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## Activation of p34<sup>cdc2</sup> Protein Kinase by Microinjection of Human cdc25C into Mammalian Cells

REQUIREMENT FOR PRIOR PHOSPHORYLATION OF cdc25C BY p34<sup>cdc2</sup> ON SITES PHOSPHORYLATED AT MITOSIS\*

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Human cdc25C protein, a specific tyrosine phosphatase that activates the p34<sup>cdc2</sup> protein kinase at mitosis, is itself a phosphoprotein that shows increased phosphorylation during the G<sub>2</sub>-M transition. *In vitro*, cdc25C protein is substantially phosphorylated by purified p34<sup>cdc2</sup>-cyclin B protein kinase. Of seven putative phosphorylation sites for p34<sup>cdc2</sup> protein kinase present in human cdc25C, five are phosphorylated by p34<sup>cdc2</sup> protein kinase *in vitro*, as assessed by tryptic phosphopeptide mapping and peptide sequencing. These same sites are also phosphorylated *in vivo* during the G<sub>2</sub>-M transition in normal mammalian fibroblasts and have been precisely mapped. The cdc25C phosphorylated *in vitro* by p34<sup>cdc2</sup> protein kinase exhibits a 2–3-fold higher activity than the nonphosphorylated cdc25C, as assayed by activation of inactive cdc2 prokinase. Microinjection of purified cdc25C proteins into living fibroblasts reveals that only the phosphorylated form of cdc25 is highly effective in activating G<sub>2</sub> cells into premature prophase in a manner similar to microinjection of purified active p34<sup>cdc2</sup> protein kinase. Together these data show that multisite phosphorylation of cdc25C by p34<sup>cdc2</sup>-cyclin B protein kinase occurs at the G<sub>2</sub>-M transition and is sufficient to induce the autoamplification of cdc2/M-phase promoting factor necessary to drive somatic mammalian cells into mitosis.

identification of two different subunits, p34<sup>cdc2</sup> and cyclin B (Labbe *et al.*, 1989a, 1989b; Lohka *et al.*, 1988). Whereas the p34<sup>cdc2</sup> subunit is responsible for the catalytic activity, cyclin B is necessary to form and maintain the active enzyme and perhaps designate cellular localization and/or substrate specificity. Prior to formation of the active kinase, the p34<sup>cdc2</sup>-cyclin B protein complex exists in an inactive form called "pre-MPF" (or "prokinase"), in which cdc2 is phosphorylated within the strongly conserved NH<sub>2</sub>-terminal region on tyrosine and additionally on threonine in vertebrate cells (GEGT(P)Y(P)GVV) (Draetta *et al.*, 1988; Krek and Nigg, 1991; Morla *et al.*, 1989; Norbury *et al.*, 1991). The phosphorylation and subsequent dephosphorylation of these residues seem to be important in coupling DNA replication and mitosis (Enoch and Nurse, 1990; Dasso and Newport, 1990). Mutation of the p34<sup>cdc2</sup> tyrosine 15 residue to phenylalanine (cdc2 F15) causes p34<sup>cdc2</sup> premature incomplete prophase events during the cell cycle of the fission yeast *Schizosaccharomyces pombe* (Gould and Nurse, 1989) and in HeLa cells (Krek and Nigg, 1991). The universality of this mechanism is unclear, since in *Saccharomyces cerevisiae*, mutations that prevent tyrosine phosphorylation do not lead to premature mitosis and do not abolish feedback controls (Sorger and Murray, 1992; Amon *et al.*, 1992). Recently, the enzymatic activities that modulate the reversible phosphorylation of p34<sup>cdc2</sup>-tyrosine 15 have been described. Phosphorylation is promoted by a kinase pathway involving *wee1* and *mik1* (Lundgren *et al.*, 1991), whereas dephosphorylation involves a phosphatase that requires the cdc25 gene product (reviewed in Millar and Russel (1992)). Various reports have demonstrated that tyrosine dephosphorylation and kinase activation could be triggered *in vitro* by bacterially expressed and highly purified human cdc25 (Strausfeld *et al.*, 1991; Gautier *et al.*, 1991; Dunphy and Kumagai, 1991; Millar *et al.*, 1991b; Lee *et al.*, 1992). Moreover, a motif (HCXXXXXR) was described, which is conserved between the cdc25 family and known tyrosine phosphatases (Moreno and Nurse, 1991; Strausfeld *et al.*, 1991). Mutations of the cysteine or arginine residues in the HCXXXXXR motif, which is localized in the active catalytic domain near the COOH terminus, abolish the *in vivo* activity of cdc25 and its phosphatase activity *in vitro* (Gautier *et al.*, 1991; Millar *et al.*, 1991b; Lee *et al.*, 1992). Despite the fact that, *in vivo*, cdc25 seems to act specifically on p34<sup>cdc2</sup>-cyclin B, other substrates used to measure phosphatase activity, such as *p*-nitrophenyl phosphate (Dunphy and Kumagai, 1991) or casein-phosphorylated or serine residues (Millar *et al.*, 1991b), can also be dephosphorylated.

The M-phase promoting factor (MPF)<sup>1</sup> is a proline-directed serine/threonine protein kinase activated during the G<sub>2</sub>-M phase transition (reviewed in Nurse (1992), Doree (1990), Pines and Hunter (1990), and Lewin (1990)). Purification of MPF by following histone H1 kinase activity had led to the

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<sup>1</sup> The abbreviations used are: MPF, M-phase promoting factor; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone; HPLC, high pressure liquid chromatography.

Since *cdc25* appears to be involved in the activation of p34<sup>cdc2</sup> kinase, various studies have examined the modalities of how this is brought about. Although some reports have described that the level of *cdc25* mRNA increases during G<sub>2</sub> in fission yeast (Ducommun *et al.*, 1990; Moreno *et al.*, 1990) and HeLa cells (Sadhu *et al.*, 1990), the abundance of *cdc25* protein is not cell cycle-regulated in *Xenopus* embryos (Jessus and Beach, 1992), HeLa cells (Millar *et al.*, 1991a), or in nontransformed rat embryo (REF-52) or human fibroblasts (HS-68) (Girard *et al.*, 1992). Therefore *cdc25*, like p34<sup>cdc2</sup>, seems to be present at a constant level throughout the cell cycle, and the question arises how *cdc25* activity is regulated with respect to the timing of MPF activation. The physical association between *cdc25* and the p34<sup>cdc2</sup>-cyclin B complex oscillates during the cell cycle, reaching a peak at M-phase (Gautier *et al.*, 1991; Jessus and Beach, 1992). However, since in fission yeast p80cdc25 is a phosphoprotein that is predominantly phosphorylated on serine (Moreno *et al.*, 1990) and *cdc25* protein appears to be heavily phosphorylated during M-phase in *Xenopus* oocytes (Gautier *et al.*, 1991), various reports have investigated whether phosphorylation might regulate *cdc25* phosphatase activity both *in vitro* and *in vivo*. In *Xenopus*, phosphorylation of *cdc25* proteins by an unidentified kinase activity present in mitotic extracts is sufficient to bring about the activation of *cdc25* phosphatase activity against both artificial substrates and p34<sup>cdc2</sup> kinase (Kumagai and Dunphy, 1992; Izumi *et al.*, 1992). More recently, Hoffmann *et al.* (1993) reported that bacterially expressed human *cdc25C* proteins could be phosphorylated by a kinase activity present in HeLa cell mitotic extracts, an event that also seems to bring about an increase in *cdc25* phosphatase activity as measured *in vitro* against artificial substrates and *in vivo* by its ability to induce *Xenopus* oocyte maturation. Based only on indirect evidence the authors suggested that p34<sup>cdc2</sup> kinase would be the kinase responsible for this mitotic phosphorylation of human *cdc25C* in HeLa cells. However, no evidence was provided for the number or location of the sites of phosphorylation on *cdc25C* or that such phosphorylation of *cdc25C* had any effect on its activity *in vivo* in mammalian cells. In the present report, we show that human *cdc25C* protein is phosphorylated *in vivo* in human fibroblasts during mitosis at the same six sites as are phosphorylated, *in vitro*, by highly purified p34<sup>cdc2</sup>-cyclin B kinase. Phosphorylation of *cdc25C* increases 2–3-fold its activation of *cdc2*-cyclin B prokinase *in vitro*. Furthermore, we demonstrate that after phosphorylation by p34<sup>cdc2</sup>-cyclin B, *cdc25C* protein is capable of activating the endogenous p34<sup>cdc2</sup> prokinase when microinjected into G<sub>2</sub> cells, an effect not observed with the nonphosphorylated protein, showing that only the phosphorylated form of *cdc25C* is effective *in vivo*.

#### EXPERIMENTAL PROCEDURES

**Enzyme Purification**—Active M-phase-specific H1 histone kinase (MPF) was prepared from starfish oocytes at first meiotic metaphase as described (Labbe *et al.*, 1991). Prokinase (pre-MPF), phosphorylated on p34<sup>cdc2</sup>-tyrosine 15/threonine 14 and inactive as H1 histone kinase, was purified as a p34<sup>cdc2</sup>-cyclin B complex from G<sub>2</sub>-arrested starfish oocytes (Strausfeld *et al.*, 1991). Human *cdc25C* gene product (Sadhu *et al.*, 1990) was overexpressed in bacteria as previously described (Strausfeld *et al.*, 1991). *cdc25C* protein was either purified from the inclusion bodies by denaturation/renaturation or from the soluble fraction of bacteria induced at low temperature. Proteins purified from the inclusion bodies were denatured in 9 M guanidine hydrochloride in buffer containing 40 mM HEPES, 5 mM EDTA, 5 mM DTT at pH 7.5. Protein renaturation consisted of extensive dialysis against 500 mM NaCl, 40 mM HEPES, 5 mM EDTA, 5 mM DTT at pH 7.5.

**Phosphorylation of *cdc25C* Protein in Competition with Histone H1 and Trypsin Inhibitor by Purified p34<sup>cdc2</sup>-Cyclin B Kinase *In Vitro***—

Trypsin inhibitor (Boehringer Mannheim) and histone H1 (Boehringer Mannheim) were mixed together with purified human *cdc25C* protein in buffer containing 50 mM HEPES, 200 mM NaCl, 10 mM MgCl<sub>2</sub>, 0.2 mM [ $\gamma$ -<sup>32</sup>P]ATP (100 cpm/pmol), 1 mM DTT in a final volume of 25  $\mu$ l. Different concentrations of substrates were used, as described in the legend for Fig. 1. Phosphorylation reaction was started by adding 0.05  $\mu$ g of active p34<sup>cdc2</sup>-cyclin B kinase. After 30, 60, 120, and 240 s of incubation at 25 °C, 3- $\mu$ l aliquots of the phosphorylation reaction were stopped in Laemmli sample buffer. Phosphorylated proteins were separated on 10% SDS-PAGE and stained with Coomassie Brilliant Blue, and dried gels were autoradiographed at -70 °C.

**Cell Culture and Labeling**—Rat embryo fibroblasts (REF-52 cells) were cultured in Dulbecco's modified Eagle's medium and supplemented with 10% fetal calf serum as described previously (Lamb *et al.*, 1990). Cells were subcultured onto acid-washed glass coverslips and allowed to grow for 2 days prior to use. Cells were synchronized into G<sub>1</sub> or G<sub>2</sub> by serum deprivation and hydroxyurea blocking prior to S-phase as described previously (Girard *et al.*, 1991). For *in vivo* labeling, cells were subcultured onto 25-mm-diameter coverglasses (~2 × 10<sup>6</sup> cells), and after synchronization into G<sub>2</sub> coverglasses were transferred to humidified chambers overlaid with 100  $\mu$ l of phosphate-free culture media containing 300–500  $\mu$ Ci of <sup>32</sup>P-labeled O<sub>4</sub>. Cells were harvested as the mitotic index attained 10% or greater. Similar results were obtained using cells mechanically detached from flasks in mitotic shakes. Labeled cells were analyzed by two-dimensional electrophoresis using an Ampholine pH gradient of 4–7 and subjected to Western immunoblotting as described previously (Girard *et al.*, 1991). Antisera used to identify *cdc25C* were the same as those described previously (Girard *et al.*, 1992) except that the antisera were affinity purified against *cdc25C* protein. The specificity of the signal for *cdc25C* was confirmed by blocking the antisera prior to use with purified *cdc25C* proteins that completely abolished the blotting signal.

**Determination of p34<sup>cdc2</sup>-Cyclin B Phosphorylation Sites on *cdc25C***—Human *cdc25C* protein (10–50  $\mu$ g/assay) were mixed with 0.5  $\mu$ g of p34<sup>cdc2</sup>-cyclin B kinase in 50 mM glycerophosphate, 10 mM MgCl<sub>2</sub>, 7 mM EGTA, 0.5 mM [<sup>32</sup>P]ATP mix (100 cpm/pmol) in 50  $\mu$ l. After phosphorylation of human *cdc25C* protein by p34<sup>cdc2</sup>-cyclin B kinase for 1 h, the reaction was stopped by addition of either 0.2 mg/ml TPCK-treated trypsin (Worthington) in 200 mM ammonium bicarbonate final concentrations or 100 mM Tris-HCl, pH 7.4, 0.1% SDS, and 0.15 mg/ml chymotrypsin (Worthington) and further incubation for 24 h at 30 °C. Incompletely digested peptides eluting late during HPLC were pooled, lyophilized, and further digested with 0.15 mg/ml trypsin. Alternatively, to ensure the absolute comparability of the *in vivo* and *in vitro* peptides, *in vitro* phosphorylation of *cdc25C* was stopped by addition of boiling sample buffer, and the proteins were analyzed by one- and two-dimensional electrophoresis before complete tryptic digestion as described for phosphopeptide mapping (see below). The peptide mixture was injected into an Aquapore C8 reverse-phase microbore column (0.21 × 10 cm) equilibrated with 0.1% trifluoroacetic acid using a Brownlee microgradient system. Elution was carried out at 0.2 ml/min with a 60-min gradient between solvent A (0.1% trifluoroacetic acid) and solvent B (0.08% trifluoroacetic acid, 60% acetonitrile). Elution was followed by monitoring the absorbance at 220 nm with a Spectraflow 773 detector (Kratos Brownlee). Radioactivity of individual fractions was determined by Cerenkov counting. Tubes containing radioactive peptides were further purified by HPLC on a C18 column (0.21 × 10 cm) using shallow gradients. Purified peptides were concentrated by vacuum centrifugation and loaded onto Polybrene-coated glass fiber discs. Microsequencing was carried out on an Applied Biosystems 470A gas phase sequencer coupled on-line to a 120A phenylthiohydantoin analyzer. Phosphoserine was detected by the presence of the dithiothreitol adduct of dehydroalanine (D-Ser) and the absence of authentic phenylthiohydantoin-Ser (Meyer *et al.*, 1991) (sites 3, 5, and 6). Phosphothreonine was indirectly determined either as unique Thr residues present in the parent peptides (sites 2 and 4) or after positive identification of other unlabeled Thr residues (site 1, Thr at steps 2 and 5).

**Two-dimensional Phosphotryptic Mapping**—*In vitro* or *in vivo* labeled *cdc25C* protein was excised from dried gels or Immobilon membranes and digested with 0.2 mg/ml trypsin (TPCK-treated) in 200 mM ammonium bicarbonate. Digestion at 37 °C overnight was continued by the addition of fresh trypsin for 12 more h. The supernatant from this complete tryptic digestion was counted (80% of initial counts were recovered) and lyophilized three times with

resuspension in distilled water. Phosphopeptides were either used for two-dimensional peptide mapping or purified by HPLC for gas phase sequencing. Individual purified phosphopeptides isolated by HPLC for sequencing were also subjected to one- and two-dimensional peptide mapping. Phosphopeptides were separated by electrophoresis at pH 1.9 for 40 min at 950 V in the first dimension followed by ascending chromatography with isobutyric acid buffer (isobutyric acid:butanol:pyridine:acetic acid:water, 65:2.5:3:29) (described by Boyle *et al.* (1991)).

**Phosphoamino Acid Analysis**—After autoradiography, phosphopeptides were individually recovered from TLC plates in 50% pyridine. Following lyophilization of the supernatant <sup>32</sup>P-labeled peptides were hydrolyzed with 5.7 M HCl for 2 h at 110 °C and dried, and phosphoamino acids were separated by one-dimensional electrophoresis at pH 3.5 (Boyle *et al.*, 1991).

**Phosphorylation of cdc25 Protein by p34<sup>cdc2</sup>-Cyclin B Kinase and Immunoprecipitation of p34<sup>cdc2</sup>-Cyclin B Kinase**—For use in microinjection studies, 12 µg of human cdc25C protein were incubated with p34<sup>cdc2</sup>-cyclin B kinase (final specific activity, 50 pmol/µl/min phosphate molecules incorporated into histone H1, ~0.015 mg/ml) (reaction 1 + 2) or alone (reaction 3), together with 1 mM ATP (reaction 1 + 3) or ADP (reaction 2) in 50 µl of HMD buffer (10 mM HEPES, pH 7.5, 10 mM MgCl<sub>2</sub>, 5 mM DTT). After 1 h of incubation at 25 °C, affinity-purified anti-starfish cyclin B antibody, coupled to 10 µl of protein A-Sepharose beads (Strausfeld *et al.*, 1991) in HMD buffer, was added (final volume, 100 µl), and each assay was further incubated for 30 min at 25 °C. After separation of beads and supernatant, beads were washed 3 times and kept in HMD buffer at 0 °C as was the supernatant.

**Western blotting**—Proteins were separated on 11% SDS-PAGE, transferred to nitrocellulose, and probed with affinity-purified antibodies as described previously (Girard *et al.*, 1991, 1992). After binding of primary antibodies (anti-EGVPSTAIRESILLKE peptide = "anti-PSTAIR" described in Strausfeld *et al.* (1991)), peroxidase-conjugated anti-rabbit antibody (Amersham Corp.) was added (1/10,000) for 1 h. Affinity-purified and biotinylated anti-human cdc25C antibody was incubated with peroxidase-conjugated ExtrAvidin (Sigma) 1:6000 for 1 h. Detection of the immune signal was performed with the ECL Western blotting detection system (Amersham Corp.).

**In Vitro Phosphatase Assay Using Prokinase Activation**—The *in vitro* activation of starfish cdc2 prokinase (the cyclin B-cdc2 protein kinase complex containing phosphate on both threonine 14 and tyrosine 15) was performed as described earlier (Strausfeld *et al.*, 1991). Essentially, purified cdc2-phosphorylated and unphosphorylated cdc25C proteins prepared as described above were dialyzed into a buffer consisting of 40 mM NaHCO<sub>3</sub>, 0.3 M NaCl, 2 mM DTT at pH 7.4 without ATP, and the concentration of cdc25C proteins was equilibrated. Prokinase activation assays were performed by incubating for the indicated times at 20 °C 1 volume of inactive starfish prokinase (5 µg/ml) with 1 volume of the cdc25C protein solution (50 µg/ml) in 6 µl of reaction mixture. After the appropriate incubation period an aliquot (2 µl) of this reaction mixture was removed and assayed for histone 1 kinase activity by the addition of 40 µl of 1.0 mg/ml histone H1 in a buffer containing 0.2 mM ATP/[<sup>32</sup>P]ATP (50 cpm/pmol), 10 mM MgCl<sub>2</sub> in 20 mM HEPES pH 7.4. Incorporation into histone H1 was measured as described before (Labbe *et al.*, 1989a).

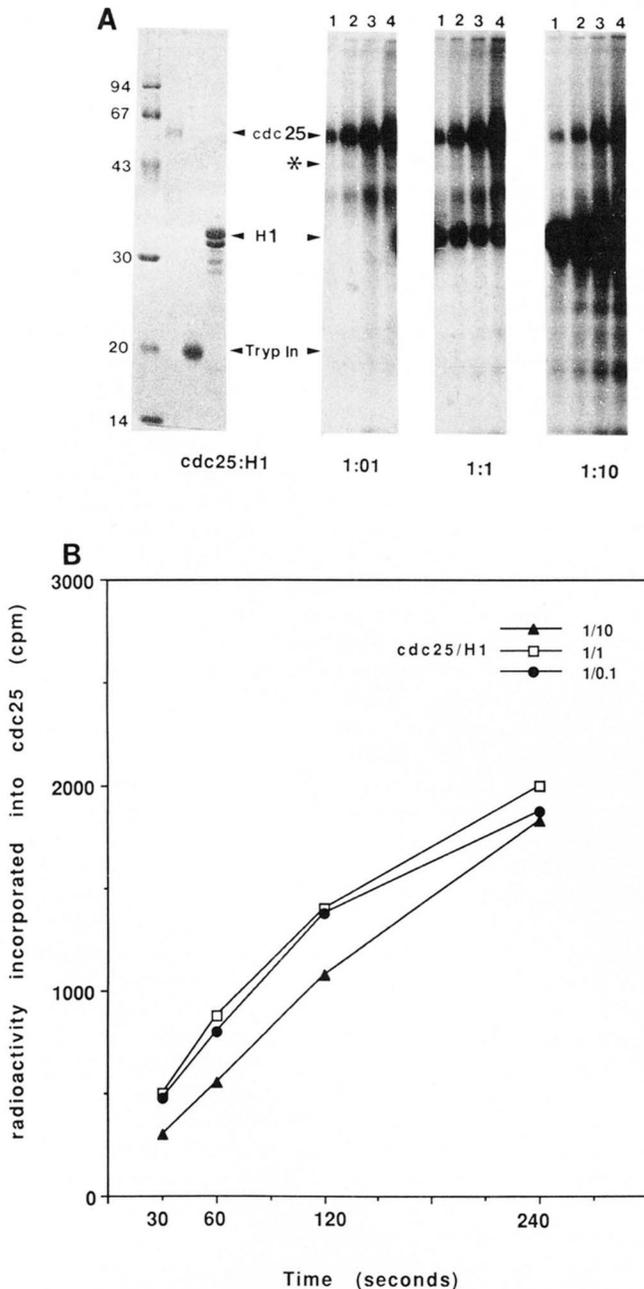
**Microinjection of cdc25C Protein and Immunofluorescence**—Cells were microinjected with 0.5-µm outer diameter micropipettes pulled on a linear horizontal puller and microinjected on Leitz inverted microscope equipment with a manual Leitz micromanipulator. 30–50 cells were injected per experiment. When indicated, cells were treated with 0.1 µM okadaic acid in Dulbecco's modified Eagle's medium applied as they were injected. Cells were stained for cdc25 as described previously (Girard *et al.*, 1992). The polyclonal anti-cdc25 antibody was monospecific for cdc25C as determined by immunoblot analysis. Cells were mounted and photographed as described previously (Lamb *et al.*, 1990).

## RESULTS

Purified human cdc25 is specifically phosphorylated by p34<sup>cdc2</sup>-cyclin B kinase *in vitro*. Analysis of the derived amino acid sequence of the cdc25C protein (Sadhu *et al.*, 1990) reveals several potential consensus sites for phosphorylation by p34<sup>cdc2</sup>-cyclin B kinase ("S/T-P" sites; shown for human cdc25C in Fig. 4). To test if these are phosphorylated, we have

performed *in vitro* phosphorylation assays in which bacterially produced and highly purified and active human cdc25C phosphatase was incubated with p34<sup>cdc2</sup>-cyclin B kinase. Highly purified cyclin B-associated cdc2 kinase was isolated from starfish oocytes as described earlier and shows only two homogeneous bands on a gel, one at 34 kDa corresponding to cdc2 kinase catalytic subunit and one at 45 kDa corresponding to cyclin B (Labbe *et al.*, 1989a, 1991). Fig. 1A shows an autoradiograph of a gel analyzing phosphorylation reaction mixtures in which 0.1 mg/ml cdc25 has been incubated with p34<sup>cdc2</sup>-cyclin B kinase (60 pmol/µl/min final) in the presence of varying amounts of the alternative substrate histone H1 and 1 mg/ml trypsin inhibitor. The first set of four lanes contains the molecular weight markers and each of the three substrate components alone stained by Coomassie Blue to show their respective molecular mass with their degradation products (in the case of cdc25 and H1). The next three sets of four lanes on Fig. 1 then represent the kinetics (30, 60, 120, and 240 s) of phosphorylation by p34<sup>cdc2</sup> protein kinase of the different substrates with the concentration of H1 in the reaction mixture being, respectively, 0.01, 0.1, and 1 mg/ml. Phosphorylation reactions were stopped by the addition of boiling SDS sample buffer. These reaction mixtures show a linear increase in phosphorylation of cdc25 protein by p34<sup>cdc2</sup> protein kinase beginning to plateau by 240 s in all three cases. Incorporation of radioactive phosphate into histone H1 and cdc25 protein reaches similar levels when equal concentrations of these two substrates were used. The quantitative representation of the kinetics of phosphorylation of cdc25 is shown in Fig. 1B. Labeling of cdc25 still reached the same level even in the presence of a 10-fold excess of H1 (Fig. 1A, lanes 1–10 and Fig. 1B, curve 1/10). Assuming a plateau value of ~2500 cpm for 3 µl of reaction mixture (*i.e.* 0.3 µg of cdc25 protein), we calculated that 4–5 mol of phosphate were incorporated per mol of cdc25C protein. The phosphorylation of cdc25 protein and histone H1 was specific, since no incorporation of phosphate into trypsin inhibitor was observed, even though it was always present in the reaction mixtures (at 1 mg/ml) and contains one potential consensus site (SP) for phosphorylation by p34<sup>cdc2</sup>-cyclin B kinase. It is to be noted that in all incubations a slight level of autophosphorylation by cdc2 kinase on its cyclin B subunit (at 45 kDa, marked by an asterisk) is detectable. In control assays in which cdc25 was omitted, no phosphorylation of the cdc25 protein band at 60 kDa and of a minor breakdown product band visible at 40 kDa occurred (data not shown). Together, these data show that cdc25 protein is phosphorylated by p34<sup>cdc2</sup>-cyclin B kinase in a highly efficient manner *in vitro* even in the presence of a 10-fold excess of competing histone H1 substrate.

**cdc25 Protein Is Phosphorylated on the Same Sites at Mitosis and by p34<sup>cdc2</sup>-Cyclin B Kinase**—We next examined the patterns of phosphorylation of cdc25 protein in growing mammalian cells. Several recent reports have described an increased phosphorylation of cdc25 proteins during the G<sub>2</sub>-M period in *Xenopus* oocytes (Kumagai and Dunphy, 1992; Izumi *et al.*, 1992) and in transformed mammalian (HeLa) cells (Hoffmann *et al.*, 1993). We have performed an analysis of the patterns of phosphorylation in human nontransformed fibroblasts (HS-268) at G<sub>2</sub> and G<sub>2</sub>-M after separation on denaturing high resolution two-dimensional gels. This analysis was essential since the molecular mass of cdc25 proteins (55–70 kDa) is coincidental with a number of cytoskeletal and structural phosphoproteins such as nuclear lamins, vimentin, and cytokeratins that increase their phosphorylation state at mitosis. Identification of the localization of cdc25 proteins was made by immunoblotting proteins after electrophoretic



**FIG. 1. Human cdc25 protein is phosphorylated *in vitro* by p34<sup>cdc2</sup>-cyclin B kinase.** Highly purified p34<sup>cdc2</sup>-cyclin B kinase was incubated with purified bacterially expressed human cdc25 protein, histone H1, and 1 mg/ml trypsin inhibitor in the presence of [<sup>32</sup>P]ATP as described under "Experimental Procedures." The protein concentration of histone H1 relative to cdc25 was varied from 0.1- to 10-fold, as indicated, where 1 is equivalent to a final protein concentration of 0.1 mg/ml. Aliquots of the reaction were stopped after 30 (1), 60 (2), 120 (3), and 240 (4) s. Proteins were separated on 11% SDS-PAGE, and the gel was stained, dried, and autoradiographed. A, in the first 4 lanes each substrate component of the phosphorylation reaction was run alone and is shown as Coomassie Blue stained on the left next to the marker protein. Lane 2, cdc25; lane 3, trypsin inhibitor (Tryp In); lane 4, histone H1. Next three sets of 4 lanes, autoradiograph of the gel showing the phosphorylation reactions. The position of starfish cyclin B subunit (45 kDa), detectable through its autophosphorylation, is shown with an asterisk. B, protein bands shown in panel A corresponding to cdc25 were excised, and their radioactivity was quantified by Cerenkoff counting.

separation using one of three different antisera raised against either human cdc25C protein expressed in bacteria or synthetic peptides corresponding to the catalytic sites of cdc25 proteins (Girard *et al.*, 1992). Fig. 2 shows a typical electrophoretic pattern obtained after blotting with anti-cdc25 (panels B and D) and the associated protein phosphorylation pattern for the same region of the blots from cells in either G<sub>2</sub> (3 h after release from hydroxyurea block) (Girard *et al.*, 1991) or G<sub>2</sub>-M (7 h after release from hydroxyurea block) (panels A and C). The position of vimentin, one of the major phosphoproteins in fibroblasts, is shown by an arrow. This position was precisely determined by reprobings the same membrane with monoclonal anti-vimentin antibodies (Boehringer Mannheim) and clearly illustrates the difficulty in studying the *in vivo* phosphorylation of cdc25 in human cells by only one-dimensional separation of the proteins. On two-dimensional separation, cdc25 in G<sub>2</sub> cells resolved as a major spot migrating at the same molecular mass as vimentin and just slightly basic of the most basic isoform of vimentin (panel B). Clearly, cdc25 proteins undergo a marked increase in phosphorylation as cells enter mitosis in a manner similar to that described for other cell systems, and this correlates with the appearance of a second more acidic spot as detected by immunoblot (compare panels A and B and C and D) in Fig. 2). It can be noted that, in the presence of urea in the sample buffer used for two-dimensional electrofocusing, the apparent decrease in electrophoretic mobility observed for the phosphorylated cdc25C proteins in one-dimensional gels is not seen. A similar result can be observed in one-dimensional gels through simple inclusion of urea in the sample or gel buffer (data not shown).

To determine if the pronounced increase in phosphorylation seen in G<sub>2</sub>-M cells occurred at the same sites as those phosphorylated *in vitro* by p34<sup>cdc2</sup> protein kinase, cdc25 phosphoproteins from HS-68 mitotic cells or cdc25C proteins phosphorylated *in vitro* by p34<sup>cdc2</sup>-cyclin B were excised from two-dimensional gels and subjected to complete tryptic digestion. Phosphopeptides were then analyzed by two-dimensional peptide mapping and HPLC separation techniques and sequenced. Phosphopeptide maps shown in Fig. 3 revealed that the patterns of distribution of phosphopeptides are very similar, with four to five predominant phosphopeptides in both cases (Fig. 3, A and B). When the two sets of peptides (from *in vitro* and *in vivo* phosphorylation) were mixed and run together with the same technique, five phosphopeptides (numbered 1, 2, 3-4, 5, and 6) were found comigrating (Fig. 3, panel C) indicating that the sites phosphorylated *in vitro* and *in vivo* are on the same tryptic peptides. To precisely identify the threonine or serine residues within the human cdc25C molecule phosphorylated by p34<sup>cdc2</sup>-cyclin B kinase, <sup>32</sup>P-labeled cdc25 protein was digested with trypsin, and the resulting phosphopeptides were purified by HPLC. Eluted peptides were individually subjected to two-dimensional mapping and to gas phase sequencing. Fig. 4 presents the complete amino acid sequence of human cdc25C with the relative position of seven predicted phosphorylation sites for p34<sup>cdc2</sup> indicated in **boldface** and numbered from 1 to 7 according to their position, starting at the NH<sub>2</sub> terminus. Along this sequence, the sequence of five phosphopeptides as identified by microsequencing is indicated. In addition the phosphorylation of peptides 1, 3-4, 2, and 6 was also confirmed for highly purified cdc25C isolated from mitotic cells through mitotic shake. To further confirm the identity of the different phosphopeptides, phosphoamino acid analysis was performed on individual peptides resolubilized from the TLC plates and showed that each phosphopeptide contained the phosphoa-

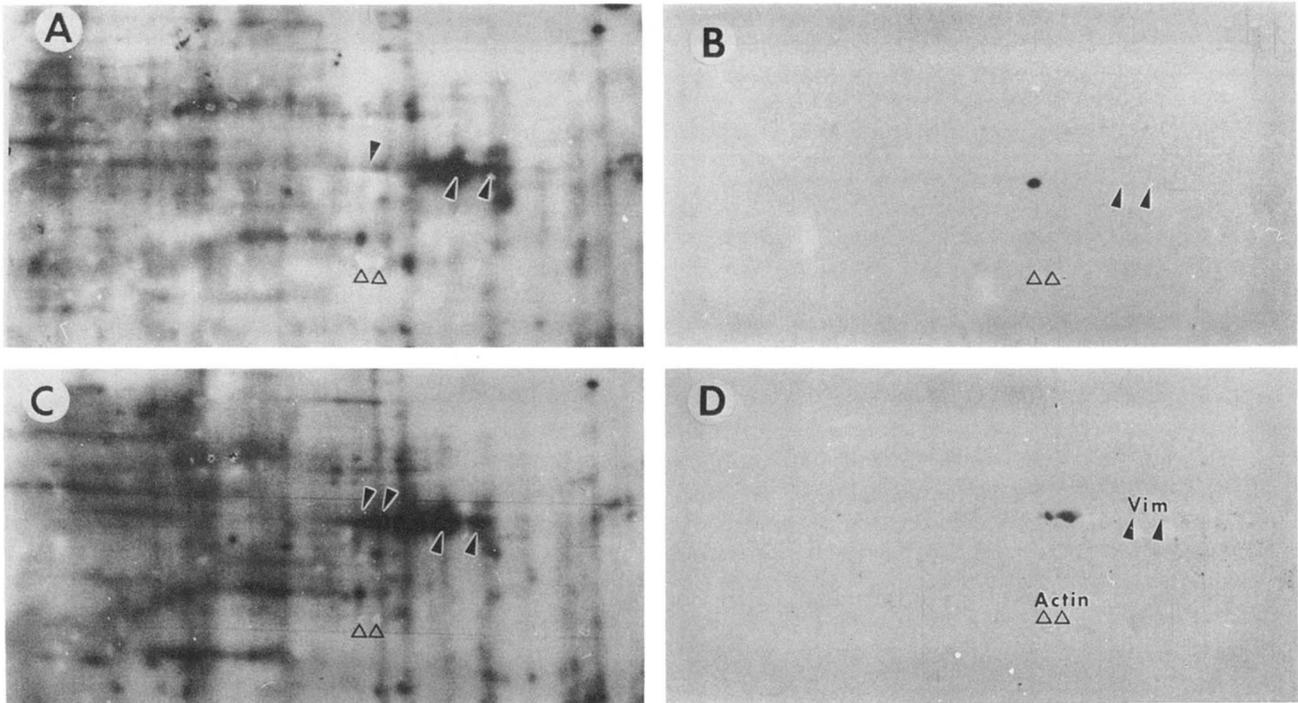


FIG. 2. Two-dimensional analysis of the electrophoretic mobility and phosphorylation of cdc25C proteins in human fibroblasts in G<sub>2</sub> and at G<sub>2</sub>-M transition. Human fibroblasts (HS-68) were grown, synchronized, and <sup>32</sup>P-labeled as described under "Experimental Procedures." After 90 min of labeling, cells were harvested in lysis buffer, and total cell extracts were analyzed by two-dimensional gel electrophoresis as described under "Experimental Procedures." Separated proteins were then transferred to nitrocellulose for immunoblotting with anti-cdc25 antibodies as described previously (Girard *et al.*, 1992). Shown are autoradiograms of the blots from cells in G<sub>2</sub> (panel A) and in late G<sub>2</sub> and M (panel C) with their corresponding immunostaining for cdc25 proteins as revealed by ECL (panels B and D, respectively). The positions of two major cytoskeletal proteins, vimentin (*Vim*) and actin, are shown by arrows.

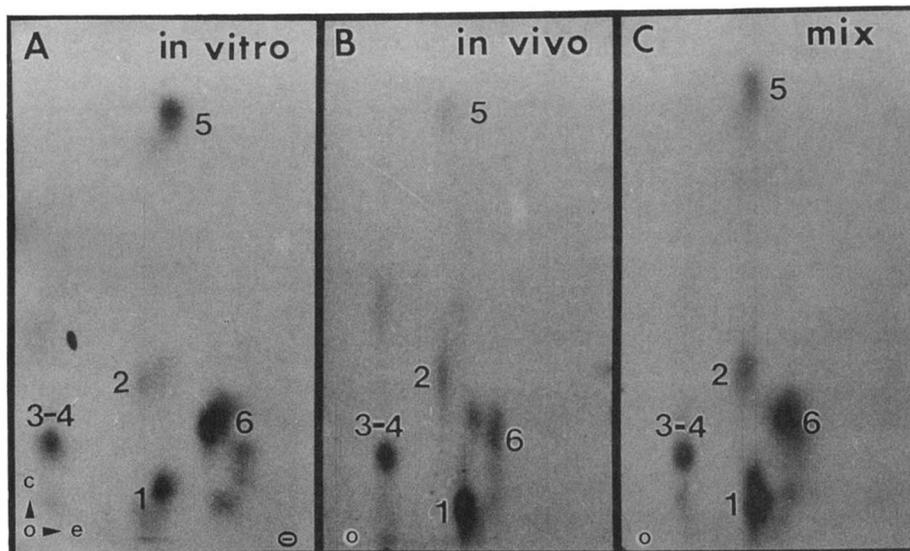
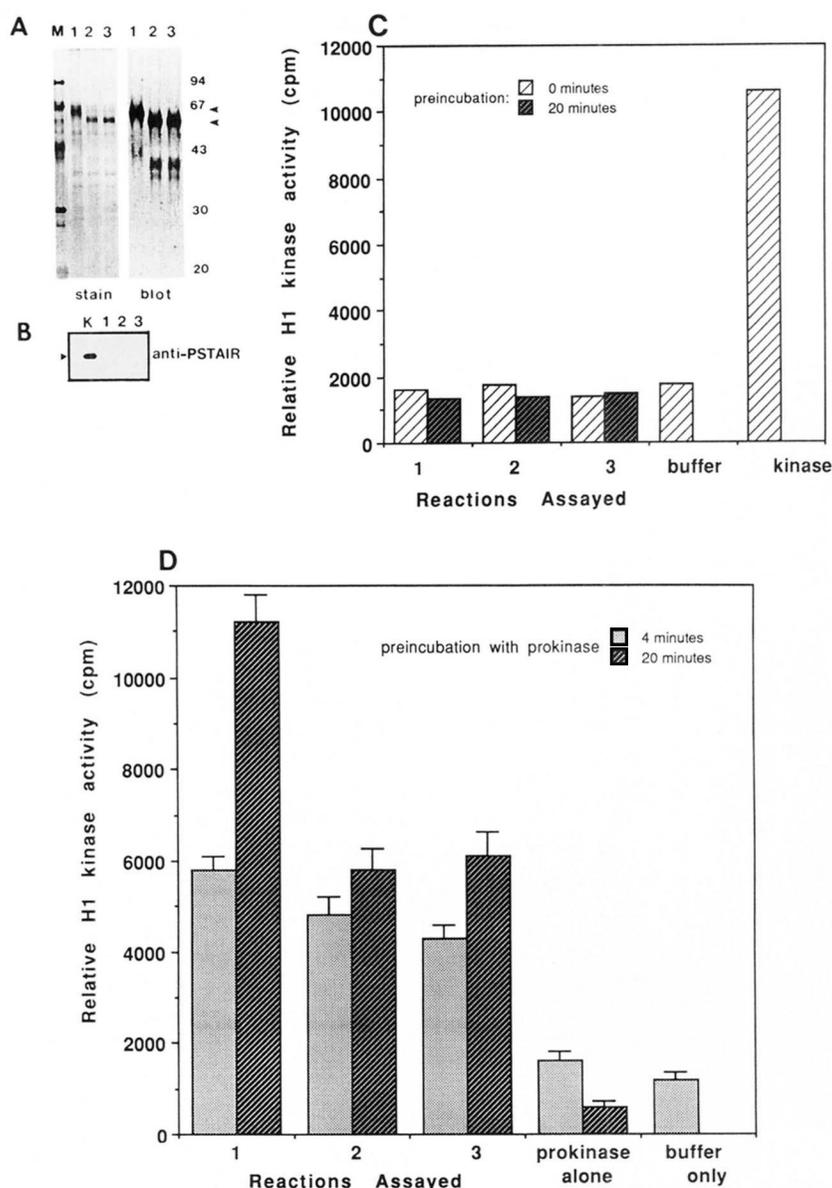


FIG. 3. Two-dimensional tryptic peptide maps from cdc25C protein. Purified cdc25C was phosphorylated *in vitro* with p34<sup>cdc2</sup>-cyclin B kinase as described under "Experimental Procedures." Alternatively, phosphorylated cdc25 was derived from <sup>32</sup>P-labeled mitotic human fibroblasts as described in Fig. 2. After labeling and two-dimensional electrophoresis, phosphorylated cdc25 was excised from the dried gels and subjected to tryptic digestion followed by two-dimensional separation of the phosphopeptides as described under "Experimental Procedures." Shown are autoradiograms of the TLC plates. Five major phosphopeptides have been labeled 1, 2, 3-4, 5, and 6 in correspondence with their location in the sequence of cdc25C as identified by microsequencing and as indicated in Fig. 4. Panel A, tryptic phosphopeptides from purified cdc25C labeled *in vitro* with p34<sup>cdc2</sup> kinase; panel B, tryptic cdc25 phosphopeptides from human fibroblasts labeled in G<sub>2</sub>-M; panel C, mixture (*mix*) of the samples described in panels A and B. Approximately 250 cpm were loaded on each plate. *o* marks the origin. The direction of migration in the first dimension (*e*, for electrophoresis, cathode on the right) and in the second dimension (*c*, for ascending chromatography) are indicated on panel A.

mino acid predicted from the sequencing data with only peptide 3-4 (see position on Figs. 3 and 4) containing both phosphoserine and phosphothreonine (data not shown). From these data it is clear that six of the seven potential S/T-P

consensus sites for phosphorylation by p34<sup>cdc2</sup>-cyclin B kinase (Thr<sup>48</sup>, Thr<sup>67</sup>, Ser<sup>122</sup>, Thr<sup>130</sup>, Ser<sup>168</sup>, and Ser<sup>214</sup>) were indeed found labeled *in vitro*. It can be noted from the sequencing data that two of the potential cleavage sites by trypsin, Arg<sup>47</sup>



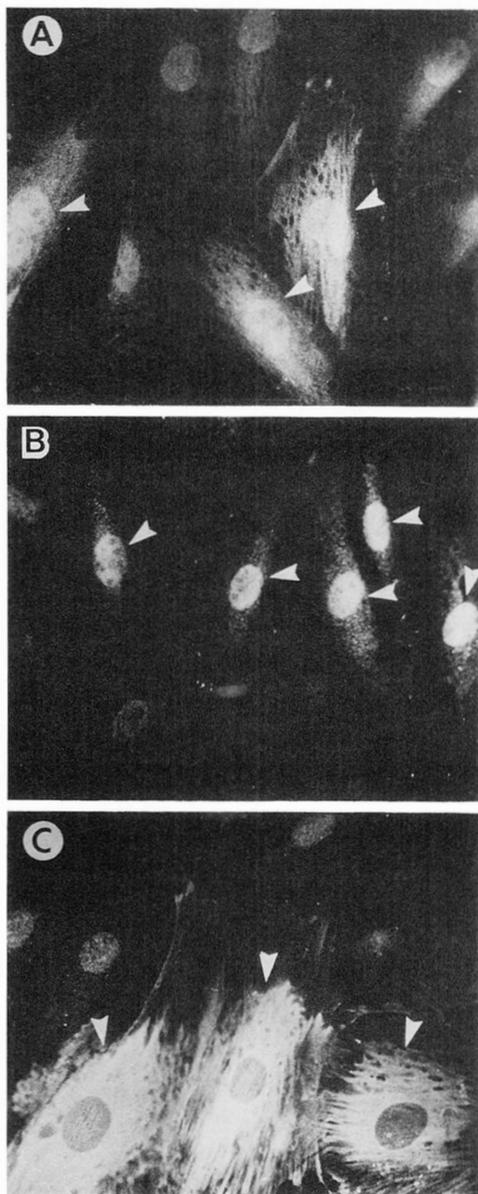


**FIG. 5. PAGE, immunoblot, and activity analysis of human cdc25C phosphorylated *in vitro* with p34<sup>cdc2</sup> protein kinase.** Human cdc25C protein was incubated together with p34<sup>cdc2</sup>-cyclin B kinase (reactions 1 and 2) or alone (reaction 3) in the presence of ATP (reactions 1 and 3) or ADP (reaction 2) as described under "Experimental Procedures." p34<sup>cdc2</sup>-cyclin B kinase was removed by immunoprecipitation with anti-cyclin B antibodies bound to protein A-Sepharose beads. Phosphorylated or nonphosphorylated cdc25 protein (reaction 1 or 2 + 3, respectively) remaining in the supernatant was analyzed on SDS-polyacrylamide gel and either silver stained (first three lanes marked *stain*) or immunoblotted with anti-cdc25 antibodies (lanes marked *blot*). The sizes of the molecular weight markers (*M*) are indicated in kDa together with the position of unphosphorylated cdc25 (lower arrow) and phosphorylated cdc25 (upper arrow). In panel B is shown an immunoblot of the supernatants from reactions 1–3 as shown in panel A, reacted with anti-PSTAIR antibodies, to detect any residual p34<sup>cdc2</sup> protein kinase. As a control, lane K shows the immunoreactivity of the same amount of p34<sup>cdc2</sup> kinase as used in reactions 1 and 2 (exposure time for the ECL detection, 15 s). Panel C, relative histone H1 kinase activity in aliquots (1/50 of the total volume) of the supernatant fractions of reactions 1–3 after immunoprecipitation of p34<sup>cdc2</sup>-cyclin B kinase. Controls were as follows: assay with 1/50 of the total amount of p34<sup>cdc2</sup>-cyclin B kinase used to phosphorylate cdc25 protein in reaction 1 and 2 (*kinase*) or with buffer only (*buffer*). Panel D, phosphorylated and unphosphorylated cdc25C proteins (reactions 1–3 above) were incubated with purified inactive cdc2-prokinase. After 4 and 20 min of incubation at 4 °C, samples were removed and assayed for histone 1 kinase activity. Shown is a histogram of the relative incorporation of counts/min into histone H1 for the different reaction mixtures. The data shown represent the mean of eight individual experiments, and standard deviations for each value are also represented. Controls showing H1 kinase activity of the prokinase alone and buffer (from the prokinase activation incubation step) are shown.

incubation time for prokinase activation by cdc25 up to 60 min did not result in significantly higher H1 kinase activity (data not shown). This 2–3-fold increase in the activity of phospho-cdc25 toward prokinase is very similar to that described previously comparing the activity of cdc25 immunoprecipitated from interphase and mitotic *Xenopus* extract using a similar assay (Izumi *et al.*, 1992; Kumagai and Dunphy,

1992). We next examined if *in vitro* phosphorylation by purified p34<sup>cdc2</sup>-cyclin B kinase brought about changes in cdc25 activity in living human and rat fibroblasts through microinjection of the phosphorylated and unphosphorylated cdc25 proteins into synchronized cells. Since one dogmatic problem in assaying the *in vitro* phosphatase activity of cdc25 has involved the extent of renaturation of the bacterially ex-

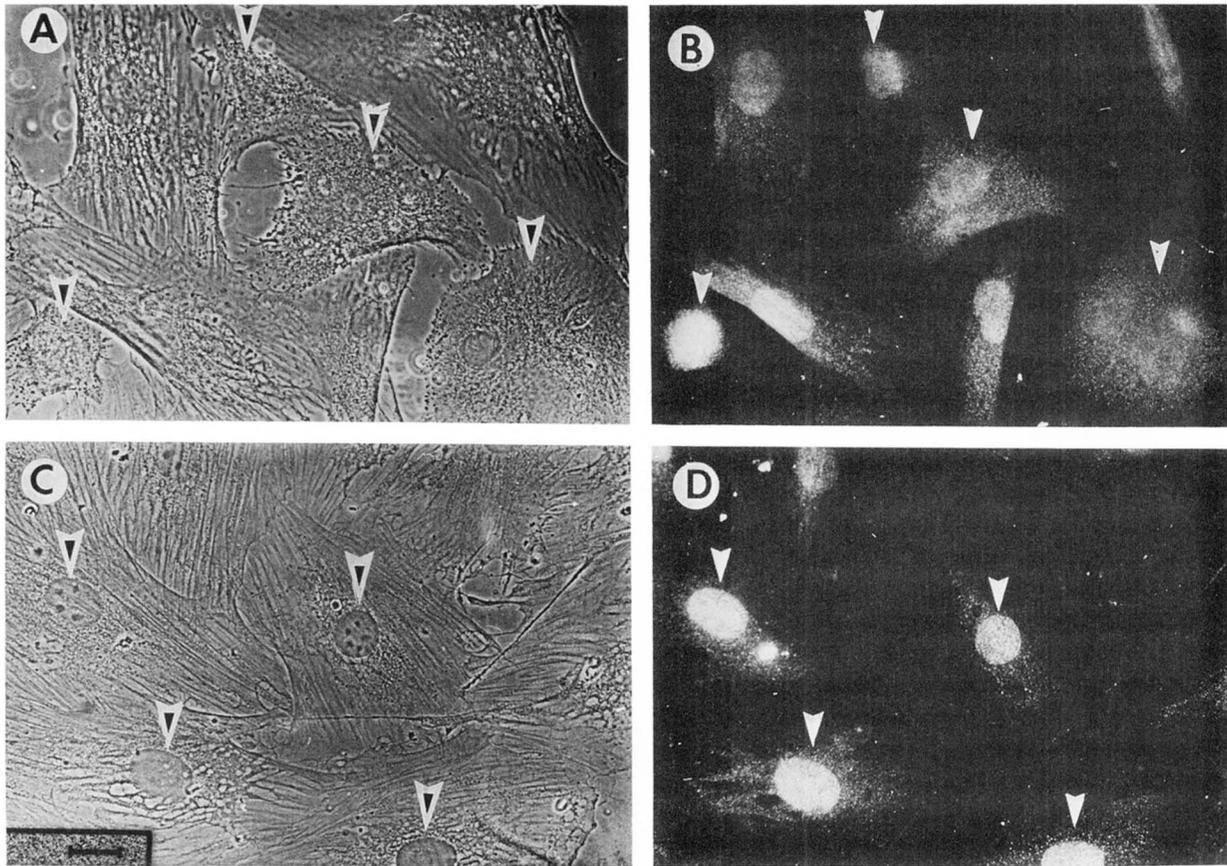
pressed cdc25 proteins, initial studies examined if cdc25 proteins could undergo the correct cytolocale after injection into growing cells. For these studies cells growing on glass coverslips were microinjected with the purified cdc25 proteins and at different times thereafter fixed and stained for the localization of cdc25. We (Girard *et al.*, 1992) and others (Millar *et al.*, 1991a) have previously shown that in rat and human fibroblasts cells, cdc25 proteins are predominantly localized in the nucleus. Fig. 6 shows the cytolocale of microinjected cdc25 proteins in REF-52 cells at 10 (Fig. 6A) and 30 min



**FIG. 6. Microinjection of purified cdc25 protein into mammalian fibroblasts.** To assess the *in vivo* effect of elevating intracellular levels of cdc25 protein, purified cdc25 protein from bacteria was microinjected into rat embryo fibroblasts at a concentration of 0.2 mg/ml. At different times thereafter, cells were fixed and stained for the distribution of cdc25 protein visualized with rabbit anti-cdc25 polyclonal antibodies as described (Girard *et al.*, 1992). *Panel A*, cells microinjected with purified cdc25 protein and fixed 10 min afterward. *Panel B*, cells microinjected with purified cdc25 protein and fixed 30 min after. *Panel C*, cells microinjected with cdc25C protein that was inactive in the prokinase activation assay due to repeated freeze-thawing and fixed 60 min after microinjection. Injected cells are shown by arrows in each panel. Nuclear staining of the endogenous cdc25 protein is visible for uninjected cells in each panel.

(Fig. 6B) after microinjection. In all cases cells were microinjected in the cytoplasm. Whereas in the initial stages after injection the protein is localized both in the cytoplasm and in the nucleus, after 30 min, proteins have essentially fully relocated into the nucleus. (Note the staining for injected cdc25 protein is brighter than the endogenous cdc25 staining in uninjected cells.) To confirm that this would not occur with cdc25C proteins that were inactive or denatured, we microinjected a sample of cdc25C proteins that had lost the capacity to induce prokinase activity *in vitro* due to repeated freeze-thawing. In this case the protein remains exclusively localized in the cytoplasm and does not show translocation into the nucleus after 60 min (Fig. 6C). These data clearly show that our bacterially expressed and purified active cdc25 protein contains the conformational information necessary for translocation to the nucleus. Hence on the basis of these observations it would appear unlikely that the cdc25 proteins we have injected are improperly refolded or denatured.

Since we have shown before that microinjection of active purified p34<sup>cdc2</sup>-cyclin B kinase produces morphological changes that mimic prophase entry into mammalian cells (Lamb *et al.*, 1990), the induction of such changes provides a good means of detecting the potential activation of endogenous p34<sup>cdc2</sup> kinase activity *in vivo*. Clearly, however, with the microinjection of a marked excess of nonphosphorylated cdc25C protein, apart from the translocation to the nucleus, little or no evidence of morphological or structural changes in injected cells was observed (Fig. 6, A and B). This would suggest that, unlike the *in vitro* system, in which addition of nonphosphorylated cdc25 is sufficient to induce p34<sup>cdc2</sup>-cyclin B kinase activation, in mammalian cells the activation of the prokinase does not occur with nonphosphorylated cdc25 protein alone. However, as shown in Figs. 7 and 8, when cells in G<sub>2</sub> were injected with the purified cdc25 protein that had been phosphorylated by p34<sup>cdc2</sup>-cyclin B kinase as described in Fig. 5, marked changes in cell morphology were observed. Within 15 min of injection of the phosphorylated cdc25 protein, injected cells began to undergo retraction of the cytoplasm and round up in a way similar to prophase entry (Fig. 8A, cells designated by arrows), an effect that reached its peak 30 min following injection (see Fig. 7, A and B and Fig. 8B) and started to reverse by 60 min after injection (Fig. 8C). Note that the cells in Fig. 8, A–C were photographed when still living, whereas the cells in Fig. 7 were photographed after fixation, immunofluorescence processing, and mounting, and this last treatment results in a poorer quality of the phase contrast images. In Fig. 8, D and E, to demonstrate that the cells showing a rounding up morphology are specifically the ones that have been injected with cdc25, injected cells have been fixed after 30 min and stained for the presence of mouse IgGs, coinjected as a marker for injected cells. As shown in Fig. 8E, two cells were injected in that field, which both show retraction of their cytoplasm and rounding up (cells designated by arrows in Fig. 8D). These effects were specific to the phosphorylated cdc25 protein since both injection of preparation 2 (cdc25 recovered after incubation with p34<sup>cdc2</sup>-cyclin B kinase and ADP) or preparation 3 (cdc25 incubated alone with ATP) showed a complete lack of effect (Fig. 7, C and D) and Table I). Furthermore, microinjection of the level of cdc2 kinase used for the *in vitro* phosphorylation experiment (~0.015 mg/ml) was on its own insufficient to induce any prophase-like changes in morphology (data not shown). This is consistent with our previous observations describing that levels of kinase 5–10-fold higher are necessary to induce such effects (Lamb *et al.*, 1990). The prophase-like phenotype induced by cdc2-phosphorylated cdc25 was exactly similar to



**FIG. 7. *In vivo* activity of phosphorylated or nonphosphorylated cdc25 protein.** We investigated if phosphorylation of cdc25 protein changed its *in vivo* activity through microinjection of purified phosphorylated cdc25C prepared as described in Fig. 5, *reaction 1* (and *reactions 2* and *3* as a control) into rat embryo fibroblasts synchronized in G<sub>2</sub>. Cells were fixed 40 min after microinjection and stained for the distribution of cdc25 and mouse marker-injected IgGs. Shown are phase contrast (*panels A* and *C*) or fluorescence micrographs of cells stained for cdc25 protein (*panels B* and *D*). *Panels A* and *B* show cells injected with cdc25 protein that had been phosphorylated by p34<sup>cdc2</sup>-cyclin B kinase and immunodepleted from the kinase as shown in Fig. 5 (*reaction 1*), and *panels C* and *D* show cells injected with nonphosphorylated cdc25 protein incubated with ATP (Fig. 5, *reaction 3*). Shown by arrows in each panel are the injected cells as identified with the staining for mouse marker IgGs (not shown).

the effects we reported before of injecting active cdc2 kinase into interphase mammalian cells (Lamb *et al.*, 1990). Like cdc2 kinase injection, phosphorylated cdc25 did not induce any detectable nuclear envelope breakdown and induced only a limited extent of chromatin condensation (data not shown). However, unlike the effects of purified active cdc2 kinase that were elicited in G<sub>0</sub>, G<sub>1</sub>, and G<sub>2</sub> cells and maintained for hours, reversing only when reaching S-phase (for G<sub>0</sub> and G<sub>1</sub> cells) or at mitosis (for G<sub>2</sub> cells) (Lamb *et al.*, 1990), the effects of phosphorylated cdc25 were only observed when injecting cells in G<sub>2</sub> (*i.e.* no effect was observed when phosphorylated cdc25 was injected in G<sub>0</sub>, G<sub>1</sub>, or S-phase cells; data not shown) and were readily reversing by 60 min after injection (Fig. 8C) and completely reversed within 90–120 min. These results could be obtained in a variety of cell lines including human embryonic and adult fibroblasts, Swiss 3T3 cells, CV1 monkey cells, and HeLa cells. In addition, the effects could be prevented if the phosphorylated cdc25C (or cdc2 kinase) was denatured by boiling prior to injection (data not shown). The reversal of this cell-rounding effect of microinjected phospho-cdc25 can be delayed through treatment of injected cells with 100 nM okadaic acid, a concentration that presumably inhibits phosphatase type 2A activity (data not shown). The last data correlate with the previous observations made on *Xenopus* oocytes for the dephosphorylation of mitotic cdc25 proteins that were found to be okadaic acid-sensitive (Kumagai and

Dunphy, 1992; Izumi *et al.*, 1992). In addition, preincubation of phosphorylated cdc25 proteins with the purified catalytic subunit of phosphatase 2A was sufficient to prevent cell rounding, further demonstrating that only the phosphorylated form of cdc25C is competent to induce changes in morphology (data not shown). Table I summarizes the overall extent of the effects observed with the injection of the three different preparations of cdc25 proteins. Taken together, these data show that only after cdc25C protein is phosphorylated by p34<sup>cdc2</sup>-cyclin B kinase is it competent to induce changes in cell morphology similar to those observed following injection of active p34<sup>cdc2</sup>-cyclin B kinase, suggesting that such phosphorylation of cdc25C phosphatase makes it highly efficient *in vivo* to activate cells into a premature prophase-like phenotype through its catalytic action on endogenous p34<sup>cdc2</sup>-cyclin B prokinase.

#### DISCUSSION

We have shown that cdc25 phosphatase is an effective substrate *in vitro* for p34<sup>cdc2</sup>-cyclin B protein kinase and shows a marked increase in phosphorylation in living human fibroblasts at the transition G<sub>2</sub>–M. Comparative phosphopeptide mapping coupled with gas-phase sequencing allowed us to identify that the same sites were phosphorylated *in vivo* at mitosis as phosphorylated *in vitro* by cdc2-cyclin B kinase. Finally, microinjection into interphase rat embryo or human

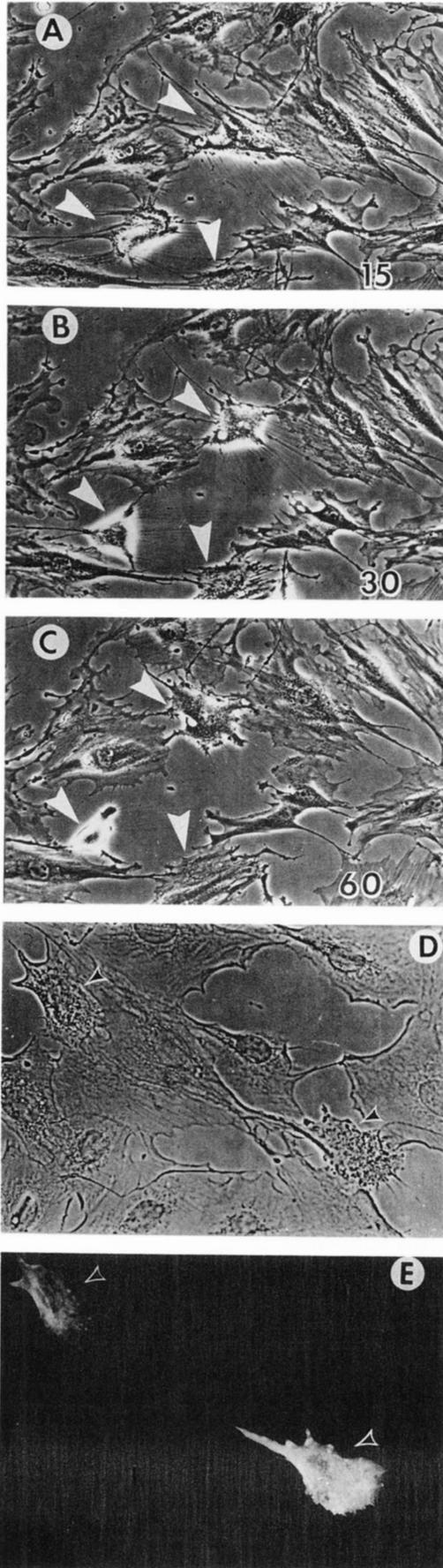


TABLE I  
Effects of phosphorylated and nonphosphorylated cdc25C protein microinjected into early G<sub>2</sub> REF-52 cells  
The values given represent the sum of four different microinjection experiments performed under "blind assay conditions" with solutions 1, 2, and 3.

Solution injected into G <sub>2</sub> cells	Cells rounded up <sup>a</sup> /total injected cells	Injected cells showing effect %
1. Phospho-cdc25 + ATP	150/153	98
2. cdc25 + ADP	0/142	0
3. cdc25 + ATP	0/89	0

<sup>a</sup> Cells lose their normal flattened morphology and substrate anchoring in a prophase-like phenotype as previously observed following injection of active cdc2 kinase.

fibroblasts showed that only the phosphorylated form of human cdc25C was capable of activating the endogenous p34<sup>cdc2</sup> prokinase *in vivo* because it induces effects similar to those observed following microinjection of active p34<sup>cdc2</sup>-cyclin B kinase.

Although genetic studies in yeast and *Drosophila* suggest that cdc25 acts in a dose-dependent manner in the activation of mitosis (Russell and Nurse, 1986; Edgar and O'Farrell 1989), in most other cell types including *Xenopus* (Jesus and Beach, 1992; Kumagai and Dunphy, 1991), HeLa (Millar *et al.*, 1991a), and two nontransformed human or rat cell lines (Girard *et al.*, 1992), cdc25 levels are invariant throughout the cell cycle. This observation implies that regulation and activation of cdc25 at mitosis must involve posttranslational modifications. Cyclic changes in *Xenopus* cdc25 electrophoretic mobility and *in vitro* tyrosine phosphatase activity could be correlated to the increased phosphorylation of sites in the amino terminus (Kumagai and Dunphy 1992; Izumi *et al.*, 1992). Similar studies using glutathione *S*-transferase-human cdc25C fusion protein confirmed that mitotic extracts from HeLa cells could phosphorylate and increase the *in vitro* activity of cdc25C and reported that immunodepletion of these M-phase extracts with anti-p34<sup>cdc2</sup> or anti-cyclin B antibodies prevented such phosphorylation of cdc25 (Hoffman *et al.*, 1993). A key issue to understanding the regulation of cdc25C was therefore the identity of the kinase responsible for its phosphorylation at mitosis.

The present study shows that cdc25C protein in human fibroblasts becomes phosphorylated at mitosis on the same sites as phosphorylated *in vitro* by highly purified cdc2-cyclin B and identifies these sites precisely by sequencing. Since direct evidence that p34<sup>cdc2</sup> protein kinase is the kinase responsible for the phosphorylation of cdc25 at mitosis relies on the comparison and identification of the sites phosphorylated *in vitro* and *in vivo*, precise analysis of these sites was essential. We have repeatedly observed five phosphopeptides when analyzing either *in vivo* or *in vitro* phosphorylated cdc25. It is unclear why Hoffman *et al.* (1993) have detected only

FIG. 8. The *in vivo* effects of phosphorylated cdc25 are rapidly reversed. In a delineated area, rat embryo fibroblast (REF-52) cells in G<sub>2</sub> were microinjected with phosphorylated cdc25 as for Fig. 7, panels A and B, and the same field of cells was photographed by phase contrast at 15, 30, and 60 min after injection. Shown by arrows are three injected cells in the field. Like for Fig. 7, marker antibodies were included in the injection, and after 90 min, cells were fixed and stained for the presence of these marker antibodies to confirm the identification of the injected cells, together with the use of time lapse video microscopy. In panels D and E, cells have been fixed after 30 min and stained for the presence of marker antibodies, to prove the cells injected. Two cells clearly rounded up and shown by arrows in panel D are shown to be injected in panel E.

two phosphopeptides for human cdc25 (which apparently resolved in opposing directions from the origin, when all tryptic peptides should migrate toward the cathode at pH 1.9). One possibility, at least concerning the *in vivo* phosphorylation, is that because these authors peptide mapped cdc25C from nocodazole-blocked cells (therefore already in metaphase), their system may have been insensitive in detecting some of the activation phosphorylation sites. In addition, as illustrated under "Results" (Fig. 2), it is very unlikely that by using only one-dimensional separation of an immunoprecipitate one can guarantee to separate human cdc25C from other more abundant and variably insoluble phosphoproteins such as members of the intermediate filament family. Indeed, even by two-dimensional analysis, such separation remained critical. Comparison of the sites found phosphorylated *in vivo* in cdc25C to the consensus sequence derived from other mapped sites on different protein substrates for the cdc2-cyclin B protein kinase (reviewed in Moreno and Nurse (1990)) shows the presence of polar/basic residues before and/or after the S/T-P site in five of the six sites we have identified. The exception, site 5 (serine 167), although undergoing substantial phosphorylation *in vitro*, shows little if any phosphorylation *in vivo*. This observation is in agreement with the conclusion that the sites we have identified *in vivo* correspond to true cdc2-cyclin B protein kinase substrates.

With the evidence that p34<sup>cdc2</sup> protein kinase phosphorylates the same sites as the ones responsible for the phosphorylation/activation of cdc25C at mitosis, it was important to examine whether such phosphorylation would bring detectable changes in the biological activity of cdc25, *i.e.* against its specific substrate, p34<sup>cdc2</sup> protein kinase inside living mammalian cells. We show that, when injected into G<sub>2</sub> nontransformed rat embryo fibroblasts, phosphorylated cdc25 proteins were extremely efficient at activating changes in cell shape and morphology, an effect not observed with even 10-fold higher concentrations of the nonphosphorylated form of cdc25. Thus although nonphosphorylated cdc25 does show phosphatase activity *in vitro*, it does not produce an effect in cells. The 2-fold increase of *in vitro* activity of cdc25C by phosphorylation underlies a profound change in its *in vivo* function.

The effects of phospho-cdc25C were very similar to those observed after injection of purified p34<sup>cdc2</sup>-cyclin B kinase in that they were mostly affecting the cell shape and cytoskeleton to mimic a prophase phenotype, without any nuclear envelope breakdown and only a limited extent of chromatin condensation (Lamb *et al.*, 1990). However, we could clearly discount that these effects resulted from contaminating p34<sup>cdc2</sup>-cyclin B kinase in a number of ways. 1) The supernatant containing phosphorylated cdc25 after immunoprecipitation of p34<sup>cdc2</sup>-cyclin B was checked to be devoid of any residual kinase by two criteria, absence of H1 kinase activity and absence of any detectable PSTAIR-reacting antigen. 2) Unlike the changes induced by injection of purified active p34<sup>cdc2</sup>-cyclin B kinase, the effects of phosphorylated cdc25 protein were only induced in G<sub>2</sub> cells and reversed within 60–90 min after injection. That injected cdc25 protein can only have an effect in G<sub>2</sub> cells is consistent with the fact that p34<sup>cdc2</sup>-cyclin B kinase activation does not only involve Tyr<sup>15</sup>/Thr<sup>14</sup> dephosphorylation but also requires its phosphorylation on threonine 161, an event that occurs only after cdc2 protein kinase becomes associated with cyclin subunits, *i.e.* from S-phase onward (Krek and Nigg, 1991).

cdc25, being a serine/threonine-rich molecule, is likely to contain a putative substrate site for various protein kinases.

*In vitro*, we have observed that cAMP-dependent protein kinase, mitogen-activated protein kinase, and calcium calmodulin-dependent protein kinase 2 can phosphorylate cdc25 albeit much less efficiently than cdc2 kinase. However, these phosphorylations appear to be without significant effect on the biological activity of cdc25, assessed by microinjection.<sup>2</sup> Among the members of the cdc2-like kinase family, we have also observed that both cdc2-cyclin A and cdk2-cyclin A can phosphorylate cdc25 *in vitro*, an observation<sup>3</sup> contrasting with that reported by Hoffman *et al.* (1993). However, several lines of evidence support that the cyclin B-complexed form of p34<sup>cdc2</sup> protein kinase is the most likely to be involved in the phosphorylation of cdc25C at mitosis. First, of the known regulatory proteins that activate cdc2 kinases, only cyclin B shows the punctual marked increase in abundance during the late G<sub>2</sub>-M phase transition. Thus if kinases of the Cdk family were involved in the activation of cdc25, they would have to complex with cyclin B. Second, cdc25, as an activator of MPF, has high affinity for the p34<sup>cdc2</sup>-cyclin B kinase and transiently forms an *in vivo* protein complex with the p34<sup>cdc2</sup>-cyclin B kinase (Gautier *et al.*, 1991; Jesus and Beach, 1992). However, there are clearly additional modulatory mechanisms acting on cdc25C during G<sub>2</sub> since at that time cells have both Thr<sup>161</sup>-phosphorylated p34<sup>cdc2</sup> prokinase and low levels of cyclin B. One possibility is that cdc25, which was found to be essentially nuclear (Millar *et al.*, 1991a; Girard *et al.*, 1992), and cdc2 proteins are spatially separated from cyclin B, which is thought to be cytoplasmically localized (Pines and Hunter, 1991). In this case some event acting on either cdc25 or cyclin B must take place to allow their interaction. In that respect, we note that injected phosphorylated cdc25C is not as highly concentrated in the nucleus as the nonphosphorylated cdc25 (Fig. 7B *versus* D). Additionally, as discussed by Kumagai and Dunphy (1992), a second enzymatic activity that regulates the activation of cdc25 is the protein phosphatase responsible for dephosphorylating cdc25. Since phosphorylation of cdc25 by p34<sup>cdc2</sup> protein kinase induces such marked changes in cells, the phosphatase that modulates this process must be of paramount importance. In *Xenopus*, both phosphate type 1 and 2A can effectively dephosphorylate cdc25 and inhibit its *in vitro* activity (Kumagai and Dunphy, 1992; Izumi *et al.*, 1992). In mammalian cells we have observed that low levels of the phosphatase inhibitor okadaic acid are sufficient to prolong the effects of phosphorylated cdc25C suggesting that *in vivo* the phosphatase activity acting on cdc25C is more likely to be the type 2A form. Although okadaic acid treatment alone is inconclusive (the levels of inhibitor that penetrate the cells cannot be determined), such a role for type 2A phosphatase would be in agreement with the observation that INH, an inhibitory activity that prevents mitotic activation in *Xenopus*, is a type 2A-related phosphatase (Lee *et al.*, 1991). However, since phosphatase 2A is a multimeric protein, further studies are still required to determine which form of the enzyme would be responsible for the inhibition of cdc25 activation.

The results obtained from the injection of the cdc25C protein phosphatase also underline an interesting finding with respect to the cytolocalization of cdc25C and of cdc2 activation at mitosis. We observed that denatured forms of the protein remain in the cytoplasm after injection, whereas active cdc25 proteins are quickly translocated into the nucleus. This observation confirms that cdc25C is predominantly a nuclear protein in mammalian cells and suggests that the events involved in the activation of cdc2 protein kinase are either

<sup>2</sup> A. Fernandez and N. J. C. Lamb, unpublished observations.

<sup>3</sup> A. Fernandez and N. J. C. Lamb, unpublished observations.

restricted within the nucleus or take place only after cdc25 has translocated to the cytoplasm. The process we have described here suggests that the phosphorylation of cdc25 by p34<sup>cdc2</sup>-cyclin B kinase may be part of a positive autocatalytic activation loop (see model Fig. 7 in Izumi *et al.*, 1992), in which cdc25 activates p34<sup>cdc2</sup>-cyclin B kinase that, in turn, phosphorylates cdc25 and activates further p34<sup>cdc2</sup>-cyclin B kinase. The interesting possibility is that different cdc25 proteins are responsible for differentially dephosphorylating the various members of the Cdk protein kinase family. For human cdc25 proteins, there is clearly a precedent since cdc25A and cdc25B appear to activate cdc25 phosphatase activity in the absence of cdc2 protein kinase but in the presence of cyclin B (Galaktionov and Beach, 1991). They would thus induce the initial activation of cdc2-cyclin B with the subsequent phosphorylation of cdc25C inducing the hyperactivation of cdc2 protein kinase. In such a process the role of the cdc25C phosphorylation would be to stimulate a sufficiently high level of cdc2 protein kinase activity to simultaneously overcome the counteracting protein phosphatases and drive cells into mitosis. However, the need for phosphorylation of cdc25 is questionable since untreated cdc25 shows *in vitro* phosphatase activity (Gautier *et al.*, 1991; Dunphy and Kumagai, 1991; Lee *et al.*, 1992). Moreover, in *Xenopus* the interphase form of cdc25X has intrinsic *in vitro* phosphatase activity (Kumagai and Dunphy, 1992). In our *in vivo* assay only the cdc25 protein phosphorylated by cdc2 showed an effect, whereas even up to a 10-fold excess of unphosphorylated cdc25C was without any detectable mitotic effect. (We have estimated from immunoblotting defined quantities of cell extracts against purified cdc25C proteins, the concentration of which was estimated from amino acid analysis, that the intracellular concentration of cdc25C protein is of the order of 10 µg/ml.) Such an all-or-nothing response *in vivo* contrasts with the data obtained *in vitro* by others (Izumi *et al.*, 1992; Kumagai and Dunphy, 1992) and in this study comparing the activity of phosphorylated and unphosphorylated cdc25C measured against cdc2 prokinase. Phospho-cdc25C exhibited only a 2–4-fold higher activity than the unphosphorylated forms, whereas when using a crude *Xenopus* cell extract instead of purified cdc2 prokinase, more than a 20-fold increase in H1 kinase activation was found with the phosphorylated form of cdc25C (Hoffmann *et al.*, 1993). These discrepancies between the data obtained *in vitro* with highly purified components and either *in vitro* data obtained with crude extracts or our *in vivo* microinjection data strongly suggest that some additional factors related to the state of phosphorylation of cdc25C are involved in the regulation of its activation *in vivo*. Unphosphorylated cdc25C may interact with proteins that would exert an inhibitory effect on it, or alternatively phospho-cdc25 may complex or draw into a complex with cdc2 factors integral to the amplification effect. Such mechanisms would allow a further level of control on MPF activation and will require further investigation. Nonetheless, the mapping of the phosphorylation

sites on cdc25 and the microinjection experiments described here clearly show that phosphorylation of cdc25C by cdc2-cyclin B kinase is a key step in the induction of mitotic activation in living mammalian cells.

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