J. Biochem. 131, 705–712 (2002)

Heterologous Expression and Catalytic Properties of the C-Terminal Domain of Starfish Cdc25 Dual-Specificity Phosphatase, a Cell Cycle Regulator[1]

Shungo Deshimaru,[*] Yasuo Miyake,[*] Tadamasa Ohmiya,[*] Yoshiro Tatsu,[†] Yasuko Endo,[†,2] Noboru Yumoto,[†] and Tetsuo Toraya[*,3]

[*]Department of Bioscience and Biotechnology, Faculty of Engineering, Okayama University, Tsushima-Naka, Okayama 700-8530; and [†]National Institute of Advanced Industrial Science and Technology (AIST), Midorigaoka, Ikeda, Osaka 563-8577

Received February 9, 2002; accepted February 27, 2002

The 3′-terminal region of starfish *Asterina pectinifera cdc25* cDNA encoding the C-terminal catalytic domain was overexpressed in *Escherichia coli*. The C-terminal domain consisted of 226 amino acid residues containing the signature motif HCxxxxxR, a motif highly conserved among protein tyrosine and dual-specificity phosphatases, and showed phosphatase activity toward *p*-nitrophenyl phosphate. The enzyme activity was strongly inhibited by SH inhibitors. Mutational studies indicated that the cysteine and arginine residues in the conserved motif are essential for activity, but the histidine residue is not. These results suggest that the enzyme catalyzes the reaction through a two-step mechanism involving a phosphocysteine intermediate like in the cases of other protein tyrosine and dual-specificity phosphatases. The C-terminal domain of Cdc25 activated the histone H1 kinase activity of the purified, inactive form of Cdc2·cyclin B complex (preMPF) from extracts of immature starfish oocytes. Synthetic diphosphorylated di- to nonadecapeptides mimicking amino acid sequences around the dephosphorylation site of Cdc2 still retained substrate activity. Phosphotyrosine and phosphothreonine underwent dephosphorylation in this order. This is the reverse order to that reported for the *in vivo* and *in vitro* dephosphorylation of preMPF. Monophosphopeptides having the same sequence served as much poorer substrates. As judged from the results with synthetic phosphopeptides, the presence of two phosphorylated residues was important for specific recognition of substrates by the Cdc25 phosphatase.

Key words: *Asterina pectinifera*, Cdc25, dual specificity phosphatase, protein tyrosine phosphatase, starfish.

The molecular mechanism of the onset of mitosis has been one of the major research subjects in modern cell biology. Maturation-promoting factor (MPF), first purified from *Xenopus oocytes* (*1*), has been established to be the target of the mitotic regulatory cascade (*2*). This key factor induces a cell to enter mitosis by phosphorylating a variety of regulatory and structural proteins. MPF is a heterodimeric enzyme that consists of the Cdc2 protein kinase, a catalytic subunit, and cyclin B, a regulatory subunit (*3, 4*). Although the level of Cdc2 remains almost constant throughout the cell division cycle, cyclin B is synthesized during the interphase and periodically degraded at the end of each mitosis cycle (*5*).

The activity of the Cdc2·cyclin B complex in the late G2 phase is still low, as judged on assaying *in vitro* with histone H1 as a substrate. Activation of the complex is needed for the initiation of mitosis. Genetic studies with the fission yeast *Schizosaccharomyces pombe* indicated that Cdc25 protein controls the timing of mitosis by activating Cdc2 (*6, 7*). Inactivation of Cdc25 results in cell cycle arrest at late G2 where Cdc2 protein phosphorylated of Tyr15 accumulates in the arrested cells (*8*). The function of Cdc25 is not required in the yeast strains with the Y15F Cdc2 mutants (*8*). These findings suggested that Cdc25 functions in the pathway leading to dephosphorylation of Cdc2 at Tyr15 in yeast. In animals, the Cdc2-cyclin B complex is kept inactive until needed by phosphorylation at both Thr14 and Tyr15 (*9*) and activated by the dual-specificity phosphatase Cdc25, which dephosphorylates both Thr14 and Tyr15 (*10, 11*). Experimental evidence for direct dephosphorylation of Cdc2 by intrinsic phosphatase activity of Cdc25 has been obtained by expressing the catalytically active C-terminal domains of *Drosophila* (*12, 13*), *S. pombe* (*14*), and human

[1] The authors wish to thank Miss Tomoko Matsumoto and Ms. Yukiko Kurimoto for preparation of the rabbit anti-starfish Cdc2 antiserum and the assistance in preparation of the manuscript, respectively.

[2] Recipient of a fellowship from NEDO.

[3] To whom correspondence should be addressed. Tel: +81-86-251-8194, Fax: +81-86-251-8264, E-mail: toraya@biotech.okayama-u.ac.jp

Abbreviations: bp, base pair(s); Cdc25ct, C-terminal domain of Cdc25; Cdk, cyclin-dependent kinase; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonate; IPTG, isopropyl-1-thio-β-D-galactopyranoside, MPF, maturation-promoting factor; Ni-NTA, nickel nitrilotriacetate; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate.

(*11*) Cdc25. Three human Cdc25 isoforms, designated as A, B, and C, are presumed to have different specificities for Cdks (*15*). Cdc25B appears to be essential in the G2/M transition in human cells (*16*). Activating phosphorylation of human Cdc25C by the Cdc2·cyclin B complex was suggested to be involved in the self-amplification of MPF at mitosis (*17*). Cdc25A is presumed to promote the G1/S transition by acting on the Cdk2·cyclin A/E complex (*18*). The crystal structures of the catalytic domains of human Cdc25A (*19*) and Cdc25B (*20*) were recently reported.

Hormonal regulation of starfish oocyte maturation has been intensively investigated (*21*), because it provides a good model system for biochemical studies on the mechanism of signal transduction as well as cell cycle progression. To investigate the mechanistic features and substrate specificity of the Cdc25 phosphatase, we cloned and sequenced a half-length cdc25 cDNA (downstream 915 nucleotides of the open reading frame) from the starfish *Asterina pectinifera* (GenBank, AF466828). The catalytic properties of the enzyme were studied by expressing the C-terminal domains of the wild type and mutant Cdc25 in a soluble form in *Escherichia coli*. Kishimoto and coworkers reported that they isolated an *Asterina pectinifera cdc25* cDNA (*22*), although the complete nucleotide and amino acid sequences have only appeared in the database very recently (DDBJ, AB076395). A part of the amino acid sequence (residues 444-553 of the catalytic domain) has been reported by Ashcroft *et al.* (*23*).

MATERIALS AND METHODS

Materials—Oligonucleotide primers were synthesized by Life Technologies. *N*-α-Fmoc-*O*-benzyl-L-phosphothreonine and *N*-α-Fmoc-*O*-benzyl-L-phosphotyrosine were purchased from Novabiochem. Other Fmoc-L-amino acids used for peptide synthesis were obtained from Shimadzu. Restriction enzymes were purchased from New England Biolabs and T4 DNA ligase from Gibco BRL. Rabbit anti-Cdc2 antiserum was raised against starfish Cdc2 purified from overexpressing *E. coli* JM109 cells. All other materials were obtained from commercial sources.

Synthesis of Phosphopeptides—Phosphopeptides were synthesized by use of a Shimadzu PSSM-8 automated peptide synthesizer according to the method of Wakamiya *et al.* (*24*). All the peptides were purified by reversed-phase HPLC, and their molecular masses were confirmed by mass spectrometry on a time-of-flight mass spectrometer (Shimadzu/Kratos Kompact MALDI II) with matrix-assisted laser desorption ionization.

Bacterial Strains, Plasmids, and DNA Manipulations—*E. coli* JM109 was used as a host for plasmid pCR2.1 and expression plasmid pUSI2ENd (*25*). Standard recombinant DNA techniques described by Sambrook *et al.* (*26*) were used. Restriction endonucleases and the enzymes for construction of plasmids were used according to the manufacturer's instructions.

PCR—DNA segments were amplified by PCR with rTaq (Toyobo) DNA polymerase using a template and the appropriate primer pairs listed in Table I. PCR products were cloned into vector pCR2.1 using a TA Cloning kit (Invitrogen).

Construction of an Ovary cDNA Library—A starfish ovary cDNA library in the pGAD10 vector was constructed using a two-hybrid cDNA library construction kit (Clonetech), as described previously (*27*).

DNA Sequencing—Template plasmid DNA was prepared by an alkaline lysis method and sequenced with an ALF DNA sequencer (Pharmacia) using a Thermo Sequenase fluorescent-labeled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Pharmacia Biotech). The oligonucleotide primers used for DNA sequencing were (Cy5)M13 Universal and (Cy5)M13 Reverse primers (Amersham Pharmacia Biotech).

Heterologous Expression of the C-Terminal Catalytic Domain of Cdc25 in E. coli—To introduce restriction sites into the 5′- and 3′-terminal regions of *cdc25* cDNA, PCR was performed using plasmid DNA from the pGAD10 cDNA library as a template with primers SC255f and SC256r (Table I). The 0.70-kb segment amplified was cloned into pCR2.1 to obtain pCR2.1(cdc25ct). The expression plasmid, pUSI2ENd(cdc25ct), for the C-terminal domain ("Cdc25ct") was constructed by ligating a 0.69-kb *Nde*I–*Eco*RI fragment from pCR2.1(cdc25ct) with the *Nde*I–*Eco*RI region of expression vector pUSI2ENd (*25*). For expression of His_{10}-tagged Cdc25ct, a 0.72-kb *Nde*I–*Bam*HI fragment from pCR2.1(cdc25ct) was ligated with the *Nde*I–*Bam*HI region of pET19b to construct pET19b-(cdc25ct). Recombinant *E. coli* JM109 and *E. coli* [BL21-(DE3)] strains transformed with pUSI2ENd(cdc25ct) and pET19b(cdc25ct), respectively, were grown and harvested as described previously (*27*).

Site-directed Mutagenesis—Site-directed mutagenesis was performed with a QuickChange Site Directed Mutagenesis kit (Stratagene), according to the manufacturer's instructions. Plasmid pCR2.1(cdc25ct) was used as a template. The mutagenic primers designed were SC25HAf and SC25HAr for H492A, SC25CSf and SC25CSr for C493S, and SC25RAf and SC25RAr for R499A (Table I). The DNA sequences of all the mutants were confirmed by nucleotide sequencing. For expression of His_{10}-tagged mutant Cdc25ct, 0.72-kb *Nde*I-*Bam*HI fragments from pCR2.1(cdc25ct-H492A), pCR2.1(cdc25ct-C493S), and pCR2.1(cdc25ct-

TABLE I. **Oligonucleotide primers used in this study.**

Name	Sequence	Location* or comments
SC255f	5′CG*GGATCCCATATG*CAGACAAATAAGTCTCCAAGG	1–24 containing *Bam*HI-*Nde*I sites
SC256r	5′CG*GAATTC*TCAGCTGACGGAGGTTTTAC	662–681 containing *Eco*RI sites
SC25HAf	5′CGGGTCATCATCTTCGCCTGCGAGTTCTCGTCC	376–408
SC25HAr	5′GGACGAGAACTCGCAGGCGAAGATGATGACCCG	376–408
SC25CSf	5′CATCATCTTCCACTCCGAGTTCTCGTCCAAGC	381–412
SC25CSr	5′GCTTGGACGAGAACTCGGAGTGGAAGATGATG	381–412
SC25RAf	5′GAGTTCTCGTCCAAGGCGGGGCCGGACCTG	397–426
SC25RAr	5′CAGGTCCGGCCCCGCCTTGGACGAGAACTC	397–426

*Numbers from the start site for expression of starfish Cdc25ct.

R499A) were ligated with the *Nde*I-*Bam*HI region of pET19b to construct pET19b(cdc25ct-H492A), pET19b(cdc25ct-C493S), and pET19b(cdc25ct-R499A), respectively. *E. coli* [BL21(DE3)] was transformed with a mutant expression plasmid, as described above for wild-type Cdc25ct.

Purification of Recombinant Starfish Cdc25ct—His_{10}-tagged wild-type and mutant Cdc25ct were purified to homogeneity by metal-chelate affinity chromatography (*28*). All the procedures were carried out at 0–4°C. About 3 g of overexpressing *E. coli* cells from 440 ml of culture were suspended in 30 ml of 0.05 M potassium phosphate buffer (pH 7.4)/2 mM phenylmethanesulfonyl fluoride/5 mM mercaptoethanol and then disrupted by sonication. After centrifugation at 100,000 ×*g* for 60 min, KCl and imidazole were added to the supernatant (crude extract) to final concentrations of 0.5 M and 10 mM, respectively. The extract was then applied to a Ni-NTA agarose (Qiagen) column (bed volume, 1 ml) that had been preliminarily equilibrated with the same buffer. The effluent was passed through the column again. The column was washed successively with 10 bed volumes of 50 mM potassium phosphate buffer/10 mM imidazole/5 mM mercaptoethanol/1 mM PMSF (pH 7.4), and with 10 bed volumes of the same buffer containing 60 mM imidazole, and then eluted by increasing the concentration of imidazole to 250 mM. Protein-containing fractions were pooled and dialyzed against 50 mM potassium phosphate buffer/10 mM mercaptoethanol/20% glycerol (pH 7.4).

SDS-PAGE—SDS-PAGE was performed on a 10.5% acrylamide gel as described by Laemmli (*29*), followed by staining with Coomassie Brilliant Blue R-250.

Analysis of the N-Terminal Amino Acid Sequence—Purified Cdc25ct was hydrolyzed with recombinant enterokinase (Novagen) to remove the His_{10} tag. The hydrolysate was subjected to SDS-PAGE and then electro-blotted onto a PVDF membrane (Perkin-Elmer). After staining with Coomassie Brilliant Blue R-250, the major polypeptide band corresponding to M_r of 28,000 was excised and analyzed as to the N-terminal amino acid sequence with a Perkin Elmer model 492 protein sequencer.

Protein Assay—Protein concentrations were determined by the method of Lowry *et al.* (*30*) with crystalline bovine serum albumin as a standard. The concentration of purified Cdc25ct was determined by measuring the absorbance at 280 nm. The molar absorption coefficient at 280 nm calculated by the method of Gill and von Hippel (*31*) was 27,010 M^{-1} cm^{-1}.

Phosphatase Assay with Synthetic Substrates—The phosphatase activity of Cdc25ct was routinely assayed using *p*-nitrophenyl phosphate as a substrate. The standard assay mixture contained 25 mM HEPES buffer (pH 7.4), 5 mM mercaptoethanol, and 10 mM disodium *p*-nitrophenyl phosphate, in a total volume of 1.0 ml. The increase in the absorbance at 410 nm upon incubation at 30°C was measured. One unit of phosphatase activity was defined as the amount of enzyme activity that catalyzed the formation of 1 μmol of *p*-nitrophenol/min under the assay conditions.

Synthetic phosphopeptides were also used as substrates for investigating the substrate specificity of Cdc25ct. This assay was carried out essentially as described by Baykov *et al.* (*32*), except that a 60-μl reaction mixture was used. The assay mixture contained an appropriate amount of phosphopeptide instead of *p*-nitrophenyl phosphate as the substrate. After incubation at 30°C for the indicated time periods, the reaction was terminated by adding 0.8 mM malachite green hydrochloride (Sigma) in 1.2 N H_2SO_4. The amount of orthophosphate (P_i) released was determined by measuring the absorbance at 630 nm (*32*).

Activation of PreMPF by Cdc25ct—Protein phosphatase activity was estimated from the ability to activate prePMF (inactive form of the Cdc2-cyclin B complex in immature oocytes). PreMPF was purified from extracts of immature oocytes of *Asterina pectinifera* by affinity chromatography using Suc1-Sepharose beads (*33*). PreMPF was incubated at 30°C for 45 min with Cdc25ct in the presence of 5 mM mercaptoethanol and 20 mM HEPES buffer (pH 7.4)/5 mM $MgCl_2$/5 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetate, in a total volume of 20 μl. Histone H1 kinase activity was then determined—that is, 5 μl of the mixture was incubated at 30°C for 30 min with 2.5 μg of histone H1 and 10 μM ATP (specific activity, 20,000 dpm/pmol) in 25 μl of the same buffer. The mixture was then subjected to SDS-PAGE on a 12% gel, and the radioactivity in the histone H1 band was determined with a BAS1000 bioimaging analyzer.

RESULTS AND DISCUSSION

Heterologous Expression of the Recombinant Cdc25 C-Terminal Domain in E. coli Cells—The *A. pectinifera cdc25* cDNA that we cloned contained a downstream 915-bp coding region encoding a C-terminal region of Cdc25 consisting of 304 amino acid residues and a 244-bp 3′-untranslated region (GenBank, AF466828). It has been established that the catalytic site of Cdc25 phosphatase is located in its C-terminal region (*11–14*). To obtain a functional phosphatase domain of starfish Cdc25, a DNA segment (nucleotides

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362  MQINKSPRARPLSRSHSMDDSTEYHARIAMALMPNVDYTKLTGDLKKPYTLPLCKGKHQD  421

422  LQSITPETLCQILRSEVSEVKEHYILDCRYPYEYNGGHIRGAQNLYTKELVSQFFLENPM  481

482  EPRDGRVIIFHCEFSSKRGPDLLRFLRNKDREANCDRYPDLYYPELYILLGGYKAFYEND  541

542  KIMCEPQSYTPMDHEGYRKECLHFKAKSKSWAGERSRLGHRKTSVS               587
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Fig. 1. **The amino acid sequence of starfish Cdc25ct.** Amino acid residues are numbered beginning with the first Met residue, which corresponds to Met362 of Cdc25 (DDBJ, AB076395). The amino acid residues constituting the signature motif HCxxxxxR, a motif highly conserved among protein tyrosine phosphatases and dual-specificity phosphatases (*37*), are indicated by arrows above the sequence. The solid, broken, and dotted lines above the sequence show the sequences characteristic of the start of the Cdc25 catalytic domain (*35*), CH2A and CH2B motifs (*19*), respectively.

235–915 of the cloned *cdc25* cDNA) encoding a C-terminal region was placed under control of the *tac* promoter in expression vector pUSI2ENd (*25*). The ATG site used as the translational start site corresponds to Met362 of starfish Cdc25 (DDBJ, AB076395). This C-terminal domain, designated as "Cdc25ct," is a polypeptide of 226 amino acid residues with a predicted molecular weight of 26,477 (Fig. 1). The predicted amino acid sequence of the domain contains the partial amino acid sequence (residues 444–553) reported by Ashcroft *et al.* (*23*), and is essentially identical with the corresponding region of the sequence that appears in the database (DDBJ, AB076395). It showed high similarity with Cdc25 of other animals as well (*23*) and contained the "cdc25 box" (I/V)FHCExSxxR (*15*), a motif highly conserved among Cdc25 proteins from animals to yeast, as well as CH2A (residues 444–466) and CH2B (residues 524–553) motifs (*19*). No consensus sequence, (S/T)PxZ (X, polar amino acid; Z, generally basic amino acid), for potential Cdc2 substrates (*34*) was found in the sequence.

E. coli JM 109 was transformed with expression plasmid pUSI2ENd(cdc25ct). When the recombinant *E. coli* cells were induced with IPTG, a thick protein band corresponding to M_r 27,000 was observed upon SDS-PAGE of the homogenates (data not shown). This M_r value was in good agreement with the predicted molecular weight of Cdc25ct. Although a major part of the peptide existed in the precipitant fraction, a significant amount of the protein was present in the supernatant fraction as well. The supernatant fraction showed distinct phosphatase activity toward *p*-nitrophenyl phosphate. When the His_{10}-tagged Cdc25ct was expressed in *E. coli* [BL21(DE3)], similar distribution in both the supernatant and precipitant fractions was observed. After purification of His_{10}-tagged Cdc25ct on a Ni-NTA column, it was subjected to limited proteolysis with enterokinase, followed by Edman sequencing. The N-terminal amino acid sequence obtained for the major band material coincided with that predicted for Cdc25ct (Fig. 1).

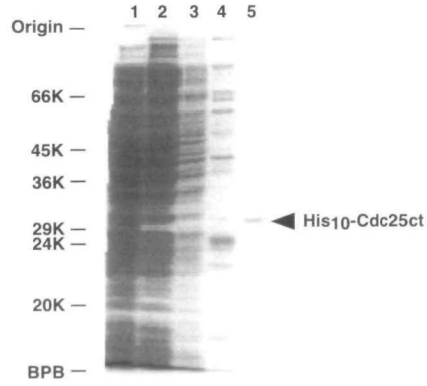

Fig. 2. **Purification of His_{10}-tagged Cdc25ct by metal-chelate affinity chromatography on an Ni-NTA agarose column.** Fractions at each purification step were subjected to SDS-PAGE, followed by protein staining, as described in the text. Molecular weight markers, SDS-7 (Sigma). BPB, bromophenol blue. Lanes: 1, crude extract; 2, flow-through from the Ni-NTA agarose column; 3, wash with the buffer containing 10 mM imidazole; 4, wash with the buffer containing 60 mM imidazole; 5, eluate from the column with the buffer containing 250 mM imidazole.

Phosphatase Activity of the C-terminal Domain of Starfish Cdc25—Starfish Cdc25ct overexpressed in *E. coli* was purified to homogeneity on an Ni-NTA column (Fig. 2). As shown in Fig. 3A, the purified Cdc25ct showed phosphatase activity toward *p*-nitrophenyl phosphate, like those of other animals (*11–14*). The K_m and k_{cat} values for this substrate were 1.5 mM and 0.13 s^{-1}, respectively. The reaction proceeded linearly with incubation time. The presence of a sulfhydryl compound, such as mercaptoethanol and dithiothreitol, had a protective effect against inactivation of the enzyme during dialysis. Glycerol also stabilized the phosphatase activity during storage.

Inhibition by SH Inhibitors—When Cdc25ct was treated with either *N*-ethylmaleimide, iodoacetamide or *p*-chloromercuribenzoate, phosphatase activity was completely lost (data not shown). This indicated that SH group(s) of the enzyme are essential for the catalytic activity of Cdc25, in accordance with the results obtained with *Drosophila* (*12*) and *S. pombe* (*14*) Cdc25cts.

Site-Directed Mutagenesis—To determine catalytically important residues, Cdc25ct mutants in which one of the conserved amino acid residues was mutated by site-directed mutagenesis were expressed, purified, and assayed for phosphatase activity. As shown in Table II, phosphatase

TABLE II. **Phosphatase activity of Cdc25ct mutants.**[a]

Mutant	Specific activity	
	(nmol/min/mg of protein)	(%)
Wild type	176	(100)
C493S	1.4	0.81
R499A	3.0	1.7
H492A	22	13

[a]Phosphatase activity was measured with 16 μg of each mutant Cdc25ct using *p*-nitrophenyl phosphate as a substrate. The experimental details are given in the text.

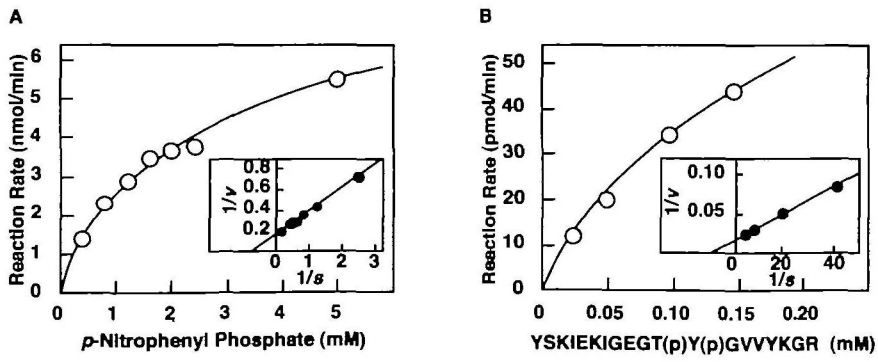

Fig. 3. **Effects of varying substrate concentrations on the rate of dephosphorylation.** Insets, Lineweaver-Burk plots. The amounts of Cdc25ct used were 5.9 munits in A (volume, 1.0 ml) and 0.11 munit in B (volume, 60 μl), respectively. A, *p*-nitrophenyl phosphate; B, phosphopeptide YSKIEKIGEGT(p)Y(p)GVVYKGR.

activity was completely abolished by mutation of Cys493 to Ser, indicating again that the SH group is absolutely required for the catalytic activity. The R499A mutant was also essentially inactive, but the H492A mutant retained partial activity. Thus, it is evident that Arg499 is essential for the activity, whereas His492 is important but not absolutely necessary.

Mechanism of Action of Cdc25 Phosphatase—According to the recently reported X-ray structure of the C-terminal domain of human Cdc25B, the guanidinium group of Arg479 (Arg499 in starfish Cdc25) and main-chain amide NH groups forming a phosphate-binding loop interact with a sulfate anion through hydrogen bonding and a salt bridge (*20*). This loop would not only fix the phosphate group in-line to the catalytic Cys473 residue (Cys493 in starfish Cdc25), but also activate it by stabilizing the oxyanion. Furthermore, the SH group of catalytic Cys473 would be activated to thiolate anion by nearby functional groups on the loop. In human Cdc25B, Glu478 on the loop was proposed to function as a general acid (*20*) and to help the phenolic oxygen in the substrate to leave. However, it seems unlikely that Lys498 in starfish Cdc25 corresponding to Glu478 in human Cdc25B serves as a general acid. Instead, Glu494 may function as a general acid in starfish Cdc25, since Glu431 in human Cdc25A was also proposed to be a general acid for human Cdc25A (*19*). The above-mentioned results of inhibition and mutation experiments, together with the high similarity in the consensus active-site sequence, suggest strongly that the starfish Cdc25-catalyzed phosphatase reaction proceeds through a two-step mechanism, as shown in Fig. 4. That is, the thiolate anion of Cys493 makes an in-line nucleophilic attack on the phosphorus atom of phosphomonoester from the backside, forming a phospho-Cys intermediate. The resulting phosphoenzyme undergoes hydrolysis, leading to the formation of orthophosphate and regeneration of the thiolate anion of catalytic Cys493. Such a mechanism seems to be common to protein tyrosine phosphatases (*36, 37*), and dual-specificity phosphatases (*38*) having the signature motif HCxxxxx-R (*39*).

Activation of preMPF—When purified Cdc25ct was incubated with starfish preMPF that had been affinity-purified from extracts of immature oocytes with Suc1-beads, histone H1 kinase activity increased markedly depending upon the amount of Cdc25ct (data not shown). This activation of preMPF was accompanied by an increase in electrophoretic mobility (data not shown). The upper band was assigned to the inactive form of Cdc2 kinase, and the lower band to the active from of Cdc2 kinase (*40*). It has been established that active MPF contains the Thr14- and Tyr15-dephosphorylated, Thr160-phosphorylated form of Cdc2 kinase, whereas all the three residues are phosphorylated in

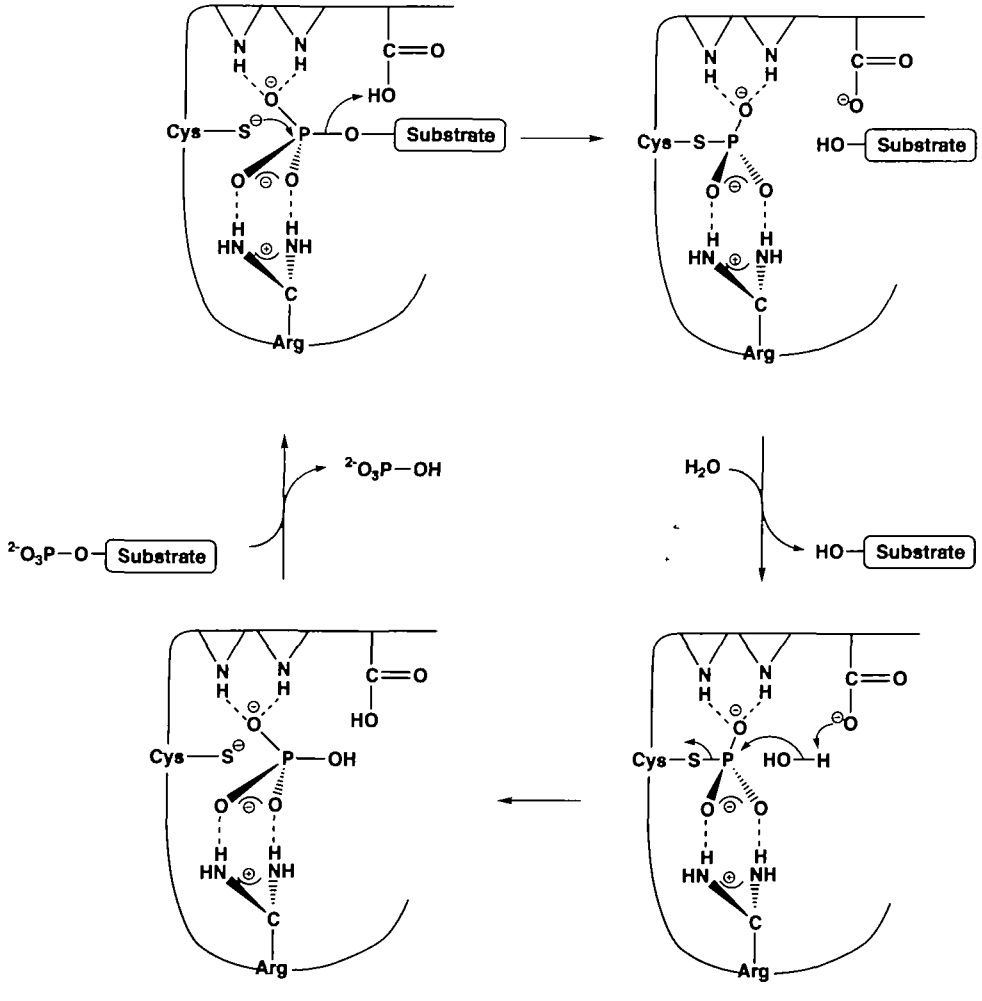

Fig. 4. **Possible mechanism of action of Cdc25.** The crystal structure of human Cdc25B suggests that the SH group of Cys473, the guanidinium group of Arg479, the COOH and the >NH groups of Glu478, and the >NH group of Ser477 are involved in catalysis.

preMPF (*41*). Thus, it was concluded that starfish Cdc25ct served as a dual-specificity phosphatase toward preMPF like those of other animals (*11–14*). Activation of preMPF did not occur with catalytically inactive Cdc25ct mutants (C493S and R499A), whereas preMPF was activated by the H492A mutant (Fig. 5). Essentially the same results have been reported for *Drosophila* and *S. pombe* Cdc25cts (*13*).

Substrate Recognition by Cdc25—Although Cdc25 phosphatases are known to be highly specific for Cdks (*11–13, 42*), their mechanism of substrate recognition remains to be elucidated. It is of general interest to study, with synthetic phosphopeptides, how Cdc25 phosphatases recognize substrates. Based on the X-ray structure of the Cdk2·cyclin A complex (*43*), we designed a series of synthetic phosphopeptides as substrates. The peptide YSKIEKIGEGT(p)Y(p)-GVVYKGR mimics the amino acid sequence near the dephosphorylation site (residues 4–22) that is conserved in both Cdk2 and Cdc2. It includes residues comprising parts of the β-strands and the loop moiety of Cdk2, phospho-Thr14 and phospho-Tyr15 of which are dephosphorylated by Cdc25 (*11–13, 42*). The results of kinetic analysis shown in Fig. 3B indicate that this diphosphopeptide underwent dephosphorylation by Cdc25ct with K_m and k_{cat} values of 0.24 mM and 0.12 s^{-1}, respectively. As judged from the k_{cat}/K_m values, the diphosphorylated substrate was an about 6-times better one than *p*-nitrophenyl phosphate. This is due to the higher affinity for the diphosphopeptide substrate, since *p*-nitrophenyl phosphate should be a better substrate due to its chemical nature (lower nucleophilicity of *p*-nitrophenolate than phenolate or alkoxide).

As shown in Fig. 6A, phospho-Tyr-containing and phospho-Thr-containing monophosphopeptides with the same sequence were much poorer substrates than the diphosphopeptide, the substrate activity decreasing in this order. Judging from these results with synthetic phosphopeptides, the presence of two phosphorylated residues is important for substrate recognition by Cdc25, and the intrinsic chemical natures of phosphoamino acid residues are also a determinant of substrate activity. When the products dephosphorylated by Cdc25 from this diphosphopeptide were analyzed by high-performance liquid chromatography, a phospho-Thr-containing monophosphopeptide was transiently and almost exclusively formed as an intermediate and then further converted very slowly to the corresponding dephosphopeptide (Fig. 7). This finding indicated that phospho-Tyr and phospho-Thr were dephosphorylated in this order. This is the reverse order to that reported for the *in vitro* dephosphorylation of preMPF by GST-Cdc25A or

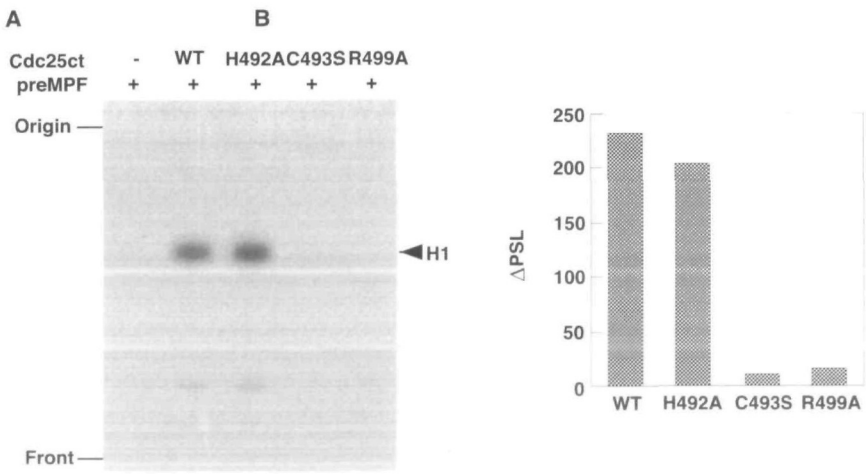

Fig. 5. **Activation of PreMPF by Cdc25ct mutants.** PreMPF was incubated with 1.1 μg of either the wild-type or a mutant Cdc25ct, and then histone H1 kinase activity was determined, as described in the text. A: Autoradiography. B: Quantitation of the radioactivity in the histone H1 band.

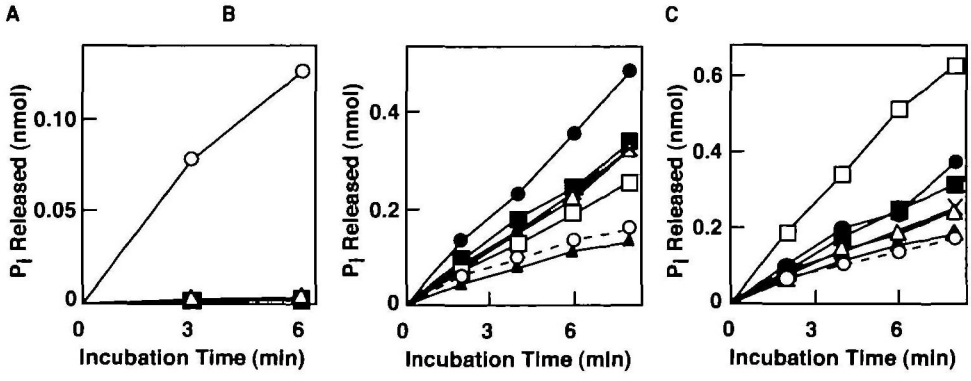

Fig. 6. **Substrate activity of synthetic phosphopeptides.** The assay was carried out essentially as described in the text using 121 μM synthetic phosphopeptides. About 0.13 munit of Cdc25ct was used in each assay mixture. A: YSKIEKIGEGT(p)-Y(p)GVVYKGR (open circles); Y-SKIEKIGEGTY(p)GVVYKGR (open triangles); YSKIEKIG-EGT(p)YGVVYKGR (closed squares). B: YSKIEKIGEGT-(p)Y(p)GVVYKGR (open circle); IEKIGEGT(p)Y(p)GVVY-NH2 (closed circles); KIGEGT(p)Y(p)-GV-NH_2 (open squares); GEGT-(p)Y(p)G-NH_2 (closed squares); EGT(p)Y(p)G-NH_2 (open triangles); GT(p)Y(p)G-NH_2 (closed triangles); T(p)Y(p)-NH_2 (crosses). C. YSKI-EKIGEGT(p)Y(p)GVVYKGR (open circles); GEGT(p)Y(p)G-NH_2 (closed circles); GEGY(p)T(p)G-NH_2 (open squares); GEGS(p)Y(p)G-NH_2 (closed squares); GEGT(p)T(p)G-NH_2 (open triangles); GAGT(p)Y(p)G-NH_2 (closed triangles); GEGT(p)GY(p)G-NH_2 (crosses). –NH_2 indicates that the C-terminals of the peptides are amidated.

GST-Cdc25C, and *in vivo* dephosphorylation during the prophase/metaphase transition in starfish oocytes (*44*). This discrepancy suggests that the diphosphopeptide substrate is not bound in the same orientation as that of the enzyme-bound loop moiety of Cdc2 to be dephosphorylated. This constitutes clear evidence for the importance of the protein-protein interaction between Cdc25 and Cdc2—that is, phospho-Thr in the dephosphorylation site of Cdc2 may be forced to bind to the catalytic residues of Cdc25 upon the formation of a transient complex between the two proteins. After the hydrolysis of phospho-Thr, phospho-Tyr of Cdc2 would then bind to the catalytic site and undergo hydrolysis without dissociation of the complex. In contrast, Fig. 7 suggests that the diphosphorylated peptide substrate first undergoes hydrolysis into a monophosphorylated intermediate and then dissociates from the enzyme. In yeast, Tyr15 phosphorylation/dephosphorylation directly regulates Cdc2 function, and phospho-Thr as well as phospho-Tyr decreases as cells enter mitosis (*8*). The protein–protein interaction between Cdc25 and Cdc2 would be important for the recognition of protein substrates in yeast as well.

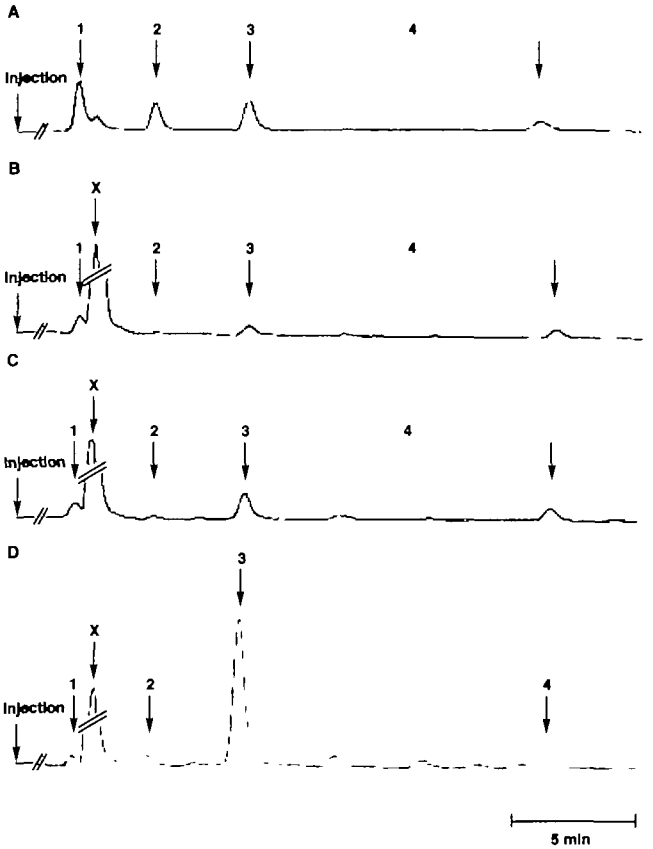

Fig. 7. **Analysis of the dephosphorylation products derived from a diphosphopeptide substrate by high-performance liquid chromatography.** About 1.5 milliunits of Cdc25ct was incubated with 121 μM YSKIEKIGEGT(p)Y(p)GVVYKGR at 30°C for 0.5, 1, and 3 h, in a volume of 60 μl. To the reaction mixture was added ethanol to a final concentration of 80%. After the denatured protein had been removed by centrifugation at 14,000 rpm for 10 min, the supernatant solution was evaporated to dryness under reduced pressure. The residue was taken up into 100 μl of water and then analyzed by chromatography of a sample on a reversed-phase column (8.0 mm i.d. × 25 cm) of Cosmosil $5C_{18}$-AR (Nacalai Tesque, Kyoto) with 20% acetonitrile containing 0.1% trifluoroacetic acid at a flow rate of 1 ml/min. The peptides were detected as to the absorption at 280 nm. A: Mixtures of YSKIEKIGEGT(p)Y(p)GVVYKGR (1), YSKIEKIGEGTY(p)GVVYKGR (2), YSKIEKIGEGT(p)YGVVYKGR (3), and YSKIEKIGEGTYGVVYKGR (4). B: 0.5 h. C: 1 h. D: 3 h. Assignments of the numbered peaks were confirmed by co-chromatography (data not shown). X was a peak due to unknown compound(s) included in the purified preparation of Cdc25ct.

Shorter diphosphopeptides were synthesized and assayed for substrate activity (Fig. 6B). The diphosphorylated tridecapeptide, which has a Rossmann fold-like sequence constituting a part of the ATP-binding site, exhibited the highest substrate activity. However, it should be noted that even diphosphorylated dipeptide T(p)Y(p) was as active as penta-, hexa- and nonapeptides, and rather more active than tetra- and nonadecapeptides, indicating that the Rossmann fold-like sequence is not essential. Thus, it was concluded again that the presence of two phosphorylated residues is important for substrate recognition by Cdc25. The specificity for the two phosphorylated residues was also investigated (Fig. 6C). GEGY(p)T(p)G was more active than GEGT(p)Y(p)G. The diphosphorylated hexapeptide containing phospho-Ser in place of phospho-Thr was as active as the phospho-Thr-containing counterpart. The hexapeptide containing two phospho-Thr also served as a substrate. The diphosphorylated heptapeptide in which phospho-Thr and phospho-Tyr were separated by a Gly residue also served as a substrate. Furthermore, substrate activity significantly decreased on substitution of Glu by Ala, suggesting that the negative charge of the conserved Glu residue in the dephosphorylation site is also effective for potentiating the substrate recognition by Cdc25.

From the data shown in Fig. 6, it is not likely that the local amino acid sequence near the dephosphorylation site is very important. But, of course, matching of the three-dimensional structure of a substrate protein with that of Cdc25 phosphatase should be a primarily important determinant for the Cdc25-catalyzed dephosphorylation. Ishida *et al.* (*45*) also reported that the primary structure around the dephosphorylation site is less important for Ca^{2+}/calmodulin-dependent protein kinase phosphatase, and concluded that the substrate specificity of the enzyme is determined by the higher-order structure of the substrate protein rather than the primary structure around its dephosphorylation site. Thus, it may be generally concluded that steric allowance and electrostatic/hydrogen bonding interactions with phosphate group(s) of proteins are essential for substrate recognition by protein phosphatases, whereas the local amino acid sequence near the dephosphorylation site is less important.

REFERENCES

1. Rohka, M.J., Hayes, M.K., and Maller, J.L. (1988) Purification of maturation-promoting factor, an intracellular regulator of early mitotic events. *Pro. Natl. Acad. Sci. USA* **85**, 3009–3013
2. Nurse, P. (1990) Universal control mechanism regulating onset of M-phase. *Nature* **344**, 503–508
3. Gautier, J., Norbury, C., Lohka, M., Nurse, P., and Maller, J. (1988) Purified maturation-promoting factor contains the product of a *Xenopus* homolog of the fission yeast cell cycle control gene *cdc2*$^+$. *Cell* **54**, 433–439
4. Gautier, J., Minshull, J., Lohka, M., Glotzer, M., Hunt, T., and Maller, J.L. (1990) Cyclin is a component of maturation-promoting factor from *Xenopus*. *Cell* **60**, 487–494

5. Evans, T., Rosenthal, E.T., Youngblom, J., Distel, D., and Hunt, T. (1983) Cyclin: A protein specified by maternal mRNA in sea urchin eggs that is destroyed at each cleavage division. *Cell* **33**, 389–396
6. Fantes, P. (1979) Epistatic gene interactions in the control of division in fission yeast. *Nature* **279**, 428–430
7. Russell, P. and Nurse, P. (1986) *cdc25*$^{+}$ functions as an inducer in the mitotic control of fission yeast. *Cell* **45**, 145–153
8. Gould, K.L. and Nurse, P. (1989) Tyrosine phosphorylation of the fission yeast cdc2^{+} protein kinase regulates entry into mitosis. *Nature* **342**, 39–45
9. Krek, W. and Nigg, E. (1991) Mutations of p34cdc2 phosphorylation sites induce premature mitotic events in HeLa cells; evidence for a double block to p34cdc2 kinase activation in vertebrates. *EMBO J.* **10**, 3331–3341
10. Honda, R., Ohba, Y., Nagata, A., Okayama, H., and Yasuda, H. (1993) Dephosphorylation of human p34cdc2 kinase on both Thr-14 and Tyr-15 by human cdc25B phosphatase. *FEBS Lett.* **318**, 331–334
11. Strausfeld, U., Labbé, J.C., Fesquet, D., Cavadore, J.C., Picard, A., Sadhu, K., Russell, P., and Dorée, M. (1991) Dephosphorylation and activation of a p34cdc2/cyclin B complex in vitro by human CDC25 protein. *Nature* **351**, 242–245
12. Dunphy, W.G. and Kumagai, A. (1991) The cdc25 protein phosphatase contains an intrinsic phosphatase activity. *Cell* **67**, 189–196
13. Gautier, J., Solomon, M.J., Booher, R.N., Bazan, J.F., and Kirschner, M.W. (1991) cdc25 is a specific tyrosine phosphatase that directly activates p34^{cdc2}. *Cell* **67**, 197–211
14. Millar, J.B.A., McGowan, C.H., Lenaers, G., Jones, R., and Russell, P. (1991) p80^{cdc25} mitotic inducer is the tyrosine phosphatase that activates p34^{cdc2} kinase in fission yeast. *EMBO J.* **10**, 4301–4309
15. Galaktionov, K. and Beach, D. (1991) Specific activation of cdc25 tyrosine phosphatases by B-type cyclins: Evidence for multiple roles of mitotic cyclins. *Cell* **67**, 1181–1194
16. Lammer, C., Wagerer, S., Saffrich, R., Mertens, D., Ansorge, W., and Hoffmann, I. (1998) The Cdc25B phosphatase is essential for the G2/M phase transition in human cell. *J. Cell Sci.* **111**, 2445–2453
17. Hoffmann, I., Clarke, P.R., Marcote, M.J., Karsenti, E., and Draetta, G. (1993) Phosphorylation and activation of human cdc25-C by cdc2-cyclin B and its involvement in the self-amplification of MPF at mitosis. *EMBO J.* **12**, 53–63
18. Hoffmann, I., Draetta, G., and Karsenti, E. (1994) Activation of the phosphatase activity of human cdc25A by a cdk2-cyclin E dependent phosphorylation at the G1/S transition. *EMBO J.* **13**, 4302–4310
19. Fauman, E.B., Cogswell, J.P., Lovejoy, B., Rocque, W.J., Holmes, W., Montana, V.G., Piwnica-Worms, H., Rink, M.J., and Saper, M.A. (1998) Crystal structure of the catalytic domain of the human cell cycle control phosphatase, Cdc25A. *Cell* **93**, 617–625
20. Reynolds, R.A., Yem, A.W., Wolfe, C.L., Deibel, M.R., Jr., Chidester, C.G., and Watenpaugh, K.D. (1999) Crystal structure of the catalytic subunit of Cdc25B required for G2/M phase transition of the cell cycle. *J. Mol. Biol.* **293**, 559–568
21. Kishimoto, T. (1996) Starfish maturation-promoting factor. *TIBS* **21**, 35–37
22. Okumura, E., Sekiai, T., Hisanaga, S., Tachibana, K., and Kishimoto, T. (1996) Initial triggering of M-phase in starfish oocytes: A possible novel component of maturation-promoting factor besides cdc2 kinase. *J. Cell Biol.* **132**, 125–135
23. Ashcroft, N.R., Kosinski, M.E., Wickramasinghe, D., Donovan, P.J., and Golden, A. (1998) The four *cdc25* genes from the nematode *Caenorhabditis elegans*. *Gene* **214**, 59–66
24. Wakamiya, T., Togashi, R., Nishida, T., Saruta, K., Yasuoka, J., Kusumoto, S., Aimoto, S., Kumagaye, K.Y., Nakajima, K., and Nagata, K. (1997) Synthetic study of phosphopeptides related to heat shock protein HSP27. *Bioorg. Med. Chem.* **5**, 135–145
25. Toraya, T. and Mori, K. (1999) A reactivating factor for coenzyme B_{12}-dependent diol dehydratase. *J. Biol. Chem.* **274**, 3372–3377
26. Maniatis, T., Fritsch, E.F., and Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
27. Miyake, Y., Deshimaru, S., and Toraya, T. (2001) Revised sequence and expression of cyclin B cDNA from starfish *Asterina pectinefera*. *Biosci. Biotech. Biochem.* **65**, 1119–1126
28. Petty, K.J. (1993) Metal-chelate affinity chromatography. in *Current Protocols in Molecular Biology* (Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K., eds.), Suppl. **24**, 10.11.8–22, John Wiley & Sons, New York
29. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685
30. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**, 265–275
31. Gill, S.C. and von Hippel, P.H. (1989) Calculation of protein extinction coefficients from amino acid sequence data. *Anal. Biochem.* **182**, 319–326
32. Baykov, A.A., Evtushenko, O.A., and Avaeva, S.M. (1988) A malachite green procedure for orthophosphate determination and its use in alkaline phosphatase-based enzyme immunoassay. *Anal. Biochem.* **171**, 266–270
33. Brizuela, L., Draetta, G., and Beach, D. (1987) p13^{suc1} acts in the fission yeast cell division cycle as a component of the p34^{cdc2} protein kinase. *EMBO J.* **6**, 3507–3514
34. Moreno, S. and Nurse, P. (1990) Substrates for p34^{cdc2}: In vivo veritas. *Cell* **61**, 549–551
35. Eckstein, J.W., Beer-Romero, P.B., and Berdo, I. (1996) Identification of an essential acidic residue in Cdc25 protein phosphatase and a general three-dimensional model for a core region in protein phosphatases. *Protein Sci.* **5**, 5–12
36. Barford, D., Flint, A.J., and Tonks, N.K. (1994) Crystal structure of human protein tyrosine phosphatase 1B. *Science* **263**, 1397–1404
37. Stuckey, J.A., Schubert, H.L., Fauman, E.B., Zhang, Z-Y., Dixon, J.E., and Saper, M.A. (1994) Crystal structure of *Yersinia* protein tyrosine phosphatase at 2.5 Å and the complex with tungstate. *Nature* **370**, 571–575
38. Yuvaniyama, J., Denu, J.M., Dixon,J. E., and Saper, M.A. (1996) Crystal structure of the dual specificity protein phosphatase VHR. *Science* **272**, 1328–1331
39. Stone, R.L. and Dixon, J.E. (1994) Protein-tyrosine phosphatase. *J. Biol. Chem.* **269**, 31323–31326
40. Ookata, K., Hisanaga, S., Okano, T., Tachibana, K., and Kishimoto, T. (1992) Relocation and distinct subcellular localization of p34^{cdc2}-cyclin B complex at meiosis reinitiation in starfish oocytes. *EMBO J.* **11**, 1763–1772
41. Gautier, J. and Maller, J.L. (1991) Cyclin B in Xenopus oocytes: implications for the mechanism of pre-MPF activation. *EMBO J.* **10**, 177–182
42. Gabrielli, B.G., Lee, M.S., Walker, D.H., Piwnica-Worms, H., and Maller, J.L. (1992) Cdc25 regulates the phosphorylation and activity of the *Xenopus* cdk2 protein kinase complex. *J. Biol. Chem.* **267**, 18040–18046
43. Jeffrey, P.D., Russo, A.A., Polyak, K., Gibbs, E., Hurwitz, J., Massague, J., and Pavletich, N.P. (1995) Mechanism of CDK activation revealed by the structure of a cyclin A-CDK2 complex. *Nature* **376**, 313–320
44. Borgne, A. and Meijer, L. (1996) Sequential dephosphorylation of p34^{cdc2} on Thr-14 and Tyr-15 at the prophase/metaphase transition. *J. Biol. Chem.* **271**, 27847–27854
45. Ishida, A., Shigeri, Y., Tatsu, Y., Endo, Y., Kameshita, I., Okuno, S., Kitani, T., Takeuchi, M., Yumoto, N., and Fujisawa, H. (2001) Substrate specificity of Ca^{2+}/calmodulin-dependent protein kinase phosphatase: Kinetic studies using synthetic phosphopeptides as model substrates. *J. Biochem.* **129**, 745–753