Purification of a Serine Kinase That Associates with and Phosphorylates Human Cdc25C on Serine 216*

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Scott Ogg‡§, Brian Gabrielli§¶, and Helen Piwnica-Worms§‖

From the ‡Department of Physiology, Tufts University School of Medicine, Boston, Massachusetts 02111 and the §Beth Israel Hospital and Department of Microbiology and Molecular Biology, Harvard Medical School, Boston, Massachusetts 02115

Human Cdc25C is a protein phosphatase that dephosphorylates and activates Cdc2-cyclin B to trigger entry into mitosis. Cdc25C is itself regulated by phosphorylation. In asynchronously growing HeLa cells, we have determined that serine 216 is the major site of Cdc25C phosphorylation. We have isolated a protein kinase that binds to Cdc25C and phosphorylates serine 216. The kinase binds within amino acids 200–256 of Cdc25C. This region is conserved in some Cdc25 homologues and contains a putative bipartite nuclear localization signal just downstream from serine 216. Finally, the Cdc25C-associating kinase was purified over 8000-fold from rat liver as a 36–38-kDa doublet of proteins.

Progression through the eukaryotic cell cycle involves the sequential activation of cyclin-dependent kinases (Cdks)[1] (reviewed in Refs. 1 and 2). As the name implies, activation of a Cdk is dependent upon its association with a cyclin regulatory subunit. Cdk-cyclin complexes can also be regulated by reversible phosphorylation, which helps to ensure the proper timing of activation during the cell cycle (reviewed in Ref. 3). Entry of cells into mitosis is regulated in part by the activity of the Cdc2-cyclin B complex, which serves as a paradigm for the Cdk family. Cyclin B is synthesized and associates with Cdc2 in the cytoplasm beginning in S-phase and continuing throughout G_2-phase (4). In higher eukaryotic cells, cyclin B association induces the rapid phosphorylation of Cdc2 on three sites. Phosphorylation on threonine 161 is required for kinase activity, whereas phosphorylation on threonine 14 and tyrosine 15 suppresses kinase activity (5–12). It is the phosphorylation of threonine 14 and tyrosine 15 that maintains Cdc2-cyclin B in an inactive state. The kinase responsible for phosphorylating threonine 14 has not been identified, but two kinases, Wee1 and Mik1, have been identified that regulate the phosphorylation of tyrosine 15 (7–9, 13–15). Dephosphorylation of threonine 14 and tyrosine 15 is the final step in the activation of Cdc2-cyclin B and is required for entry of cells into mitosis (6, 11, 16, 17).

Dephosphorylation and activation of Cdc2-cyclin B is dependent upon the Cdc25 phosphatase. Cdc25 was first identified as a cell cycle mutant in *Schizosaccharomyces pombe* that acts antagonistically to Wee1 (18, 19). Homologues of Cdc25 have been reported in several other eukaryotes, including three in humans (denoted A, B, and C) (20, 21). Cdc25 proteins have a conserved carboxyl-terminal domain that has limited homology to phosphotyrosine-specific protein phosphatases (22). The homology region includes the "signature" motif (HC*XXXXX*R, where *X* represents any amino acid) that is conserved among the active sites of phosphotyrosine-specific protein phosphatases. Cdc25 proteins possess intrinsic phosphatase activity toward tyrosine 15-phosphorylated Cdc2 complexed with cyclin B (23–27). Cdc25 will also dephosphorylate threonine 14- and tyrosine 15-phosphorylated Cdk2 complexed with cyclin A (28). Furthermore, mutation of the cysteine residue within the signature motif of Cdc25 abolishes its phosphatase activity (23–25).

The Cdc25C protein is present throughout the cell cycle in higher eukaryotes, whereas its substrate (Cdc2-cyclin B) accumulates throughout the S- and G_2-phases of the cell cycle (20, 29, 30). The interesting question arises as to how Cdc25C and Cdc2-cyclin B are prevented from functionally interacting until the appropriate time in the cell cycle. Cyclin B is synthesized during S-phase and accumulates in the cytoplasm in a complex with Cdc2 (4). As HeLa cells (4) and starfish oocytes (31) enter mitosis, Cdc2-cyclin B complexes enter the nucleus. Although Cdc2-cyclin B becomes activated in the cytoplasm of starfish oocytes (31, 32) and *Xenopus* oocytes (33, 34), it is less clear where Cdc2-cyclin B becomes activated in mammalian tissue culture cells. Consistent with the activation of Cdc2-cyclin B in the cytoplasm of *Xenopus* oocytes, it has been found that *Xenopus* Cdc25 is exclusively localized in the cytoplasm (35). The subcellular localization of Cdc25C in mammalian tissue culture cells is less clear. In rat and human fibroblasts (29) and HeLa cells (20), endogenous Cdc25C has been found in the nucleus during interphase. Other reports suggest that Cdc25C is localized in the cytoplasm in hamster cells (36) or when human Cdc25C is overexpressed in HeLa or baby hamster kidney cells (37).[2] A number of Cdc25 proteins contain a region with homology to bipartite nuclear localization signals. There are a number of potential phosphorylation sites near the putative nuclear localization signal, suggesting the possibility that the subcellular localization, and therefore interaction with Cdc2-cyclin B, may be controlled by phosphorylation.

Phosphorylation has been found to directly activate the enzymatic activity of Cdc25C (35, 38–40). Cdc25C becomes phosphorylated and activated as cells progress into M-phase (35, 38, 40–43). Studies have suggested that Cdc2-cyclin B phosphorylates and activates Cdc25C leading to an "autoactivation loop"

[1] The abbreviations used are: Cdk, cyclin-dependent kinases; GST, glutathione S-transferase; KWB, kinase wash buffer; HPLC, high performance liquid chromatography.

[2] L. Paige and H. Piwnica-Worms, unpublished observation.

(39, 40, 44). However, other studies suggest the existence of other kinases that phosphorylate and activate Cdc25 proteins (35, 39). To understand the regulation of Cdc25C, it is important to identify phosphorylation sites of Cdc25C *in vivo* and to identify the responsible kinases.

In this study, we identify serine 216 as the major phosphorylation site in Cdc25C isolated from asynchronously growing HeLa cells. In addition, we report the purification of a protein kinase from rat liver that phosphorylates Cdc25C *in vitro* on serine 216. Interestingly, this kinase not only phosphorylates Cdc25C, but it also binds to a region (bordered by amino acids 200–256) located within the amino terminus of Cdc25C. This binding region of Cdc25C contains a putative bipartite nuclear localization sequence positioned just downstream of serine 216 and is conserved among several Cdc25 family members.

EXPERIMENTAL PROCEDURES

Tissue Culture and ^{32}P Labeling of Cdc25C—HeLa cells were grown in Dulbecco's modified Eagle's medium supplemented with 2 mM glutamine and 10% fetal calf serum. HeLa cells were ^{32}P-labeled as follows. Cells were grown to approximately 80% confluence on 10-cm² tissue culture dishes. Cells were washed with prewarmed phosphate-free Dulbecco's modified Eagle's medium. Cells were labeled for 3 h at 37 °C in 1.5 ml of phosphate-free Dulbecco's modified Eagle's medium supplemented with 2 mCi/ml ^{32}P-labeled inorganic phosphate, 2 mM glutamine, and 10% dialyzed calf serum. Labeled cells were washed with phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄·7H₂O, 1.4 mM KH₂PO₄, pH 7.3) and lysed in 1.2 ml of NETN buffer (20 mM Tris, pH 8.0, 0.5% Nonidet P-40, 1 mM EDTA, 100 mM NaCl) adjusted to 1 M NaCl and supplemented with 2 mM phenylmethylsulfonyl fluoride, 0.15 units/ml aprotinin, 20 μM leupeptin, 20 μM pepstatin, 5 μM microcystin, 10 mM NaF, and 0.5 mM sodium vanadate. The cell lysates were clarified by centrifugation at 10,000 × g for 15 min. Lysates were precleared with 100 μl of Sepharose CL-4B protein A beads. Immunoprecipitation reactions were performed using 10 μg of either nonimmune IgG or antibody purified to the amino-terminal 258 amino acids of human Cdc25C. Antibody-antigen complexes were purified with 20 μl of Sepharose CL-4B protein A beads and washing 3 times with radioimmune precipitation buffer (20 mM Tris, pH 7.4, 137 mM NaCl, 10% glycerol, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, 2 mM EDTA), 2 times in NETN buffer, and once more with radioimmune precipitation buffer (each supplemented as described above). Proteins were released from the beads by boiling in 100 μl of 1% SDS in phosphate-buffered saline for 5 min. The beads were pelleted and the supernatant diluted into 900 μl of NETN buffer. Cdc25C was reimmunoprecipitated using this same protocol and solubilized from the beads in SDS sample buffer by boiling for 5 min.

Affinity Purification of Cdc25C(N258) Antibody—GST and GST-25(N258) (27) were coupled to activated CH-Sepharose 4B (Pharmacia Biotech Inc.) according to the manufacturer's instructions. Polyclonal GST-25C antisera were passed over a GST-Sepharose column to remove antibodies specific for GST. Unbound antisera were loaded onto a GST-25(N258)-Sepharose column. Nonspecific antibodies were removed by washing with radioimmune precipitation buffer, and then NETN buffer was adjusted to 1 M NaCl. Antibodies specific for Cdc25(N258) were eluted first with 100 mM glycine (pH 2.5) and then with 1% triethylamine (pH 11). All fractions were neutralized with 1 M Tris (pH 8.0). Bovine serum albumin was added to a final concentration of 0.1 mg/ml to each fraction containing antibody. Proteins were precipitated with 50% ammonium sulfate (w/v) and centrifugation. Samples were resuspended in Tris-buffered saline (10 mM Tris, pH 8.0, 0.5 M NaCl) and then dialyzed against Tris-buffered saline.

Purification of GST Fusion Proteins—Expression and purification of GST fusion proteins were accomplished essentially as described (27). Routinely, 100 ml of overnight culture was used to inoculate 1 liter of Luria broth. After a 1-h incubation at 37 °C, isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 0.5 mM. Following a 2-h incubation at room temperature, the cells were collected by centrifugation at 3800 × g for 10 min. The cells were resuspended in phosphate-buffered saline, aliquoted, and then recentrifuged at 5000 × g. Each aliquot represented 20 ml of bacterial culture. Bacterial pellets were stored at −80 °C until use. For use, each frozen bacterial pellet was resuspended in 5 ml of NETN buffer supplemented as described above and containing 0.5 mg/ml lysozyme. The suspensions were incubated at 4 °C for 10 min and sonicated 10 times for 1 s each using a probe tip

sonicator at its maximum setting. The sonicates were centrifuged at 12,000 × g for 10 min. The clarified supernatants were incubated with glutathione (GSH)-agarose beads at 4 °C for 30 min. The beads were then washed 4 times with NETN buffer. Purification of insect cell-produced GST fusion proteins was performed as described (12).

Cdc25-associating Kinase Assays—2–20 μg of bacterially produced GST fusion proteins were purified on 15 μl of GSH-agarose as described above. The beads were rocked with either 0.1 mg of HeLa cell lysate or column fractions diluted into 0.5 ml of NETN buffer for 1–2 h at 4 °C. The beads were pelleted and washed twice with 0.7 ml of NETN buffer and twice with 0.5 ml of kinase wash buffer (KWB; 50 mM Tris, pH 7.4, 10 mM MgCl₂). The beads were pelleted, and reactions were carried out in 25 μl of KWB containing 2 mM dithiothreitol, 20 μM ATP, and 10 μCi of [γ-³²P]ATP (>4000Ci/mmol). In some experiments, soluble substrates were also added. After a 5-min incubation at 30 °C, the reaction was terminated by the addition of 20 μl of 3 × SDS sample buffer and boiling for 5 min. After electrophoresis on SDS-polyacrylamide gels, the proteins were visualized by staining with Coomassie Brilliant Blue and autoradiography. Radioactivity incorporated into protein bands was quantitated by excising the band and Cerenkov counting.

Phosphopeptide Mapping and Phosphoamino Acid Analysis—Tryptic digests were performed essentially as described (45). Digested peptides were resolved by electrophoresis in the first dimension and ascending chromatography in the second as described (7). Two-dimensional phosphoamino acid analysis was performed as described (7).

Sequencing of Cdc25C Phosphorylation Site—75 μg of GST-25(N258) was purified from bacteria using 100 μl of GSH-agarose as described. 1 ml of rat liver P100 (see below) was solubilized in 10 ml of NETN buffer for 10 min and clarified at 10,000 × g for 10 min. The supernatant was incubated with the immobilized GST-25(N258) for 2 h. After 5 washes with NETN buffer and 3 washes with KWB, the beads were incubated in KWB containing 2 mM dithiothreitol, 0.5 μM ATP, and 10 μCi of [γ-³²P]ATP for 30 min at 30 °C. The reaction was stopped by addition of 3 × SDS sample buffer and boiling for 5 min. GST-25(N258) was resolved from the unincorporated radioactivity using an SDS-polyacrylamide gel. GST-25(N258) was excised and incubated in 5 ml of 30% methanol at 37 °C, changing into fresh methanol 4 times over a 24-h period. The gel slice was then treated with 0.5 mg of sequencing grade trypsin (Sigma) in 4 mg/ml ammonium bicarbonate for several hours at 37 °C. Fresh trypsin was added, and the digestion was allowed to continue overnight. Peptides released from the gel slice were stored at −80 °C. The gel slice was treated with fresh trypsin solution once more. Released peptides were pooled and dried *in vacuo*. Peptides were then washed with decreasing volumes of water (100, 100, 50, and 25 μl) and dried *in vacuo*.

Washed peptides were separated using a Hewlett Packard 1090 high pressure liquid chromatography system with a Vydac 2.1-mm C18 reverse phase column. Fractions were Cerenkov counted to identify phosphorylated peptides. One quarter of the major phosphopeptide was subjected to automated Edman degradation and analysis using an Applied Biosystems 477 protein sequencer and Applied Biosystems 120. The rest of the phosphopeptide was subjected to semi-automated amino-terminal sequence analysis using a Beckman 890C spinning cup sequencer (46).

Construction of GST-25(200–256)—Sequences encoding amino acids 200–256 of Cdc25C were amplified by polymerase chain reaction using pGC52(cdc25Hs) as the template (27). The primers were as follows: NH₂-terminal, 5′-GGCAGATCTCCGGATCCAAAGATCAAGAAGCAA-AGG-3′ (contains *Bgl*II and *Bam*HI sites); carboxyl-terminal, 5′-GGGAATTCGTCGACCTATAAGCCCTTCCTGAGCTTTCC-3′ (contains *Eco*RI and *Sal*I sites). The polymerase chain reaction product was digested to completion with *Bgl*II and *Eco*RI and was cloned into *Bam*HI/*Eco*RI-linearized pGEX-3X.

Construction of pGEX-2TN25CHamster—pET3a-hamster Cdc25C was digested with *Nde*I and *Xma*I. The insert encoding full-length hamster Cdc25C was ligated into the *Nde*I/*Xma*I site of pGEX-2TN to generate pGEX-2TN25CHamster.

Purification of Cdc25C-associating Kinase from Rat Liver—The entire purification was done at 4 °C or on ice. 100 g of frozen rat livers (Pel-Freez Biologicals, Rogers, AR) were homogenized using a Waring blender in 200 ml of 20 mM Hepes, pH 7.6, 100 mM NaCl, 1 mM dithiothreitol, 1 mM EGTA, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 20 μM pepstatin, 20 μM leupeptin. The homogenate was clarified using successive centrifugation spins at 1000 × g for 10 min, 10 × kg for 20 min, and twice at 100 × kg for 1 h each. The 100 × kg pellet (P100) contained some Cdc25C-associating kinase activity and was used for certain experiments. The supernatant (S100) was passed through 0.8-micron and then 0.45-micron Nalgene syringe filters. The sample was

loaded onto a 60-ml S-Sepharose Fast Flow column (Pharmacia) equilibrated in buffer A (20 mM Hepes, pH 7.0, 1 mM EGTA, 1 mM EDTA) containing 0.1 M NaCl. After washing with buffer A containing 0.1 M NaCl, bound proteins were eluted with a 100-ml linear gradient of NaCl (0.1–0.6 M). 5-ml fractions were collected and assayed for Cdc25C-associating kinase activity with GST-25(200–256) as the affinity reagent and kinase substrate. Fractions with the highest Cdc25C-associating kinase activity were pooled and buffer exchanged into buffer B (10 mM Tris, pH 8.0, 0.05% Brij-35) containing 20 mM NaCl using a 190-ml Sephadex G-25 column (Pharmacia). Appropriate fractions were pooled and loaded onto a 25-ml Q-Sepharose column (Pharmacia) pre-equilibrated in buffer B containing 20 mM NaCl. Proteins were eluted with a 120-ml linear gradient of NaCl (20–320 mM). 5-ml fractions were collected and assayed under standard conditions. The peak fractions were pooled and diluted with buffer B to reduce the NaCl concentration to 0.1 M. This material was loaded onto a 5-ml ATP-agarose column (Sigma, A-6888) equilibrated in buffer B containing 0.1 M NaCl. After extensive washing, the bound proteins were eluted in buffer B containing 0.8 M NaCl. Eluted proteins were buffer exchanged into buffer C (20 mM Hepes, pH 7.6, 1 mM EGTA, 1 mM EDTA, 0.05% Brij-35) containing 50 mM NaCl using the Sephadex G-25 column described above. Appropriate fractions were pooled and loaded onto a 1-ml Resource S column (Pharmacia). Proteins were eluted with a 5-ml linear gradient of NaCl (50–300 mM). 0.5-ml fractions were collected and assayed under standard conditions. Fractions with Cdc25C-associating kinase activity were pooled and concentrated to 0.7 ml using a Centricon-10 concentrator (Amicon). 0.5 ml was loaded onto a Superose-12 10/30 column (Pharmacia) in buffer C plus 50 mM NaCl. 0.5-ml fractions were collected and assayed under standard conditions. Molecular weight standards were run on the Superose-12 10/30 column at a concentration of approximately 1 mg/ml using the same buffer conditions (bovine serum albumin, 66 kDa; carbonic anhydrase, 29 kDa; cytochrome *c*, 12.4 kDa).

In Gel Kinase Assays—In gel kinase assays were performed essentially as described (47). Samples were resolved on SDS-polyacrylamide gels that had been polymerized in the absence or presence of 0.1 mg/ml GST-25 (200–256). After electrophoresis, the gels were washed twice in 100 ml of 20% isopropanol, 50 mM Tris, pH 8.0 (v/v), for 1 h. The gels were then washed in 100 ml of 50 mM Tris, pH 8.0, 5 mM β-mercaptoethanol for 1 h. The proteins were denatured with two 100-ml washes in 6 M guanidine HCl, 50 mM Tris, pH 8.0, 5 mM β-mercaptoethanol for 1 h each. The proteins were renatured at 4 °C using several 250-ml washes with 50 mM Tris, pH 8.0, 5 mM β-mercaptoethanol, 0.04% Tween-20 over a 16-h period. The gels were then incubated for 30 min at room temperature in renaturation kinase buffer (50 mM Hepes, pH 7.6, 1 mM dithiothreitol, 0.1 mM EGTA, 3 mM magnesium acetate, 0.01% Nonidet P-40). Kinase assays were performed in renaturation kinase buffer containing 20 μM ATP and 40 μCi/ml [γ-^{32}P]ATP. After 1 h at room temperature, the kinase assays were terminated with 5% trichloroacetic acid, 1% sodium pyrophosphate in water (w/w/v). Unincorporated radioactivity was washed away in the same solution using several changes over a 16-h period. The gels were rinsed with water, dried, and subjected to autoradiography.

RESULTS

Phosphorylation State of Cdc25C from Asynchronously Growing HeLa Cells—The phosphorylation state of human Cdc25C was examined by immunoprecipitation of Cdc25C from ^{32}P-labeled HeLa cells. Cdc25C was immunoprecipitated using antibodies affinity purified against its amino-terminal 258 amino acids, released by boiling in 1% SDS, and then re-immunoprecipitated. A phosphoprotein with an apparent molecular mass of 55–57 kDa was specifically immunoprecipitated with anti-Cdc25C antibodies (Fig. 1A, *lane 2*) but not with control antibodies (Fig. 1A, *lane 1*). Cdc25C was eluted from the gel and subjected to tryptic digestion and two-dimensional analysis as well as to phosphoamino acid analysis. One major phosphopeptide was detected (Fig. 1B), and phosphoamino acid analysis revealed phosphoserine (Fig. 1C).

Association of Cdc25C with a Protein Kinase in Vitro—We tested if the kinase responsible for phosphorylating Cdc25C *in vivo* would stably bind to Cdc25C *in vitro* (Fig. 2). GST, GST-25C, GST-25(N258), and GST-25(C215) were purified on GSH-agarose beads (Fig. 2A). Immobilized GST, GST-25C, GST-25(N258), and GST-25(C215) were incubated with HeLa cell
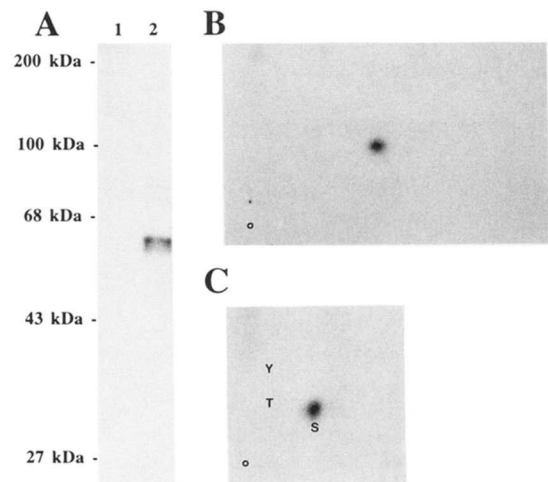

FIG. 1. **Analysis of Cdc25C immunoprecipitated from asynchronously growing HeLa cells.** Asynchronously growing HeLa cells were ^{32}P-labeled and lysed as described under "Experimental Procedures." Either preimmune antibody (*lane 1*) or anti-Cdc25C(N258) antibody (*lane 2*) was used for an immunoprecipitation reaction, and samples were resolved using a 10% SDS-polyacrylamide gel. ^{32}P-labeled proteins were visualized by autoradiography (*panel A*). Cdc25C was eluted from the gel, and portions were subjected to either tryptic digestion and two-dimensional analysis (*panel B*) or phosphoamino acid analysis (*panel C*).

extracts, washed, and assayed for kinase activity either in the absence of added substrate (Fig. 2B, *lanes 1*) or in the presence of soluble GST (Fig. 2B, *lanes 2*) or GST-25C (Fig. 2B, *lanes 3*). A protein kinase activity was detected that bound to and phosphorylated both GST-25C and GST-25(N258). Neither GST nor GST-25(C215) bound the kinase. This experiment localized the kinase binding site and the phosphorylation site to within the first 258 amino acids of Cdc25C. The enhanced phosphorylation of GST-25(N258) compared with GST-25C was not routinely observed in all experiments (see Fig. 6).

Two-dimensional tryptic phosphopeptide mapping of GST-25C phosphorylated *in vitro* by the associating kinase revealed a single phosphopeptide (Fig. 2C), and phosphoamino acid analysis revealed phosphoserine (Fig. 2D). Using this assay, Cdc25C-associating kinase activity has been detected in other human cell lines, *Xenopus* egg extracts,[3] and rat liver (see below).

Serine 216 Is Phosphorylated by the Cdc25C-associating Kinase—To identify the serine in Cdc25C that was phosphorylated by the associating kinase, GST-25(N258) was used as an affinity reagent and substrate for the associating kinase. The P100 fraction from rat liver (see below) was used as the source of kinase. ^{32}P-labeled GST-25(N258) was digested with trypsin, and the resulting peptides were purified by HPLC. Phosphorylated species were identified by Cerenkov counting. One major (15,000 cpm) and three minor (2900, 700, and 200 cpm) radioactive peaks were identified. These different peaks were taken to be different oxidative states of the same phosphopeptide and/or incomplete tryptic digestion products because performic acid treatment (omitted in this experiment) and complete tryptic digestion of either GST-25C or GST-25(N258) gives rise to a single phosphopeptide (see Fig. 2C). The major peak was divided and used for different methods of amino-terminal sequence analysis. The sequence of the phosphopeptide was determined by automated Edman degradation using polybrene-coated glass fiber discs (Fig. 3). Although the site of phosphorylation could be inferred by the lack of serine in

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[3] S. Ogg and H. Piwnica-Worms, unpublished observations.

Purification of a Cdc25C-associating Kinase

A

GST   GST-25C   GST-25(N258) GST-25(C215)

1 2 3   1 2 3   1 2 3   1 2 3

GST-25C -

GST-25(N258) -

GST-25(C215) -

GST -

B

GST   GST-25C   GST-25(N258) GST-25(C215)

1 2 3   1 2 3   1 2 3   1 2 3

GST-25C -

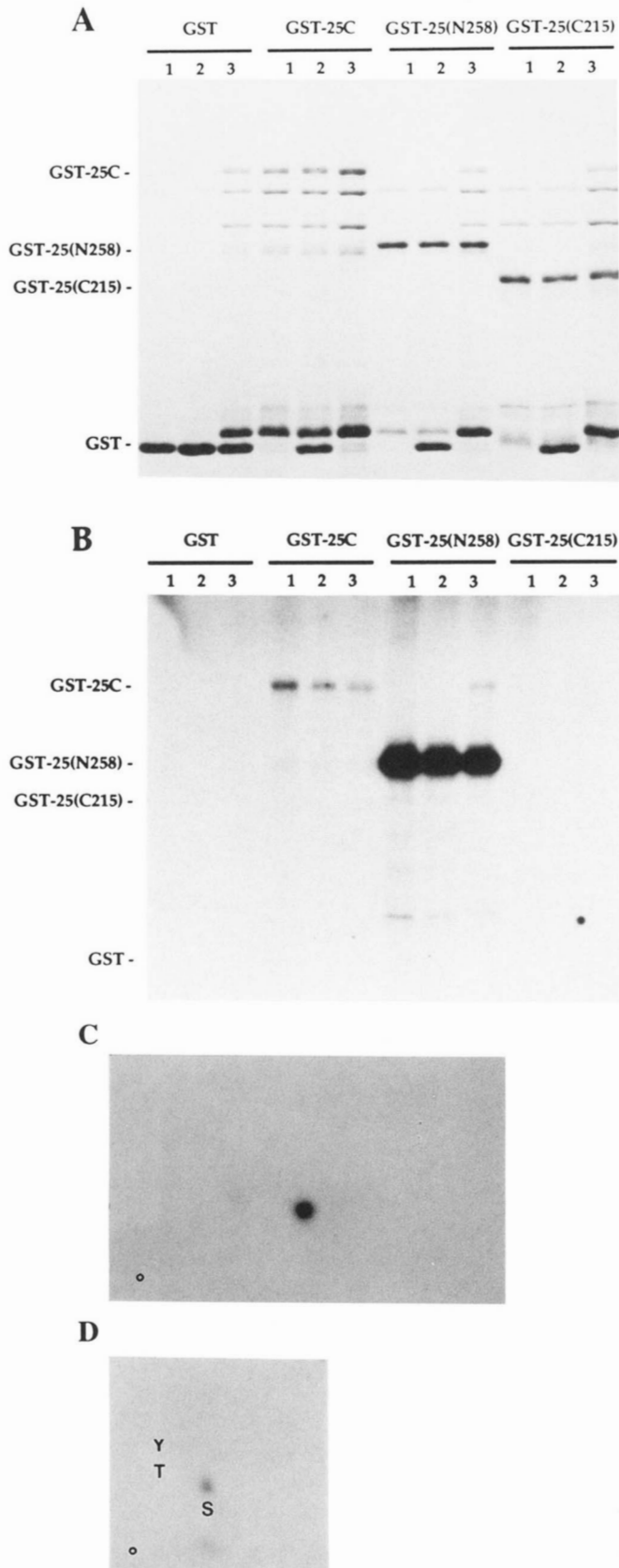GST-25(N258) -

GST-25(C215) -

GST -

C

D

Y
T
S
o

FIG. 2. **Binding of a cellular protein kinase to Cdc25C.** HeLa cell lysate was incubated with GST, GST-25C, GST-25(N258), or GST-25(C215) that was immobilized on GSH-agarose beads. The beads were washed and divided into three, and kinase assays were performed in the presence of buffer only (*lanes 1*) or 2 μg of soluble GST (*lanes 2*) or GST-25C (*lanes 3*). Proteins were resolved on an 11% SDS-polyacrylamide gel. Proteins were visualized by Coomassie staining (*panel A*)

cycle 8, this method does not allow for positive identification of phosphoserine. The phosphorylation site was determined more directly using semi-automated amino-terminal sequence analysis. The radioactivity released in each cycle was determined by Cerenkov counting (Fig. 3). Consistent with phosphorylation at serine in cycle 8, the radioactivity was released in cycle 8. These data demonstrated that Cdc25C was phosphorylated on serine 216 by the associating kinase *in vitro*.

Cdc25C Is Phosphorylated on Serine 216 in Vivo—The tryptic peptide of Cdc25C that contains serine 216 has an additional serine at position 214. It has been reported that Cdc25C can be phosphorylated on serine 214 *in vitro* by Cdc2-cyclin B and that the tryptic peptide containing serine 214 is phosphorylated in mitotic cells. Since serine 214 and serine 216 are within the same tryptic peptide it was necessary to determine if the major site of phosphorylation in Cdc25C from asynchronously growing HeLa cells was serine 214 or serine 216. To determine whether peptide phosphorylated on serine 214 could be resolved from peptide phosphorylated on serine 216, two-dimensional tryptic phosphopeptide mapping was performed.

To generate peptide containing phosphorylated serine 216, GST-25(200–256) was phosphorylated *in vitro* using Cdc25C-associating kinase purified from rat liver followed by tryptic digestion (Fig. 4A; see below). To generate peptide containing phosphorylated serine 214, GST-25(200–256) was phosphorylated *in vitro* using Cdc2-cyclin B followed by tryptic digestion (Fig. 4B). Although the site of phosphorylation has not been directly sequenced, serine 214 is the only serine/proline motif found within amino acids 200–256 of Cdc25C. We have also found that Cdc2-cyclin B can phosphorylate a peptide containing serine 214 but cannot phosphorylate the corresponding peptide in which serine 214 has been changed to alanine.[3] In addition, serine 214 was previously shown to be a substrate of Cdc2-cyclin B *in vitro* (44). As seen in Fig. 4C, a mixture of tryptic peptides phosphorylated on either serine 214 or serine 216 was resolved in two dimensions under the conditions employed in this study. Serine 216-phosphorylated peptide migrated faster in the second dimension than did serine 214-phosphorylated peptide. Peptides phosphorylated at either serine 214 or 216 could also be distinguished by secondary digestion with aminopeptidase M.[4] To determine whether Cdc25C from asynchronously growing HeLa cells was phosphorylated on serine 214 or serine 216, tryptic phosphopeptide mapping studies were performed on GST-25C phosphorylated on serine 216 by the associating kinase *in vitro* (Fig. 4D) and Cdc25C isolated from ^{32}P-labeled HeLa cells (Fig. 4E). A mixture of these two tryptic digestions demonstrated that the peptides comigrated in two dimensions (Fig. 4F). These data demonstrated that Cdc25C was phosphorylated on serine 216 in asynchronously growing HeLa cells.

Serine 216 Is Present within a Region That Is Conserved among Several Cdc25 Family Members—Although the amino-terminal, non-catalytic portion of Cdc25 proteins is less well conserved than the catalytic half, there are noted regions of homology. Serine 216 lies within one of these regions. As demonstrated in Fig. 5, amino acids located between 200 and 256 of human Cdc25C are conserved among a number of Cdc25 proteins including human Cdc25A and Cdc25B, rat A and B, mouse M2, and *Xenopus* 1, 2, and 3. This region is notably lacking in the hamster Cdc25C and mouse M1 proteins, which have most similarity to human Cdc25C. Within this region is a stretch of

[4] B. Gabrielli and H. Piwnica-Worms, unpublished data.

and autoradiography (*panel B*). Phosphorylated GST-25C was eluted from the gel and subjected to either tryptic digestion and two-dimensional analysis (*panel C*) or phosphoamino acid analysis (*panel D*).

FIG. 3. **Serine 216 of Cdc25C is phosphorylated by the Cdc25C-associating kinase.** GST-25(N258) was used as both affinity reagent and substrate for an associating kinase assay. GST-25(N258) was digested with trypsin, and radiolabeled peptides were purified by HPLC. The amino acid sequence of the major phosphopeptide was determined by microsequencing. The position of the phosphorylation was determined by monitoring the radioactivity released in each cycle. The sequence of the peptide and position of the phosphorylation (cycle 8, serine 216) are shown compared with the sequence of human Cdc25C.
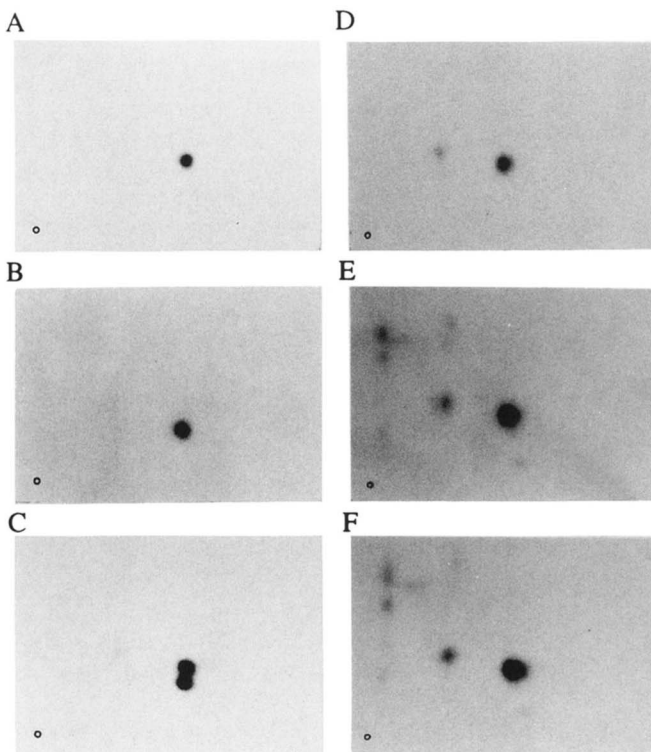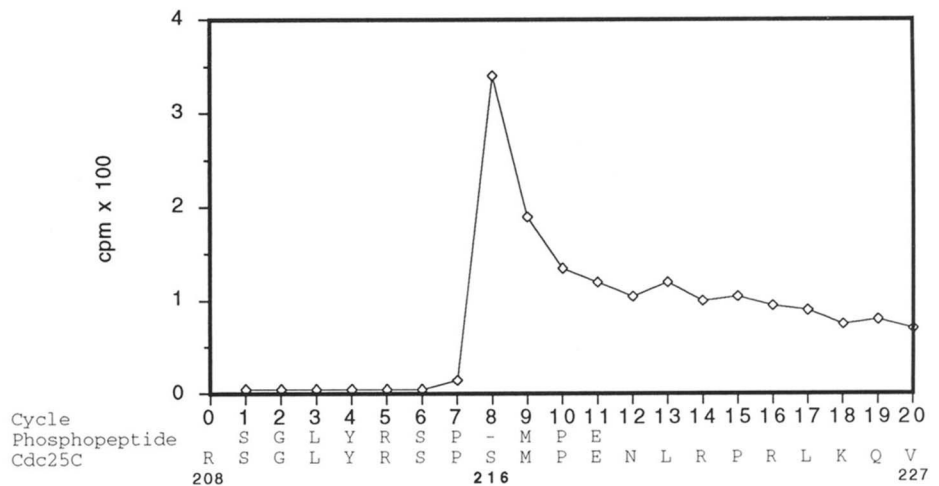

FIG. 4. **The major tryptic phosphopeptide of Cdc25C from HeLa cells comigrates with the tryptic phosphopeptide containing phosphorylated serine 216 of Cdc25C.** Tryptic digestions and two-dimensional analyses were performed on GST-25(200–256) phosphorylated *in vitro* by Cdc25C-associating kinase purified from rat liver (*panel A*), GST-25(200–256) phosphorylated *in vitro* by Cdc2-cyclin B (*panel B*), GST-25C phosphorylated *in vitro* by the Cdc25C-associating kinase from HeLa cells (*panel D*), or Cdc25C from radiolabeled HeLa cells (*panel E*). Mixtures of these tryptic digestions were separated in two dimensions to demonstrate the relative migration of the phosphopeptides. *Panel C*, mixture of *A* and *B*; *panel F*, mixture of *D* and *E*.

amino acids that has homology to bipartite nuclear localization signals.

Amino Acids 200–256 of Cdc25C Are Sufficient for Binding to the Associating Kinase—We determined whether amino acids surrounding serine 216 (amino acids 200–256) were sufficient for binding to the Cdc25C-associating kinase (Fig. 6). HeLa cell extracts were incubated with GST, GST-25C, GST-25(N258), GST-25(C215), GST-25(200–256), or GST-25(Hamster) immobilized on GSH-agarose beads. After washing, the beads were divided and assayed for kinase activity toward GST or GST-25(200–256). A protein kinase activity was detected that bound to GST-25C, GST-25(N258), and GST-25(200–256) but not to GST, GST-25(C215), or GST-25(Hamster). This kinase activity phosphorylated GST-25(200–256) but was unable to phosphorylate GST. These results indicated that amino acids 200–256 of human Cdc25C are sufficient to bind to the associating kinase. Furthermore, hamster Cdc25C, which lacks a corresponding region, was unable to bind Cdc25C-associating kinase activity.

Purification of a Cdc25C-associating Kinase from Rat Liver—The Cdc25C-associating kinase phosphorylated Cdc25C *in vitro* at the major site of phosphorylation detected *in vivo* and showed interesting binding properties, suggesting that it may function as an important Cdc25C regulator *in vivo*. To study the interactions between Cdc25C and the associating kinase, we purified the kinase. After homogenization and clarification of rat livers, 50–80% of Cdc25C-associating kinase activity was associated with the $100 \times kg$ supernatant (S100) fraction with the remainder in the pellet (P100). The solubility of kinase activity was dependent upon the concentration of NaCl since higher NaCl concentrations led to more activity partitioning in the S100 fraction. The kinase was purified from the S100 fraction using a number of chromatographic steps. Fractions from each step were assayed for kinase activity, which would both bind to and phosphorylate GST-25(200–256). The S100 fraction was chromatographed on an S-Sepharose column at pH 7.0 using a linear gradient of NaCl. The peak of activity eluted at approximately 340 mM NaCl and was purified over 50-fold. To maintain soluble kinase activity, it was necessary to include 0.05% Brij-35 in all buffers for the remaining steps of the purification. The peak of kinase activity from the S-Sepharose column was buffer exchanged before being purified a further 5-fold on a Q-Sepharose column. At pH 8.0, the peak of kinase activity eluted from the Q-Sepharose column at approximately 130 mM NaCl, with variable minor peaks of activity eluting at higher NaCl concentrations. These minor peaks of activity were not reproducible and may have represented either modified (*e.g.* proteolytic fragments) or aggregated forms of the kinase. The major peak of kinase activity was chromatographed using an ATP-agarose column and eluted with a step gradient of 0.8 M NaCl. This step provided a 3.4-fold purification. The peak fractions were buffer exchanged to pH 7.6 and chromatographed on a Resource S column. The peak of activity eluted at approximately 190 mM NaCl and was purified almost 2-fold. The peak fractions were concentrated and then separated using a Superose-12 10/30 gel filtration

FIG. 5. **The region surrounding serine 216 of human Cdc25C is conserved in other Cdc25 proteins.** A schematic of human Cdc25C illustrates the loosely conserved amino-terminal 258 amino acids and the highly conserved catalytic domain that contains the active site (HCEFSSER). The sequence of amino acids 200–256 of human Cdc25C is aligned with other Cdc25 proteins. Serine 216 is denoted by an _asterisk_. A putative bipartite nuclear localization signal is indicated by the _bars_. GenBank accession numbers are as follows: _Human C_, M34065; _Swine_, X78317; _Hamster_, S54051; _Mouse M1_, L16926; _Human A_, M81933; _Rat A_, D16236; _Human B_, M81934; _Mouse M2_, S93521; _Rat B_, D16237; _Frog A_, M96857; _Frog 1 (B)_, M94262(M96858); _Frog 2_, M94263; _Frog 3_, M94264.

Cdc25C

A protein kinase activity was present in fractions 17–19 with an apparent molecular mass of 36–38 kDa. This kinase activity bound to GST-25(200–256) but not to GST. The kinase activity was specific for Cdc25C since if GST was used as a substrate, less than 2% of the kinase activity was detected.[3] These experiments identified the 36–38-kDa doublet as the Cdc25C-associating kinase.

teins were solubilized in SDS sample buffer by boiling. A portion of each sample was resolved on an 11% SDS-polyacrylamide gel and visualized by silver staining (Fig. 8A). Each band of the 36–38-kDa doublet bound to GST-25(200–256) but not to GST. No other proteins from fractions 17–19 bound to either GST or GST-25(200–256). Another portion of each sample was used for an in gel kinase assay. The samples were resolved on an SDS-polyacrylamide gel that had been polymerized in the presence of GST-25(200–256). The proteins were subjected to an in gel denaturation and renaturation protocol followed by a kinase assay. The gel was washed, and phosphorylated proteins were visualized using autoradiography of the dried gel (Fig. 8B).
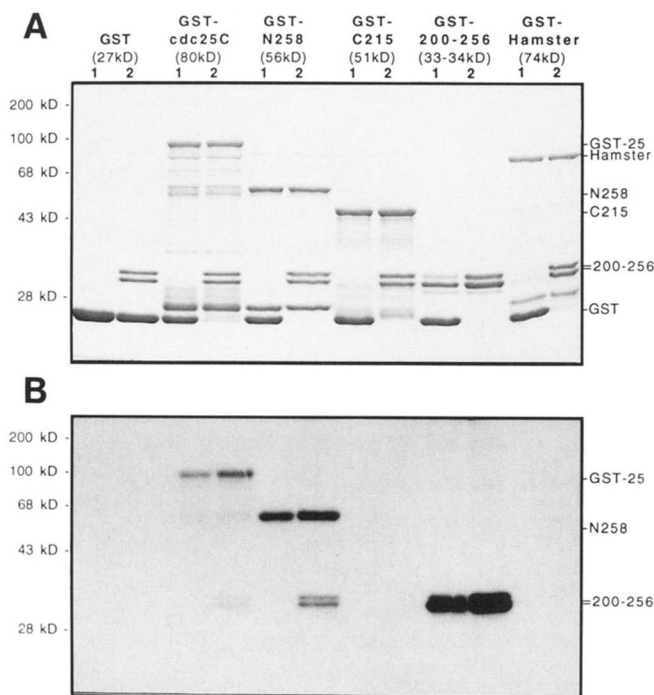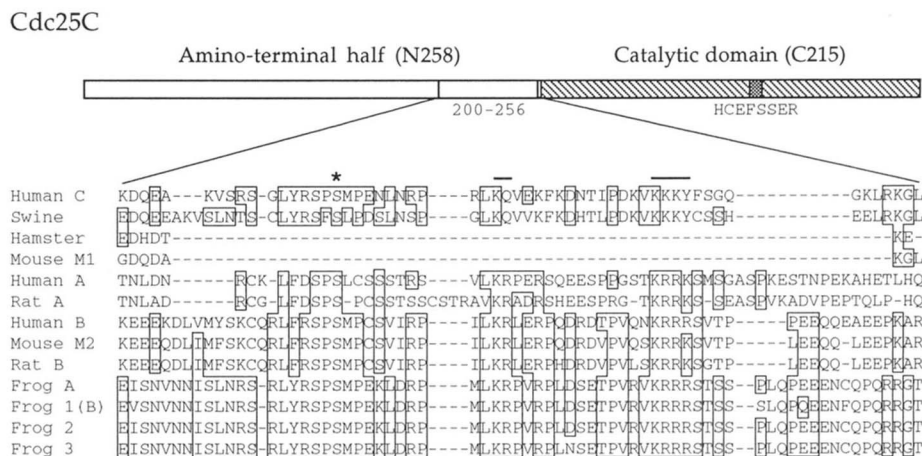
FIG. 6. **Amino acids 200–256 of human Cdc25C are sufficient for binding to Cdc25C-associating kinase activity.** HeLa cell lysate was incubated with GST, GST-25C, GST-25(N258), GST-25(C215), GST-25(200–256), or GST-25(Hamster) that was immobilized on GSH-agarose beads. The beads were washed and divided into two, and kinase assays were performed in the presence of 2 μg of either GST (_lanes 1_) or GST-25(200–256) (_lanes 2_). Proteins were resolved on an 11% SDS-polyacrylamide gel. Proteins were visualized by Coomassie staining (_panel A_) and autoradiography (_panel B_).

DISCUSSION

In this study, we demonstrate that serine 216 is the major site of Cdc25C phosphorylation in asynchronously growing HeLa cells. We describe a kinase that binds to Cdc25C and phosphorylates Cdc25C on serine 216. We have determined that amino acids 200–256 of Cdc25C are sufficient for binding to the associating kinase. Within this region is a putative bipartite nuclear localization signal that is positioned just downstream of serine 216. In addition, we purified a Cdc25C-associating kinase over 8000-fold and identified two proteins with apparent molecular masses of 36 and 38 kDa that have Cdc25C-associating kinase activity.

In a variety of species, Cdc25 proteins are underphosphorylated during interphase and become hyperphosphorylated during M-phase (35, 38, 40–43). This hyperphosphorylation correlates with the activation of Cdc25 enzymatic activity (35, 38–40). Cdc25 is a substrate of Cdc2-cyclin B _in vitro_, and phosphorylation activates Cdc25 phosphatase activity (39, 40, 44). Tryptic phosphopeptide mapping revealed that many of the same peptides phosphorylated _in vitro_ by Cdc2-cyclin B are also phosphorylated _in vivo_ (39, 44). This has led to the hypothesis that a self-amplification loop exists in which accumulation of active Cdc2-cyclin B causes the phosphorylation and activation of Cdc25, which then activates more Cdc2-cyclin B. This hypothesis would explain the abrupt and irreversible nature of M-phase and the ability of maturation-promoting factor to activate prematuration-promoting factor in _Xenopus_ oocytes (48, 49). However, it still remains unclear as to the nature of the
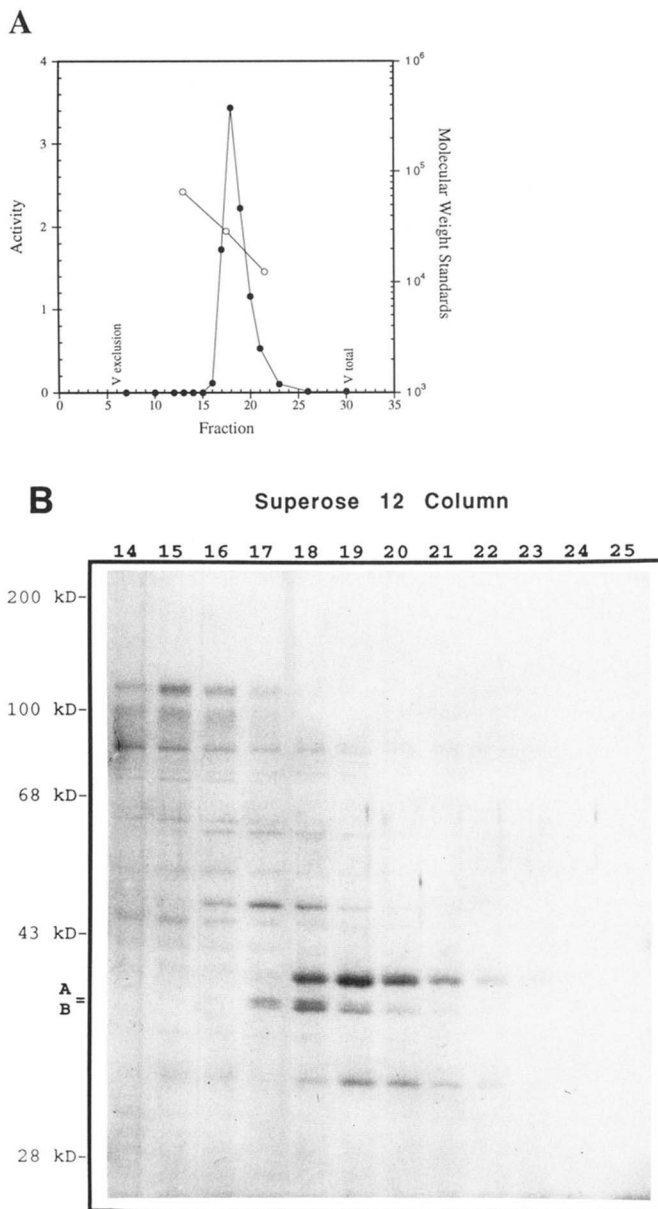
column. The peak of kinase activity eluted with an apparent molecular mass of approximately 30–40 kDa (Fig. 7A). The entire purification resulted in over an 8000-fold purification with greater than 3% yield (Table I). Column fractions from the Superose-12 10/30 column were resolved on an 11% SDS-polyacrylamide gel and visualized by silver staining (Fig. 7B). Two proteins of approximately 36–38 kDa coeluted with the peak of activity. Thus, it is likely that one or both of the proteins at ~36–38 kDa represented the Cdc25C-associating kinase.

If the 36–38-kDa proteins are Cdc25C-associating kinases, then they should bind to and phosphorylate GST-25(200–256). Either buffer alone (Fig. 8, _lanes 1_) or pooled fractions 17–19 from the Superose-12 10/30 column (_lanes 2_) were incubated with immobilized GST or GST-25(200–256). After washing, pro-

A

B

Superose 12 Column

FIG. 7. **Cdc25C-associating kinase activity from rat liver copurifies with two proteins with apparent molecular masses of 36 and 38 kDa.** Cdc25C-associating kinase activity was purified as described under "Experimental Procedures." The final column was a Superose-12 10/30 gel filtration column. Protein samples from each fraction were either assayed for Cdc25C-associating kinase activity (*panel A*) or resolved on an 11% SDS-polyacrylamide gel and visualized by silver staining (*panel B*). Molecular mass standards for the Superose-12 10/30 column were run under the same conditions used for the purification (bovine serum albumin, 66 kDa; carbonic anhydrase, 29 kDa; cytochrome *c*, 12.4 kDa). Two proteins that copurify with Cdc25C-associating kinase activity are indicated by *A* and *B*.

initial trigger that facilitates the interaction between Cdc25C and inactive Cdc2-cyclin B.

Serine 214 was among the sites reported to be phosphorylated by Cdc2-cyclin B *in vitro* (44). Based on the migration of tryptic phosphopeptides, Strausfeld *et al.* (44) proposed that serine 214 is also phosphorylated in mitotic human nontransformed fibroblasts (HS-268). Both serine 214 and serine 216 are present within the same tryptic phosphopeptide, and it is unclear whether the researchers were able to distinguish peptides phosphorylated on serine 214 from those phosphorylated on serine 216. Under the experimental conditions employed in this study, we were able to distinguish these phosphopeptides and

were able to identify serine 216 as the major site of Cdc25C phosphorylation in asynchronously growing HeLa cells (Fig. 3). With this added knowledge, it will be important to determine whether serine 214, serine 216, or both are phosphorylated during M-phase and, in addition, whether the phosphorylation of one site effects phosphorylation of the other.

Some studies suggest that there is a mechanism for activation of Cdc25 proteins that is independent from Cdk-cyclin complexes. Treatment of *Xenopus* interphase extracts with the phosphatase inhibitor, okadaic acid, causes premature activation of Cdc2 (50, 51). In this system, Cdc25 becomes hyperphosphorylated and activated prior to activation of cyclin A- and cyclin B-associated H1 kinase activities and nuclear envelope breakdown (35). These data suggest that a mechanism other than phosphorylation of Cdc25 by Cdc2-cyclin complexes is responsible for facilitating the initial interaction between Cdc2-cyclin B and Cdc25. Due to the poor enzymatic activity of recombinant Cdc25C and the limited quantities of available purified Cdc25C-associating kinase, we have been unable to adequately address whether phosphorylation at serine 216 effects the enzymatic activity of human Cdc25C. This will be more easily addressed once a recombinant source of the kinase is available.

Increased phosphatase activity of Cdc25 may not be sufficient to cause the activation of Cdc2-cyclin B. Microinjection of Cdc25C that has been phosphorylated by Cdc2-cyclin B *in vitro* into G$_2$-phase mammalian tissue culture cells causes a premature prophase-like phenotype (44). Phosphorylated Cdc25C is 2–3 times more active *in vitro* than unphosphorylated Cdc25C, but microinjection of 10 times more unphosphorylated Cdc25C protein has no effect *in vivo*. Thus, under these experimental conditions, phosphatase activity alone is insufficient for the activation of Cdc2-cyclin B by Cdc25C *in vivo*. One possibility to explain these results would be if unphosphorylated Cdc25C is sequestered away from Cdc2-cyclin B, whereas phosphorylated Cdc25C localizes differently and is able to interact with Cdc2-cyclin B. Consistent with this hypothesis is the observation that microinjected phosphorylated Cdc25C does not accumulate in the nucleus as readily as unphosphorylated Cdc25C (44). Therefore, the subcellular compartmentalization of Cdc25C may significantly contribute to the regulation of its substrate interactions.

We have purified a serine kinase that specifically binds to the region bordered by amino acids 200–256 of human Cdc25C that is lacking in hamster Cdc25C. This region is sufficient for binding the Cdc25C-associating kinase and, in addition, may be necessary since hamster Cdc25C is unable to bind to the kinase. Human Cdc25A and Cdc25B proteins contain a region corresponding to amino acids 200–256 of human Cdc25C, and all three bind to a kinase activity that will phosphorylate a serine 216 containing peptide.[3] It remains to be determined if all three human Cdc25 proteins interact with distinct Cdc25-associating kinases.

It is important to note that the interactions we have examined between Cdc25C and the associating kinase have been *in vitro*. We have not directly demonstrated that Cdc25C stably associates with the serine 216 kinase *in vivo*. Studies are currently underway to directly address this question. In addition, studies are currently underway to determine whether the putative bipartite nuclear localization signal of Cdc25C functions as an authentic nuclear localization signal *in vivo*. However, given that the associating kinase phosphorylates Cdc25C on the major site of phosphorylation detected *in vivo* and binds to a region conserved in other Cdc25 family members, we purified the activity from rat liver. The purification of the Cdc25C-associating kinase identified two proteins with apparent mo-

Step	Total activity	Protein	Specific activity	Purification	Yield
	units	*mg*	*units/mg*	*-fold*	*%*
100,000 × g supernatant	12900	7500	1.7	1	100
S-Sepharose	5726	62	92	54	44
Q-Sepharose	1465	3.3	440	260	11
ATP-agarose	1012	0.66[a]	1500	880	7.8
Resource S	453	0.17[a]	2700	1600	3.5
Superose-12	427	0.03[a]	14000	8200	3.3

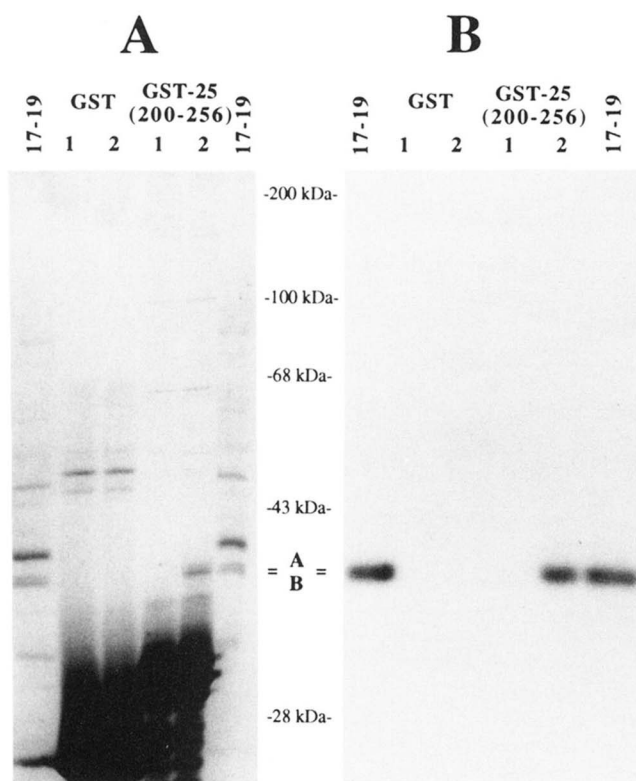[a] Estimated from $A_{280 nm}$ tracing.

FIG. 8. **Rat liver Cdc25C-associating kinase purifies as a 36–38-kDa doublet.** Fractions 17–19 from the Superose-12 10/30 column (see Fig. 7) were pooled. A portion of the pooled fractions was collected into sample buffer (*outside lanes*). Either buffer only (*lanes 1*) or pooled fractions (*lanes 2*) were incubated with GST or GST-25(200–256) immobilized on GSH-agarose beads. After washing, proteins were solubilized with SDS sample buffer and boiling. A portion of each sample was resolved on an 11% SDS-polyacrylamide gel and visualized by silver staining (*panel A*). Another portion was resolved on an 11% SDS-polyacrylamide gel that had been polymerized in the presence of GST-25(200–256). Protein samples were resolved on the gel and subjected to a denaturation/renaturation protocol followed by an in gel kinase assay. Kinase activity was visualized by autoradiography (*panel B*). Two proteins that copurify with Cdc25C-associating kinase activity are indicated by *A* and *B*.

lecular masses of 36–38 kDa. A characteristic of the purified kinase activity is that it phosphorylated a peptide corresponding to amino acids 210–231 of human Cdc25C but not the equivalent peptide in which serine 216 had been changed to a threonine.[3] cAMP-dependent protein kinase shared this characteristic, but the purified Cdc25C-associating kinase activity was not inhibited by PKI, a specific inhibitor of cAMP-dependent protein kinase.[3] Calcium/calmodulin-dependent kinase II and protein kinase Cα were distinguished from the purified kinase because they phosphorylated both serine and threonine at position 216 in the context of the peptide.[3] Cdc2-cyclin B can phosphorylate the peptide at the position corresponding to

serine 214 but not serine 216. It will be important to identify the Cdc25C-associating kinase and to characterize its interactions with Cdc25C throughout the cell cycle.

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