lane 5), C215 (lane 6), C173 (lane 7). *Species 1* and *2* are copurifying bacterial proteins.

assays performed in this study. These results suggest that the functional domain of the cdc25Hs protein resides within its C terminus between residues 258 and 435.

To further purify the cdc25Hs assay system, GST, $p80^{GST\text{-}cdc25}$, and each of the mutant proteins were tested for their ability to hydrolyze PNPP, a commonly used artificial substrate for monitoring tyrosine-specific protein phosphatase activity. Using standard assay conditions for measuring PNPP hydrolysis by tyrosine-specific protein phosphatases, GST and mutants C173, N300, and N258 were negative (at the 40-min time point, the OD units were <0.01). However, mutant C215 hydrolyzed PNPP at a significant rate under similar assay conditions (Figure 6). We calculated the specific activity of C215 to be ~300 units/mg of recombinant protein compared with 3600 units/mg for bacterially produced PTP1B (Figure 6B). For reasons that are not understood, the solubility of bacterially produced C215 protein is much greater than that of $p80^{GST\text{-}cdc25}$ or the other mutant proteins. When compared over a concentration range from 0.1 to 1 μg of protein, C215, $p80^{GST\text{-}cdc25}$, N459, and N435 were found to be indistinguishable in their ability to hydrolyze PNPP.

DISCUSSION

To study the contribution made by the cdc25Hs protein to the regulation of $p34^{cdc2}$, we have overproduced the cdc25Hs protein as well as several deletion mutants of cdc25Hs in bacteria. The recombinant proteins were assayed for function in three independent systems: 1) *Xenopus* oocytes were used to monitor the ability of recombinant cdc25 protein to induce meiotic maturation as well as to activate $p34^{cdc2}$ (both in extracts and in immunoprecipitates); 2) recombinant cdc25Hs protein was monitored for its ability to dephosphorylate $p34^{cdc2}$ (on tyrosine 15), that had been isolated from overproducing insect cells using a baculoviral expression system; and 3) recombinant cdc25Hs protein was assayed using PNPP as an artificial substrate. Results from each of these systems suggest that the cdc25Hs protein pos-

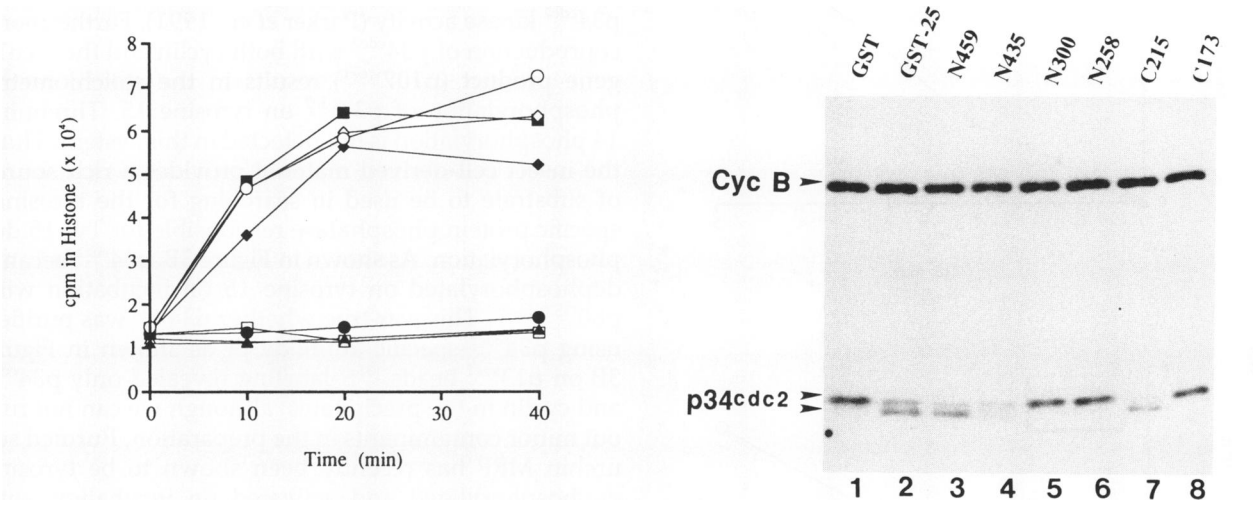

Figure 5. The C terminus of the cdc25 protein is required for function. (A) Extracts were prepared from *Xenopus* oocytes as described in MATERIALS AND METHODS. Extracts were incubated with bacterially produced proteins bound to glutathione beads and at the times indicated (0, 10, 20, and 40 min), aliquots were removed, and histone H1 kinase assays were performed. Reaction products were resolved by SDS-PAGE and analyzed by autoradiography. Histone H1 was excised from the gel and quantitated by Cerenkov counting. Results are expressed as cpm incorporated into histone H1 vs. time. ○, C215; ◇, N435; □, GST; △, C173; ●, N258; ◆, N459; ■, p80$^{GST\text{-}cdc25}$; ▲, N300. (B) ^{35}S-labeled p34^{cdc2}/cyclin complexes were precipitated from insect cells coproducing p34^{cdc2}, cyclin B, and p107^{wee1} using p13^{suc1} beads. The precipitates were divided equally and incubated with the following recombinant proteins bound to glutathione beads: glutathione S-transferase (GST, Lane 1), p80$^{GST\text{-}cdc25}$ (lane 2), N459 (lane 3), N435 (lane 4), N300 (lane 5), N257 (lane 6), C215 (lane 7), or C173 (lane 8). After 90 min, samples were boiled in the presence of SDS and analyzed by SDS-PAGE and autoradiography.

sesses intrinsic phosphatase activity and that it functions as a positive regulator of the cell cycle by dephosphorylating p34^{cdc2}.

Bacterially produced cdc25Hs protein-induced meiotic maturation of *Xenopus* oocytes in the absence of protein synthesis. Over the course of this study, 464 oocytes were injected with 2–32 ng of p80$^{GST\text{-}cdc25}$ (final concentrations varied between 22 and 400 nM). The time for 50% GVBD varied between experiments from 2.5 to 5 h but consistently occurred 1 h before that induced by progesterone. This response is slower than that seen when the cyclins are microinjected into oocytes (Swenson *et al.*, 1986; Westendorf *et al.*, 1989; Roy *et al.*, 1991). This difference may be due to the fact that p80$^{GST\text{-}cdc25}$ acts on preassembled pre-MPF whereas there is a recruitment of more p34^{cdc2} by the microinjected cyclins that amplifies the response. Alternatively, bacterially produced p80$^{GST\text{-}cdc25}$ may lack some post-translational modification required for maximal activity.

p80$^{GST\text{-}cdc25}$ activated pre-MPF when added to extracts prepared from G2/prophase-arrested *Xenopus* oocytes. The activation, measured as phosphate incorporation into exogenously added histone H1, varied between four- and eightfold. The inclusion of an ATP-regenerating system decreased the time required for the cdc25Hs-dependent activation but not the amplitude of the response. Sodium vanadate, an inhibitor of known phosphotyrosine-specific protein phosphatases, inhibited the cdc25Hs-dependent activation. The results reported in this study using the human cdc25 protein in *Xenopus* oocyte extracts are similar to those reported using the *Drosophilia* cdc25 homolog (string) (Kumagai and Dunphy, 1991).

Microinjection of cdc25Hs protein into intact oocytes and addition of cdc25Hs protein to whole-cell extracts were both crude assay systems. To further purify the assay system, pre-MPF was immunopurified from *Xenopus* oocyte extracts using an antibody specific for *Xenopus* p34^{cdc2}. The immunoprecipitates were then stringently washed using both RIPA buffer and 0.5 M LiCl and were used to test for the ability of recombinant cdc25Hs protein to activate the histone H1 kinase activity of p34^{cdc2}. As seen in Figure 1B, p80$^{GST\text{-}cdc25}$ activated the histone H1 kinase activity of p34^{cdc2} 15-fold over background. Sodium vanadate inhibited the cdc25Hs-dependent activation indicating that tyrosine-dephosphorylation was required at least in part for the activation. However, tyrosine dephosphorylation was not the only requirement as PTP1B catalyzed the tyrosine dephosphorylation of p34^{cdc2} but did not detectably activate its histone H1 kinase activity. Interestingly, p80$^{GST\text{-}cdc25}$ was capable of activating the tyrosine-dephosphorylated form of p34^{cdc2} (Figure 1C). That tyrosine dephosphorylation is not sufficient for activation

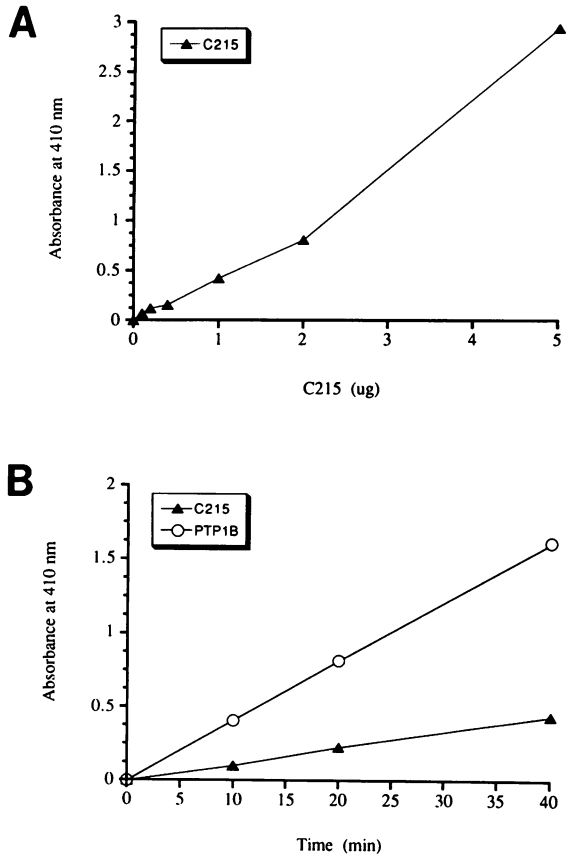

Figure 6. Hydrolysis of PNPP by cdc25Hs protein. (A) PNPP assays were performed as described in MATERIALS AND METHODS. Varying amounts of C215 mutant protein were incubated with PNPP for 45 min at which time the reaction was stopped by the addition of NaOH and absorbance at 410 nm was measured. (B) Recombinant C215 protein (2 μg) and PTP1B (0.63 μg) were incubated with PNPP as described in MATERIALS AND METHODS. At the times indicated the reactions were terminated and absorbance at 410 nm was measured.

has been reported for human $p34^{cdc2}$ as well (Morla *et al.*, 1989). One explanation for the activation observed with $p80^{GST\text{-}cdc25}$ but not with PTP1B is that the cdc25Hs protein may function in the dephosphorylation of $p34^{cdc2}$ on both threonine 14 and tyrosine 15.

The cdc25 protein has been reported to be a phosphoprotein but the role of phosphorylation in regulating the cdc25 protein has not been defined. However, because bacterially produced cdc25Hs protein was functional in the immune complex kinase assays (in the absence of ATP), phosphorylation must not be an absolute requirement for activity. The assays reported in this study should facilitate determining the contribution made by phosphorylation to the activity of the cdc25Hs protein.

As an independent means of assaying cdc25Hs function, we used a baculoviral expression system to overproduce the tyrosine 15-phosphorylated form of $p34^{cdc2}$. We have previously reported that coproduction of $p34^{cdc2}$ with cyclin in insect cells results in the formation of functional $p34^{cdc2}$/cyclin complexes as judged by coprecipitation, cyclin phosphorylation, and activation of $p34^{cdc2}$ kinase activity (Parker *et al.*, 1991). Furthermore, coproduction of $p34^{cdc2}$ with both cyclin and the $wee1^+$ gene product ($p107^{wee1}$) results in the stoichiometric phosphorylation of $p34^{cdc2}$ on tyrosine 15. Threonine 14 phosphorylation is not detected in this system. Thus, the insect cell-derived material provides a rich source of substrate to be used in searching for the tyrosine-specific protein phosphatase responsible for Tyr 15 dephosphorylation. As shown in Figure 3B, $p34^{cdc2}$ became dephosphorylated on tyrosine 15 on incubation with $p80^{GST\text{-}cdc25}$. This was true whether $p34^{cdc2}$ was purified using $p34^{cdc2}$-specific antibody or as shown in Figure 3B on $p13^{suc1}$ beads. ^{35}S-labeling revealed only $p34^{cdc2}$ and cyclin in the precipitates, although we can not rule out minor contaminants in the preparation. Purified sea urchin MPF has recently been shown to be tyrosine dephosphorylated and activated on incubation with bacterially produced cdc25Hs protein (Strausfeld *et al.*, 1991).

Unlike $p34^{cdc2}$ isolated from *Xenopus* oocytes, we were unable to detect any activation of insect cell-derived $p34^{cdc2}$ coincident with its tyrosine dephosphorylation. As previously published, the tyrosine phosphorylated form of $p34^{cdc2}$ produced in insect cells (on coproduction of $p34^{cdc2}$ with cyclin and $p107^{wee1}$) lacks any appreciable threonine phosphorylation (Parker *et al.*, 1991). Thus, the activating threonine phosphorylation (presumably threonine 161) may be absent in this population of $p34^{cdc2}$ and could account for the apparent lack of activation.

To identify the minimal domain of cdc25Hs capable of functioning in these assays, a series of N- and C-terminal mutants of the cdc25Hs protein were constructed. We identified a region in the C terminus of the cdc25Hs protein (bordered by amino acids 258 and 435) that was fully functional. Thus, the amino terminus of $cdc25^+$, at least in these assays, was completely dispensible for function. This finding is consistent with data reported for both string and MIH1 (Russell *et al.*, 1989; Kumagai and Dunphy, 1991).

Taken together, these results suggested that either the cdc25Hs protein possessed intrinsic phosphatase activity or alternatively that it activated a phosphatase that copurified with $p34^{cdc2}$/cyclin complexes isolated both from *Xenopus* oocytes and from insect cells. cdc25Hs lacks any obvious homology to known protein phosphatases, although Moreno and Nurse (1991) recently noted homology between *S. pombe* cdc25 and VH1, a phosphatase encoded by vaccinia virus that is capable of hydrolyzing phosphoserine and phosphotyrosine (Guan *et al.*, 1991). The cdc25Hs protein contains a motif (HCXAGXXR) within its C terminus that is conserved in the active site of all-known tyrosine-

specific protein phosphatases. Mutation of the cysteine residue within this motif ablates the phosphatase activity of VH1 (Guan *et al.*, 1991). In an attempt to establish a biochemically pure assay system for determining whether cdc25Hs protein was indeed a phosphatase, we tested the ability of PNPP to be hydrolyzed by recombinant cdc25Hs protein. As shown in Figure 6, PNPP was hydrolyzed at a rate of nearly 10% that of PTP1B, indicating that the activity associated with p80$^{GST\text{-}cdc25}$ was a biochemically significant activity. In addition, in this assay we utilized affinity-purified bacterially derived cdc25Hs protein and an artificial substrate (PNPP), thus ruling out the possibility that a phosphatase other than cdc25 itself was responsible for the activity. These results indicate that the phosphatase activity is intrinsic to the cdc25Hs protein and that it may directly dephosphorylate p34^{cdc2}.

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