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The cdc2Ms Kinase Is Differently Regulated in the Cytoplasm and in the Nucleus[1]

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To study a cyclin-dependent kinase (CDK) from alfalfa (*Medicago sativa* L.), an antibody was raised against the C-terminal 16 amino acids of the protein cdc2aMs. The cdc2Ms protein was immunopurified with this antibody and its histone kinase activity was measured. The cdc2Ms kinase is activated at the G1/S transition when phosphate-starved cells from the G0 phase re-enter the cell cycle and remain active as cells transit the S, G2, and M phases, indicating that the same CDK regulates all of these phases in alfalfa. In contrast, when cdc2Ms kinase was purified by binding to $p13^{suc1}$, it was active only in the G2 and M phases. In immunoblots the C-terminal antibody detected an equal amount of the cdc2Ms protein in the cytoplasm and in the nucleus. By indirect immunofluorescence, however, the cytoplasmic form of cdc2Ms could not be found in the S phase of the cells, indicating that the epitope for the cdc2 antibody is not accessible. Binding of putative inhibitor proteins to cdc2 was shown by inactivation of purified plant CDK when cell extracts were added. Furthermore, purified CDK inhibitors, such as the mouse $p27^{kip1}$ and the yeast $p40^{sic1}$, blocked the purified plant CDK activity.

By irradiation of root meristems it was found that the two active phases of the cell cycle, the DNA synthesis (S) phase and segregation of chromosomes (M) phase, are each preceded by a regulatory phase called the G1 and G2 gap phases, respectively (Howard and Pelc, 1953). Within the gap phases the cell cycle can be interrupted at specific points at which developmental signals, the accomplishment of previous events in the cell cycle, as well as DNA damage caused by irradiation are signaled to the cell cycle regulatory circuit to start or stop proliferation. Genetic studies on yeast and biochemical analysis concerning animal cells and oocytes have uncovered the basic mechanism of cell cycle regulation. In the center of this regulatory network is a family of protein kinases complexed with regulatory cyclin subunits named CDKs. CDK activity is controlled by an association with cyclins or other regulatory proteins such as CDK inhibitors, and by the phosphorylation of the CDK (reviewed by Morgan 1995).

Regulation of the cell cycle is highly conserved among eukaryotes, and many of the genes involved have been isolated from a wide range of organisms such as yeasts, animals, and plants. Whereas in yeasts a single CDK is involved both in the G1/S and G2/M transitions, in higher eukaryotes several related enzymes have evolved (Nasmyth, 1993; Hirt and Heberle-Bors, 1994). The plant sequences can be categorized according to the similarity of the conserved PSTAIRE sequence motif. Only those plant CDK genes in which the PSTAIRE sequence is perfectly conserved are able to complement *Schizosaccharomyces pombe cdc2* and the *Saccharomyces cerevisiae CDC28* mutants (Colosanti et al., 1991; Ferreira et al., 1991; Hashimoto et al., 1991; Hirayama et al., 1991; Hirt et al., 1991, 1993; Fobert et al., 1996). Therefore, these genes are considered as functional homologs. It is interesting that in alfalfa (*Medicago sativa* L.) two highly similar *cdc2* genes can complement different yeast *CDC28* alleles. The *CDC28-4* allele, which is compromised in the G1/S transition, was only complemented by the *cdc2bMs* gene, whereas the G2/M function, abrogated by the *CDC28-1N* allele, was complemented only by the *cdc2aMs* gene (Hirt et al., 1993). It is not known, however, whether these two closely related plant CDKs have a distinct role in the plant cell cycle.

Some of the CDK-related genes contain a slightly altered form of the PSTAIRE motif. In yeast or animal cells only a part of the *cdk* genes with an altered PSTAIRE motif is involved in cell cycle regulation, whereas other CDKs regulate processes such as transcription or phosphate metabolism (Kaffman et al., 1994). Some of the plant *cdk* genes in this class, however, may also regulate the cell cycle, since

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Abbreviations: cdc2CT, cdc2 C-terminal antibody; CDK, cyclin-dependent kinase; DAPI, 4,6-diamidino-2-phenylindole.

the mRNA transcripts of these genes are restricted to certain phases of the cell cycle (Fobert et al., 1996; Segers et al., 1996). A rice CDK-related sequence contains the motif NFTAIRE (Hashimoto et al., 1991; Hata, 1991). Recently, putative homologs of this gene have been identified in animals and in yeast, and it was demonstrated that they activate CDK through phosphorylation of the Thr-161 residue (Poon et al., 1993). The Thr-14/Tyr-15 amino acids are the other conserved phosphorylation sites that were also found in the plant sequences. The expression of a mutated Arabidopsis CDK, in which the Thr-14/Tyr-15 residues were exchanged to nonphosphorylatable Ala, had no observable phenotype, suggesting that the phosphorylation of these positions may not be critical in plants (Hemerly et al., 1995).

In alfalfa *cdc2aMs* mRNA and protein are present in dividing cells throughout the cell cycle (Magyar et al., 1993), indicating that the regulation of CDK activity is primarily accomplished by posttranslational modifications. So far, CDK complexes have been isolated from plants through their ability to bind to the yeast regulatory protein $p13^{suc1}$ (John et al., 1989, 1991; Bako et al., 1991; Colosanti et al., 1991; Feiler et al., 1990). Using this assay CDK activities have been detected in both the S phase and mitosis (Magyar et al., 1993; Bako et al., 1994). However, in other studies the mitotic $p13^{suc1}$-bound CDK activity was prevalent (Prennes et al., 1993; Grafi and Larkins, 1995). Active CDK in the S phase has been shown to bind to the animal and viral S-phase-specific transcription factors E2F and E1A (Grafi and Larkins, 1995). Plant CDK was also found in a complex bound to the E2F binding site (Bako et al., 1994). Initial biochemical characterization of CDKs in plants suggests a number of complexes with different sizes, biochemical properties, and substrate specificities (Bako et al., 1994).

In addition to activity, another aspect of the regulation of CDK function is its localization. In one study using an antibody against the conserved PSTAIRE motif, an intense cytoplasmic labeling was observed (Mineyuki et al., 1991). However, nuclear staining was detected with a specific antibody against the maize cdc2aZm (Colosanti et al., 1993). A possible reason for the discrepancy in the results could be that the antibody against the PSTAIRE region is able to recognize only free CDK because the PSTAIRE region of CDK is normally concealed when cyclin is bound.

Since the yeast $p13^{suc1}$ protein binds a number of CDKs as well as other CDK-related kinases (Meyerson et al., 1992), it was necessary to produce antibodies that are specific to a given plant CDK and cyclin proteins and to use these antibodies to purify and study the regulation of CDK activity during the plant cell cycle.

MATERIALS AND METHODS

Plant Tissue Culture, Synchronization, and Measurement of Cell Cycle Parameters

A suspension culture derived from the *Medicago sativa* A2 line (Bögre et al., 1988) was maintained in Murashige-Skoog liquid medium (Murashige and Skoog, 1962) supplemented with 1 mg L^{-1} 2,4-D and 0.2 mg L^{-1} kinetin. The culture was diluted 1:20 in fresh medium weekly. The tobacco BY2 suspension culture was maintained as described previously (Nagata et al., 1992).

Synchronization started with the 1:5 dilution of a 7-d-old culture. After 8 h, 10 $\mu g\ L^{-1}$ aphidicolin was added to the medium. Incubation continued for 16 h, and then the drug was removed by washing the cells five times with medium before resuspending it in the original volume. Samples were collected at various intervals, frozen in liquid N_2, and kept at $-70°C$ until analysis. Synchronization of suspension cultures by phosphate starvation was done as described previously (Meskiene et al., 1995).

Flow cytometric analysis was performed as described by Pfosser (1989). Aliquots of 0.5 to 1 mL of suspension culture were collected at the given intervals, and 0.2 mL of cell wall-degrading enzyme solution (2% cellulase R10 [Onozuka, Yakult, Tokyo], 1% pectinase dissolved in 0.6 M mannitol, 5 mM $CaCl_2$, and 3 mM Mes [pH 5.7]) was added to the pelleted cells. After incubation at 37°C for 30 min, nuclei were released in 0.4 mL of staining solution (10 mM Tris-HCl [pH 7.5], 0.1% Triton X-100, and 4 $\mu g\ mL^{-1}$ DAPI) by passing through a needle. The released nuclei were directly measured in a flow cytometer (PAS2, Partec, Münster, Germany).

For determination of the mitotic index, cells were fixed in three parts ethanol and one part acetic acid, and then washed with 70% ethanol. DNA was stained with DAPI, as for flow cytometry, and observed by epifluorescence microscopy. The number of metaphase cells was counted among 1000 cells.

Production and Purification of Antibodies

A peptide (RITARGALEHEYFKDIK) corresponding to the last 16 amino acids of cdc2aMs (Hirt et al., 1991) and the peptide (EGVPSTAIREISLLKE), representing the conserved region of the cdc2 protein, were synthesized. An additional Cys residue was added to the N terminus of each of the peptides to allow coupling to BSA. Rabbits were immunized according to standard procedures (Harlow and Lane, 1988). For immunoblotting and indirect immunofluorescence, the antibody was affinity-purified on the peptide and cross-linked to cyanogen bromide-activated Sepharose CL 4B (Pharmacia) (Harlow and Lane, 1988). For immunoprecipitation, the antibody was purified on a protein A-Sepharose column (Harlow and Lane, 1988). The bound IgG was eluted from the protein A column using 0.1 M Gly (pH 4.0), 0.5 M $MgCl_2$ and dialyzed against PBS.

Purification of $p13^{suc1}$ Protein and Preparation of $p13^{suc1}$ Beads

The fission yeast $p13^{suc1}$ protein was overproduced in *Escherichia coli* and purified by DEAE-Sepharose, Phenyl-Sepharose, and Sephacryl S200 chromatography as previously described (Meijer et al., 1989). The $p13^{suc1}$ protein was coupled to cyanogen bromide-activated Sepharose CL 4B at a concentration of 5 mg protein 1 mL^{-1} swollen

beads, according to the manufacturer's instructions, and stored as a 50% suspension in suc1 buffer (50 mM Tris-HCl [pH 7.5], 250 mM NaCl, 5 mM EDTA, 5 mM EGTA, 5 mM NaF, 0.1% Nonidet P-40, and 0.5 mM PMSF).

Preparation of Cell Extracts and Separation of CDK Forms by DEAE-Sepharose FF and Sephacryl S300 Column Chromatography

The samples were homogenized in 3 volumes of homogenization buffer containing 25 mM Tris-HCl (pH 7.5); 15 mM $MgCl_2$; 15 mM EGTA; 75 mM NaCl; 1 mM DTT; 0.1% Nonidet P-40; 5 mM *p*-nitrophenylphosphate; 60 mM β-glycerophosphate; 0.1 mM Na_3VO_3; 1 mM NaF; 1 mM PMSF; 10 μg mL^{-1} leupeptin, aprotinin, and soybean trypsin inhibitor; and 5 μg mL^{-1} antipain, chymostatin, and pepstatin. The crude extract was centrifuged at 40,000*g* for 15 min and subsequently at 200,000*g* for an additional 1 h at 4°C. The cleared supernatant was used immediately for $p13^{suc1}$-binding, immunoprecipitation, or column chromatography. For immunoblotting, the protein concentration of the extract was determined and adjusted to 5 mg mL^{-1}, the SDS sample buffer was added, and the samples were heated to 95°C for 2 min and stored at −20°C.

For anion-exchange chromatography, the extract containing 10 mg of protein was loaded onto a 5-mL DEAE-Sepharose Fast Flow column (Pharmacia) equilibrated with chromatography buffer (20 mM Tris-HCl [pH 7.8], 75 mM NaCl, 5 mM $MgCl_2$, 5 mM EGTA, 5 mM β-glycerophosphate, 1 mM DTT, 0.01% Nonidet P-40, 1 mM NaF, 0.1 mM $NaVO_3$, and 0.25 mM PMSF). Unbound proteins in the column flow-through were saved, the column was washed with 10 volumes of chromatography buffer, and the bound proteins were eluted with 3 volumes of 0.3 M NaCl in chromatography buffer. Size-exclusion chromatography was done on a Sephacryl S300 (Pharmacia) column equilibrated with chromatography buffer. A 1-mg protein sample in 0.2 mL of extract was applied to the column. The fractions were analyzed by a protein kinase assay. For immunoblotting, the fractions were concentrated by acetone precipitation.

Preparation of Extracts from Yeast Cells

Yeast cells expressing the *cdc2aMs* and *cdc2bMs* genes were grown for 2 h on Gal-containing liquid medium as described previously (Hirt et al., 1993). The cells were broken by stirring them with glass beads in an extraction buffer. Further treatment of the extracts was as for the plant cells.

Immunoprecipitation, $p13^{suc1}$-Binding, and Measurement of Protein Kinase Activity

For the measurement of CDK activity after immunoprecipitation or $p13^{suc1}$-binding, 0.1 mg of protein in 0.1 mL of extract was used. All operations were done at 4°C. For immunoprecipitation, 10 μg of protein A-purified IgG of the alfalfa cdc2 antibody was added to the extract. After 1 h, 50 μL of the protein A beads was added from a 50% protein A-Sepharose suspension stored in the suc1 buffer. The tubes were rotated for an additional 1 h, and the beads were washed four times with 1 mL of suc1 buffer and two times with the kinase buffer (20 mM Hepes [pH 7.4], 15 mM EGTA, and 1 mM DTT). All of the buffer was aspirated using a needle, and the kinase assay was performed on the beads. $p13^{suc1}$-binding was done in essentially the same way, and 50 μL of suc1 beads was added to the samples and allowed to bind for 2 h. The kinase assays were performed with proteins immobilized on $p13^{suc1}$-Sepharose or protein A-Sepharose in a final volume of 15 μL. The reaction was started by adding the assay buffer (20 mM Hepes [pH 7.5], 15 mM $MgCl_2$, 5 mM EGTA, 1 mM DTT, 0.5 mg mL^{-1} histone H1 [Sigma type III], and 2 μCi of [γ-^{32}P]ATP). The reaction was incubated at room temperature for 30 min and was terminated by the addition of 5 μL of 4× SDS sample buffer. The samples were analyzed by SDS-PAGE and subsequent autoradiography. When immunoblotting was done after the $p13^{suc1}$-binding, 1 mg of total protein was bound to 0.2 mL of suc1 beads. After washing, the bound proteins were released by boiling in SDS sample buffer.

Purification of $p27^{kip1}$ and $p40^{sic1}$ from Bacteria and Inhibition of the Plant CDK

The $p27^{kip1}$ protein was overexpessed in bacteria, and the protein was purified from the inclusion body as described previously (Polyák et al., 1994). The bacterially produced $p40^{sic1}$ protein was purified from the soluble fraction of *E. coli* lysate according to published methods (Schwob et al., 1994). The immunoprecipitated or $p13^{suc1}$-bound plant CDK, immobilized on beads, was preincubated for 20 min at room temperature with 1 μg of $p27^{kip1}$ or $p40^{sic1}$ in 10 μL of kinase buffer. Subsequently, the protein kinase assay was started by the addition of 7.5 μg of histone H1 and 2 μCi of [γ-^{32}P]ATP and was continued for an additional 30 min at room temperature.

Immunoblotting

Samples containing 50 μg of protein were loaded on 12.5% SDS-polyacrylamide gels with the dimensions of 1.5 mm × 16 cm × 10 cm. With the help of a prestained molecular-mass marker (Rainbow, Amersham), the gel was run until the 30-kD marker protein was 1 cm from the front. A long run was essential for resolving the CDK forms. For immunoblotting, the SDS-polyacrylamide gels were transferred onto PVDF (Millipore) membranes in 50 mM Tris base, 50 mM boric acid buffer (pH 8.3) in a liquid electroblotting system (Hoefer, San Francisco, CA) at 30 V overnight with cooling. After Ponceau staining, the filters were blocked in 5% milk powder, 0.05% Tween 20 in a phosphate-saline buffer for 2 h. The affinity-purified first antibody was applied at 1 to 2 μg mL^{-1} IgG in a blocking buffer. Alkaline phosphatase-conjugated goat anti-rabbit IgG (Sigma) was used as a second antibody, and the reaction was visualized by the nitroblue tetrazolium-5-bromo-4-chloro-3-indolyl-phosphate substrates.

Cell Fractionation

Nuclear isolation was done as described previously (Hadlaczky et al., 1983). Protoplasts were isolated from 10 mL of suspension-cultured cells by incubating them for 1 h in 2% cellulase (R10, Onozuka), 0.5% driselase (Sigma), 0.5% macerozyme (Serva, Heidelburg, Germany), 1% pectinase (Serva), and 0.1% pectolyase (Sigma) dissolved in 0.18 M mannitol, 0.18 M sorbitol, 3.5 mM $CaCl_2$, 0.4 mM NaH_2PO_4, and 0.5% Mes (pH 5.6). The protoplasts were purified by sieving through a mesh (50 μM) and washing twice with UM I solution (0.38 M Glc, 1.36 mM $CaCl_2$), followed by sedimentation of the cells at 200*g* for 3 min. The cells were resuspended in 10 mL of GH buffer (0.1 M Glc, 0.1% [v/v] hexylene glycol, 4.7% [w/v] Suc, 0.3 mM spermine, and 1 mM spermidine [pH 8.3], set with $Ca[OH]_2$) and incubated on ice for 5 min. The nuclei were released by the addition of 0.1% Triton X-100 and vigorous pipetting. After 5 min of incubation, the nuclei were sedimented by centrifugation at 1000*g* for 10 min. The protein concentration of the supernatant was determined, and this cytoplasmic fraction was used to measure CDK activity or for immunoblotting. The pelleted nuclei were washed two times by resuspension in GHT buffer (GH buffer supplemented with 0.1% Triton X-100). For the measurement of CDK activity the proteins were extracted from the nuclei using 1 M NaCl in the extraction buffer. For immunoblotting the nuclei were either directly placed in SDS sample buffer, or proteins were sequentially extracted with 10 mM EDTA, 0.5 M NaCl, 1 M NaCl, and 7 M urea in a buffer consisting of 25 mM Tris-HCl [pH 7.5], 0.5 mM PMSF, 1 mM DTT, and 10 μg mL^{-1} leupeptin.

Indirect Immunofluorescence

Alfalfa cells were synchronized by aphidicolin, and samples were fixed in 3.7% formaldehyde in PBS with 0.1% Triton X-100 for 1 h. After washing in PBS, the samples were treated with an enzyme solution (1% cellulase R10 [Onozuka]) and 0.5% macerozyme (Calbiochem) in PBS for 20 min. After washing in PBS the cells were attached to slides coated with poly-L-Lys (Sigma) and extracted with 1% Triton X-100 for 30 min and with 100% methanol (−20°C) for 10 min. After washing with PBS and BPBS (PBS plus 1% [w/v] BSA), samples were processed for immunostaining. The affinity-purified antibody against the C-terminal peptide of cdc2aMs was used at a concentration of 1 μg IgG mL^{-1} overnight at 4°C. After washing with PBS and BPBS, samples were incubated with secondary anti-rabbit fluorescein isothiocyanate or tetramethylrhodamine isothiocyanate-conjugated antibody (Sigma) at a dilution of 1:200 for 45 min at room temperature. Samples were washed with PBS, and DNA was stained with 1 μg mL^{-1} DAPI in PBS. Slides were mounted in an antifade mounting medium (Dako, Glostrup, Denmark). Preparations were examined using an Olympus epifluorescence microscope equipped with standard fluorescence filter sets. Photographs were taken on T-MAX 400 film (Kodak). A control experiment was also performed in which 10 μg of antibody was preadsorbed with 1 μg of the peptide that was used for immunization; no immunofluorescence was observed.

RESULTS

Specificity of the Alfalfa cdc2 Antibody

An antibody was raised against a synthetic peptide that encodes the last 16 amino acids of the alfalfa cdc2aMs protein (Hirt et al., 1991). This cdc2CT specifically recognizes a 31-/32-kD protein doublet in alfalfa whole-cell extracts (Fig. 1A, lanes 1). The same proteins could be detected after purification of CDKs by binding to the yeast protein $p13^{suc1}$ (Fig. 1A, lanes 2). Only a subfraction of the CDK amount bound to $p13^{suc1}$, indicating that different CDK forms might be present in the extract. An antibody against a peptide containing the conserved PSTAIRE amino acids in CDKs reacted with the same 31-/32-kD doublet in the cell extract and in the $p13^{suc1}$-bound fraction (Fig. 1A). The PSTAIRE antibody also reacted with the yeast CDK in extracts that were prepared from *S. cerevisiae* (Fig. 1A, lanes 3). The binding to the antibodies was specific, because preincubation of the antibodies with an excess of the peptides that were used for immunization competed the immunoreaction to these proteins.

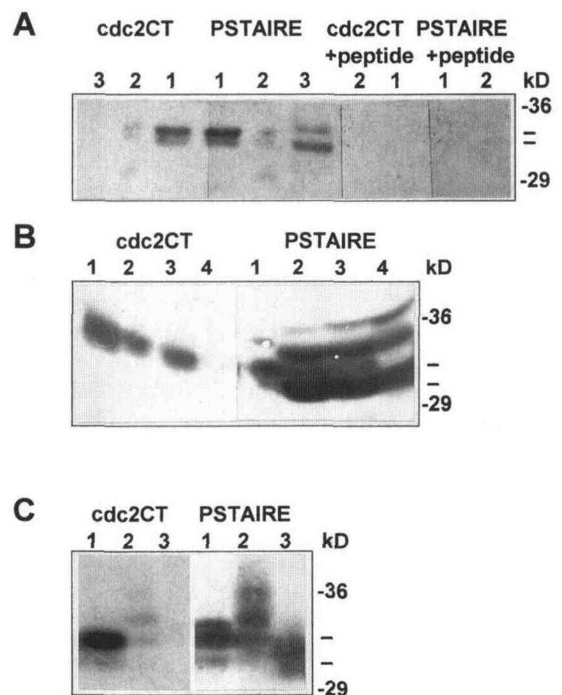

Figure 1. Specificity of the alfalfa cdc2CT and the PSTAIRE antibodies. A, Immunoblotting of proteins with the cdc2CT and the PSTAIRE antibodies and with the same antibodies preadsorbed with 2 μg of the peptides used for immunization. Lanes 1, Fifty-microgram proteins prepared from alfalfa cells; lanes 2, $p13^{suc1}$-bound proteins from alfalfa cell extract; lanes 3, *S. cerevisiae* protein extract. B, Protein extracts from alfalfa cells (lanes 1) and from *S. cerevisiae* transformed with the expression vector pYES-cdc2aMs (lanes 2), pYES-cdc2bMs (lanes 3), and pYES (lanes 4) were immunoblotted with the cdc2CT and PSTAIRE antibodies. C, Extracts prepared from alfalfa cells (lanes 1), from tobacco BY2 cells (lanes 2), and from wheat germ (lanes 3) were immunoblotted with the cdc2CT and PSTAIRE antibodies. The positions of molecular mass markers as well as of the cdc2Ms forms with a calculated mass of 31 and 32 kD are indicated.

There are two closely related CDK genes cloned from alfalfa, *cdc2aMs* and *cdc2bMs*. In the C-terminal region, against which the antibody was raised, there is a difference of 2 out of 16 amino acids. Therefore, we determined if the antibody against the cdc2aMs would cross-react with the cdc2bMs protein. Both genes were expressed in yeast, and the specificity of the antibody was studied by immunoblotting. The cdc2CT also reacted with the cdc2bMs protein (Fig. 1B, lanes 2 and 3, respectively). The yeast-expressed proteins have a molecular mass that is similar to that of the native protein in alfalfa extracts (Fig. 1B, lanes 1). In this article we refer to the proteins detected by the cdc2CT as cdc2, but bear in mind that it might equally be cdc2aMs or cdc2bMs. The PSTAIRE antibody reacted with the same alfalfa proteins as well as with the yeast CDC28 protein (Fig. 1B, lanes 1–4).

We were also interested in determining if these antibodies recognize the cdc2 in different plant species. The antibody raised against the alfalfa cdc2aMs binds tobacco cdc2, although with a lower affinity (Fig. 1C, lanes 2), but it cannot react with wheat cdc2 (Fig. 1C, lanes 3). The PSTAIRE antibody reacted with CDKs in all three of the plants tested (Fig. 1C).

The cdc2CT Immunoprecipitates a Histone Kinase Activity from Alfalfa Extracts That Co-Fractionates with p13^{suc1}-Bound Histone Kinase Activity

Since the cdc2CT specifically recognized the alfalfa cdc2 homologs, it was used to determine cdc2 activity in alfalfa extracts by immunoprecipitation from cell extracts and histone H1 phosphorylation (Fig. 2A, lane 1). The immunopurification of the histone kinase by the cdc2CT was specific, since no histone kinase activity was measured when the purification was done with an antibody preadsorbed with the peptide that was used for immunization (data not shown). Until now, cdc2 activity was determined in plant extracts by virtue of the binding of CDKs to the yeast cell cycle regulatory protein p13^{suc1}. Recently, it has been shown that two prevalent p13^{suc1}-bound histone H1 kinase activities can be separated by anion-exchange chromatography of alfalfa extracts. One of the kinases does not bind to the DEAE-Sepharose FF matrix and can be found in the flow-through fraction, whereas the other histone H1 kinase activity was recovered from the column by 0.3 M NaCl elution (Bako et al., 1994). The p13^{suc1} protein, however, was shown to bind to several members of the CDK family. Therefore, the identity of these kinases is not certain. To clarify this question, we compared the histone H1 kinase activities isolated by p13^{suc1}-binding or by immunoprecipitation with the cdc2CT from the cell extracts fractionated by anion-exchange chromatography (Fig. 2A, lanes fth and 0.3). As with the p13^{suc1} affinity matrix, histone kinase activity was immunoprecipitated from both of these fractions. This indicates that the alfalfa cdc2 is a part of at least two different complexes.

The alfalfa cell extract was also fractionated by size-exclusion chromatography on Sephacryl S200, and CDK activity was determined after p13^{suc1}-binding or immunoprecipitation with the cdc2CT. The peak histone H1 kinase activity was found in the same fraction, eluted at 200 kD by both methods. The proteins in the DEAE-Sepharose FF and Sephacryl S200 chromatography fractions were separated by SDS-PAGE, and the cdc2 was detected by western blotting (Fig. 2B). The cdc2CT recognized two proteins with apparent molecular masses of 31 and 32 kD and reacted weakly with a 34-kD protein (p31^{cdc2}, p32^{cdc2}, and p34^{cdc2}, respectively). The PSTAIRE antibody bound to each of these three proteins with equal affinity (Fig. 2B). p34^{cdc2} was found exclusively in the flow-through fraction of the DEAE-Sepharose FF column, and the majority of the p32^{cdc2} also fractionated there. Slightly more p31^{cdc2} was separated in the 0.3 M salt eluate. During the size-exclusion chromatography, the majority of p31^{cdc2} detected by the cdc2CT separated as part of a 200-kD complex and co-eluted with the active cdc2 fractions (Fig. 2B). The p34^{cdc2} form separated between 66 and 29 kD, which might be indicative of a cdc2 monomer. These fractions had low or no histone kinase activities. The majority of the p32^{cdc2} detected by the cdc2CT also eluted in later fractions with low histone kinase activity. However, a considerable amount of the p32^{cdc2} form could also be found in fractions with high histone kinase activities. We conclude that the p31^{cdc2} form is part of a complex that is the prevalent histone kinase in the extract but that p32^{cdc2} might also be an active histone kinase. Currently, we cannot tell if these

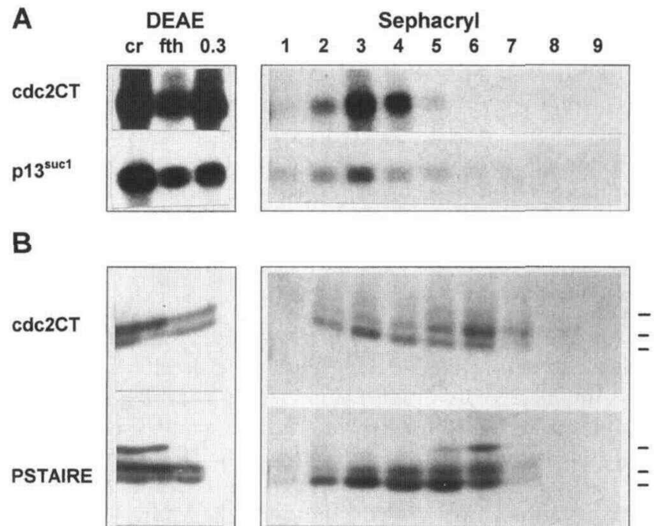

Figure 2. Separation of alfalfa CDKs by anion-exchange and size-exclusion chromatography. A, Histone kinase activity purified by immunoprecipitation with the cdc2CT or by binding to the p13^{suc1} protein from alfalfa crude extracts (cr), from the flow-through fraction (fth), or from the 0.3 M NaCl eluate (0.3) after DEAE-Sepharose chromatography of the crude extract. The crude cell extract was also separated on a Sephacryl S300 column. The fractions analyzed for histone kinase activity are shown (1–9). The Sephacryl S300 column was calibrated with standard proteins: fraction 2, 443 kD; fraction 3, 200 kD; fraction 4, 150 kD; fraction 5, 66 kD; fraction 7, 29 kD; and fraction 9, 12.4 kD. The phosphorylation of histone H1 is shown after SDS-PAGE and autoradiography. B, The same fractions shown in A were immunoblotted with the cdc2CT and the PSTAIRE antibodies. The mobilities of the three separated 31-, 32-, and 34-kD forms of alfalfa cdc2 are indicated on the right.

cdc2 forms with different apparent mobility on SDS-PAGE are the products of different genes (since the alfalfa cdc2 antibody recognizes the cdc2aMs and cdc2bMs gene products equally well) or whether they represent different phosphorylation forms of the alfalfa cdc2 protein. Treatment of the extracts with potato acid phosphatase failed to change the mobility of these proteins (data not shown).

The Regulation of cdc2 Activity in Synchronously Dividing Cell Suspension Culture

An alfalfa suspension culture was synchronized by the release of cells after blocking with aphidicolin. Incubation of alfalfa cells in aphidicolin (which inhibits the DNA polymerase) for 16 h arrested the cells at the G1/S transition. The removal of the inhibitor allowed a large fraction of the cells to proceed through the S, G2, and M phases synchronously, which was followed by flow cytometry (Fig. 3C) and by counting the number of cells in mitosis (Fig. 3D). Three hours after the release most of the cells were in the S phase, whereas between 6 and 9 h the majority of the cells were in the G2 phase. The sudden rise in the mitotic index at 12 h indicated that the cells were in the M phase (Fig. 3D). At 24 h, a large fraction of the cells had returned to the G1 phase. Samples were collected at the indicated intervals after the release from aphidicolin, and CDK complexes were purified either by p13^{suc1}-binding or by immunoprecipitation with the cdc2CT (Fig. 3A). Whereas a prevalent mitotic histone kinase activity was found in association with the p13^{suc1} beads, the activity immunoprecipitated with the cdc2CT was found at similar levels in the S, G2, and M phases, and the activity decreased only when cells entered the G1 phase. Several other proteins were co-purified with the CDK and became phosphorylated in a cell cycle-regulated manner during the in vitro kinase reaction. Although the S-phase CDK isolated by p13^{suc1} affinity chromatography was less active as a histone H1 kinase compared with the immunoprecipitated cdc2, both preparations equally phosphorylated an endogenous 40-kD protein. The cell cycle-regulated phosphorylation, as well as the fact that proteins with the same apparent mobility on SDS-PAGE co-purified with CDK both during immunoprecipitation with the cdc2CT and during p13^{suc1}-binding, indicated that these proteins are part of a CDK complex. Further purification of the CDK complex would be required to reveal the identity of the alfalfa proteins that became phosphorylated in our assays. No protein with the same apparent mobility as histone H1 was phosphorylated by the immunoprecipitated or p13^{suc1}-purified CDK in an in vitro kinase assay (data not shown). Immunoblotting of the protein extracts of the synchronized cells with the cdc2CT showed no change in cdc2 protein levels in different phases (Fig. 3B).

cdc2 Is Activated at the G1/S Transition When Phosphate-Starved Cells Reenter the Cell Cycle

Aphidicolin blocks DNA replication, and the progression of the cell cycle is blocked by a negative feedback control, which prevents the cells from entering mitosis. Therefore,

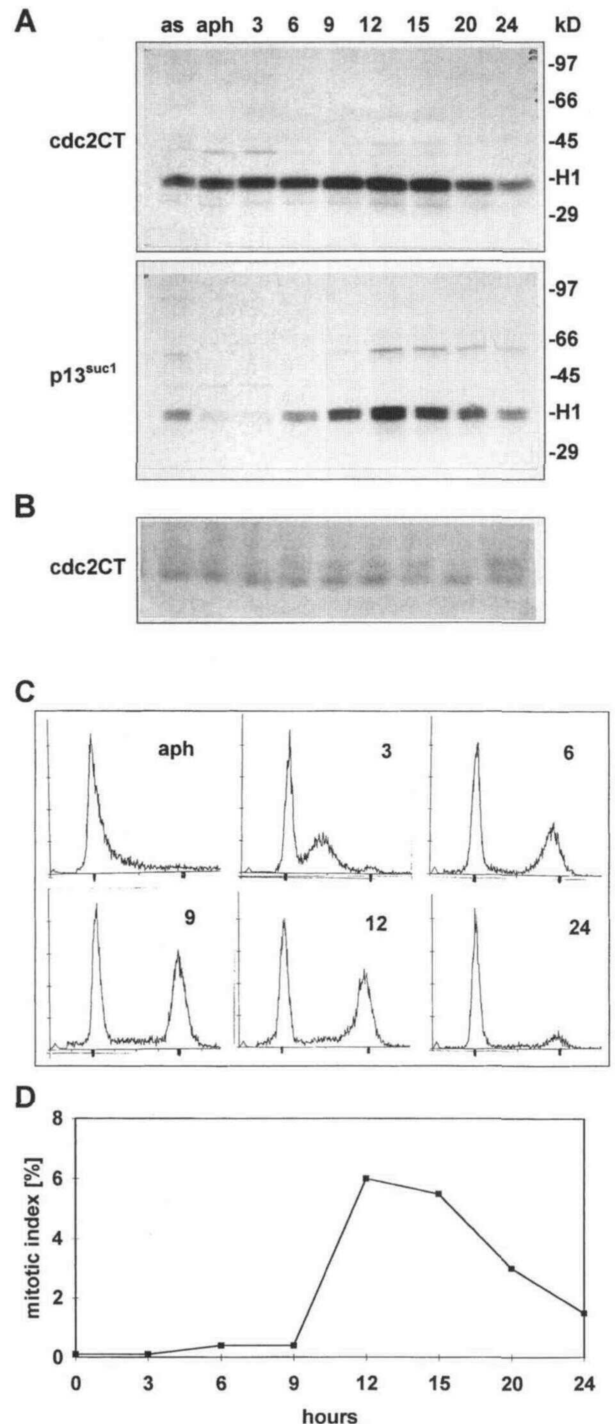

Figure 3. Cell cycle-regulated activity of the immunoprecipitated or p13^{suc1}-purified cdc2 kinase. A, Histone H1 kinase activity of cdc2 immunoprecipitated by the cdc2CT antibody or purified by binding to p13^{suc1} beads from extracts prepared from asynchronously growing cells (as), from cells arrested in the G1/S transition with the drug aphidicolin (aph), or from cells after the release from the block at 3 to 24 h. After in vitro kinase reaction, the phosphorylated proteins were detected by autoradiography. B, Immunoblotting of the same samples with cdc2CT. C, Flow cytometry measurement of the cell cycle phases at the indicated times. The lines mark the positions of cells with G1 and G2 DNA content. D, Mitotic index during the synchronized cell division.

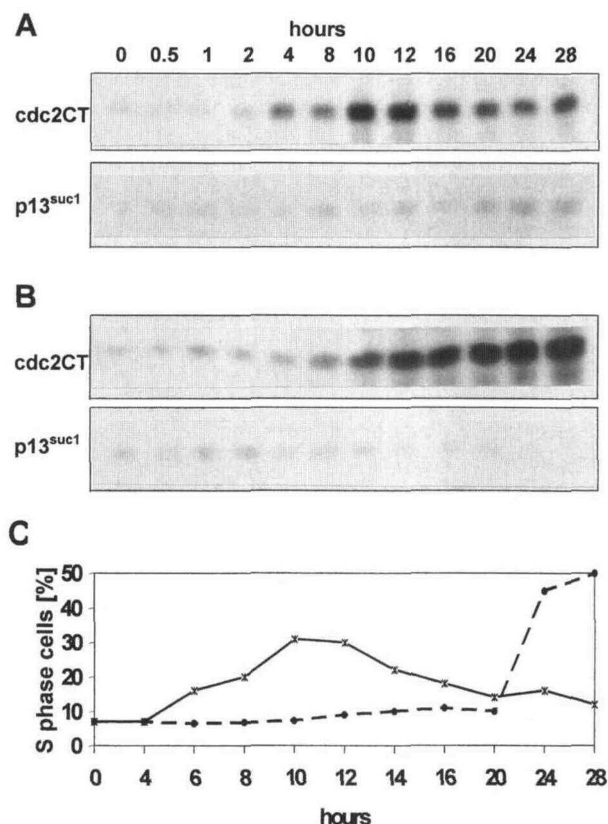

Figure 4. The cdc2 kinase is activated at the G1/S transition when phosphate-starved cells reenter the cell cycle. A, cdc2 kinase was purified by immunoprecipitation with cdc2CT or by binding to the p13^{suc1} beads from samples prepared from cells arrested in the G1 phase by phosphate starvation (0) or after the readdition of phosphate at the indicated time intervals. B, Histone kinase activities purified as before from cells arrested at the G1/S transition with 10 μg L^{-1} aphidicolin after readdition of phosphate. After the protein kinase assay, the samples were subjected to SDS-PAGE and subsequent autoradiography. The phosphorylation of histone H1 is shown. C, Flow cytometry measurement of the percentage of cells in the S phase after the readdition of phosphate to phosphate-starved cells (solid line) or the readdition of phosphate in the presence of aphidicolin (broken line).

synchronization with aphidicolin does not allow us to study how the cells enter the S phase in a natural situation. It was shown, however, that phosphate is one of the first components that becomes limiting for cell growth, resulting in cell cycle arrest in the G1 phase (Amino et al., 1983). This block is reversible, and upon readdition of phosphate, cells pass through the G1 phase and enter the S phase within 10 h (Fig. 4C). Addition of aphidicolin together with the readdition of phosphate caused cells to accumulate at the G1/S boundary. Samples were taken at the indicated times, and histone H1 kinase activity was measured after immunoprecipitation with the cdc2CT or p13^{suc1}-binding (Fig. 4, A and B). Four hours after the readdition of phosphate to the medium an increase in the histone kinase activity of the immunopurified cdc2Ms was observed; the activity continued to rise until 12 h, when a large fraction of cells were in the S phase (Fig. 4A). Contrary to this, the highest histone kinase activity was purified from cells by p13^{suc1}-binding between 20 and 28 h after the readdition of phosphate (Fig. 4A). At this time the number of cells in mitosis increased in the culture (data not shown). To determine if the increase in cdc2 kinase activity occurred before or after cells entered the S phase, the cell cycle was blocked by aphidicolin at the G1/S transition. At the same time as in the previous experiment the activity of the immunopurified cdc2 kinase began to rise, but in this case it continued to increase after 12 h as cells accumulated at the G1/S boundary in the presence of aphidicolin (Fig. 4B). This was not the case, however, when CDK was isolated by p13^{suc1}-binding, indicating that p13^{suc1}-bound CDK remained inactive (Fig. 4B), as was found when cells were synchronized with aphidicolin (Fig. 3). We conclude that active histone kinase could be immunopurified from cells at the G1/S transition, but that the p13^{suc1}-bound histone kinase is inactive at this stage.

The Detection of cdc2 by Indirect Immunofluorescence Microscopy Changes in a Cell Cycle Phase-Specific Manner

Another important aspect of the cdc2 function is its localization within the cell. This was visualized by indirect immunofluorescence microscopy using the cdc2CT in synchronized alfalfa cells (Fig. 5). cdc2Ms was detected exclu-

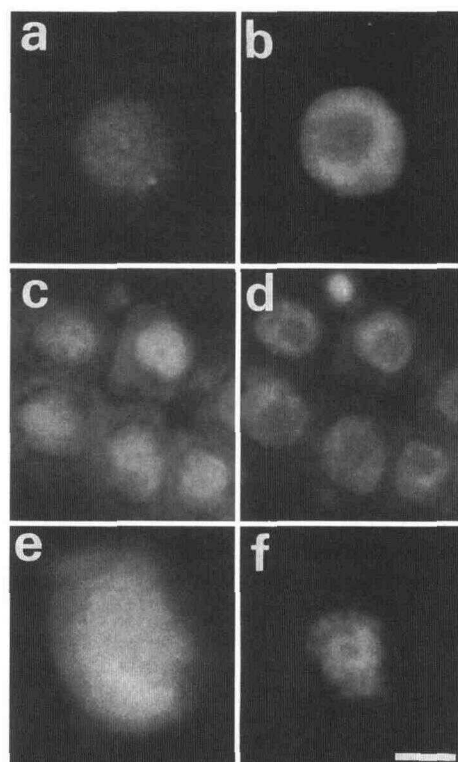

Figure 5. Localization of cdc2 within cells in the S, G2, and M phases by indirect immunofluorescence with cdc2CT. a and b, Aphidicolin-arrested cell; c and d, cells 8 h after the release from aphidicolin arrest in the G2 phase; e and f, cell in mitosis. a, c, and e, Indirect immunofluorescence of cells with cdc2CT; b, d, and f, DAPI staining of nuclei in the same cells. Bar = 10 μm.

sively in the nucleus when cells were arrested in the S phase with aphidicolin (Fig. 5, a and b). In G2 cells cytoplasmic cdc2 staining became apparent in addition to the bright nuclear signal (Fig. 5, c and d). When cells entered mitosis and the nuclear envelope broke down, a bright signal was found throughout the cytoplasm (Fig. 5, e and f). Although the total amount of cdc2 detected by western blotting did not increase when cells entered mitosis (Fig. 3b), the elevated immunofluorescence in the M phase indicates that the epitope for the cdc2CT became more accessible at this stage. The immunofluorescence signal correlates with cdc2 kinase activity, which was also highest in mitosis (Fig. 3a). Localization to cytoskeletal structures, such as to the preprophase band and the phragmoplast, was also seen in a small portion of these cells (data not shown).

Direct Measurement of the Amount of cdc2 Protein and Histone H1 Kinase Activity in Nuclear and Cytoplasmic Fractions

To confirm the changes of the intracellular localization of cdc2 during the cell cycle, cells in the S phase were fractionated to the cytoplasm and the nucleus, and the amount of cdc2 in these fractions was determined by immunoblotting with the cdc2CT (Fig. 6A). The purity of the cytoplasmic fraction was confirmed by the presence of nucleolus-localized NucMs1 protein only in the nuclear extract. In contrast to the results obtained by immunofluorescence, cdc2 was detected in both the cytoplasm and the nucleus (Fig. 6A, lanes C and N, respectively). One explanation for this observation could be that the epitope for the cdc2CT is masked on the cdc2 protein present in the cytoplasm of the S-phase cells. In whole-cell extracts both the $p31^{cdc2}$ and $p32^{cdc2}$ forms were visible. However, the nuclear fraction is highly enriched for the $p31^{cdc2}$ form, which co-eluted with the active cdc2 kinase complex during size-exclusion chromatography (Fig. 2).

We also investigated how tightly cdc2 is associated with the nuclear matrix. After isolation of nuclei, a sequential elution of the proteins with increasing salt concentrations (0.5 and 1 M NaCl, 7 M urea) was performed. A considerable amount of cdc2 was found by immunoblotting in all fractions, suggesting that cdc2 associates with different nuclear structures (data not shown).

To determine the activity of cdc2 in the cytoplasm and in the nucleus, synchronously dividing cells were fractionated and histone H1 kinase activity of $p13^{suc1}$-bound CDK and immunopurified cdc2 was measured (Fig. 6B). The highest activity was immunoprecipitated from nuclear extracts that were isolated from cells arrested by aphidicolin, from S-phase cells, and from cells in the early G2 phase (Fig. 6B). At later times, the cdc2 kinase activity decreased and almost no activity was attached to chromatin in M-phase cells. The immunoprecipitated cdc2 from the cytoplasm was equally active at all phases of the cell cycle. In contrast to the result with crude cell extracts, active CDK was also found in S-phase nuclear extracts by $p13^{suc1}$-binding. However, in agreement with the data from crude extracts, low histone kinase activity was purified by $p13^{suc1}$-binding from the cytoplasm of these cells (Fig. 6B).

cdc2Ms Kinase Binds to $p13^{suc1}$ but in an Inactive Form in the S Phase

Since the cdc2 in the $p13^{suc1}$-bound fraction could be detected by immunoblotting with the cdc2CT (Fig. 1A), the difference in histone kinase activity that was purified from S-phase cells by immunoprecipitation with the cdc2CT and by $p13^{suc1}$-binding was surprising. To determine if $p13^{suc1}$

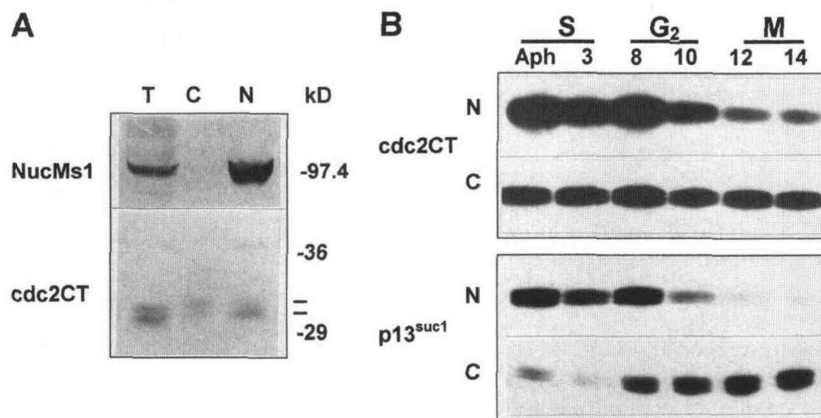

Figure 6. cdc2 amount and histone kinase activity in cytoplasmic and nuclear fractions. A, Samples were prepared from S-phase cells, and 50-μg proteins of whole-cell extract (T), cytoplasmic fraction (C), and nuclear fraction (N) were immunoblotted with cdc2CT and with an antibody against the alfalfa nucleolin NucMs1 (Bögre et al., 1996). B, cdc2 kinase activity measured in cytoplasmic and nuclear extracts prepared from synchronized cells. Cells were synchronized at the G1/S transition by aphidicolin arrest (Aph) and then released; samples were analyzed at the indicated hours after release. The cell cycle stages were determined by flow cytometry and by counting the mitotic index. The synchrony of the cell cycle was comparable to that shown in Figure 3. The cell cycle stages S, G2 (G_2), and M are indicated. Cells were fractionated into nuclei (N) and cytoplasm (C), and the histone kinase activity was measured after immunoprecipitation of cdc2 by cdc2CT or by $p13^{suc1}$-binding. The phosphorylation of histone H1 was detected by autoradiography.

binds to the same complex as the cdc2CT, extracts prepared from S- and M-phase cells were first depleted of CDK by incubating them with an excess of p13^{suc1} beads, and then the remaining cdc2 was immunoprecipitated by the cdc2CT and its activity was determined. After depletion of p13^{suc1}-bound CDK, almost no cdc2 kinase activity could be found by immunoprecipitation with the alfalfa cdc2CT in either S- or M-phase extracts (Fig. 7A). These results are consistent with the idea that the same CDK is recognized by the cdc2CT and by p13^{suc1} but the S-phase complex is inactive when it is isolated by binding to p13^{suc1}. However, p13^{suc1} protein does not inactivate the cdc2 directly, because the addition of p13^{suc1} to immunopurified cdc2 kinase did not affect its activity (data not shown).

One possibility to explain the difference in S-phase kinase activities after immunopurification by the cdc2CT or isolation by p13^{suc1}-binding is the presence of an inhibitory molecule in the latter complex. This was also suggested by the observation that the relatively inactive CDK isolated by p13^{suc1}-binding from S-phase cells could be reactivated by the addition of the cdc2CT to the purified CDK prior to the enzyme assay, whereas the antibody showed no effect on histone kinase activity isolated from M-phase cells (Fig. 7B). An explanation for this result could be that the binding of the antibody to the C-terminal part of cdc2 released an inhibitor molecule.

The Inactivation of Alfalfa cdc2 by Heat-Sensitive Factors Present in Cell Extracts and by the *S. cerevisiae* p40^{sic1} and Mouse p27^{kip1} CDK Inhibitors

CDK inhibitors have been found in animal and yeast cells but have not been identified in plants. We searched for CDK inhibitors in alfalfa by adding cell extracts from S- and M-phase cells to active CDK isolated from M-phase cells by p13^{suc1}-binding or immunoprecipitation with the cdc2CT (Fig. 8A). These extracts abolished the histone kinase activity. However, in this assay no difference in inhibition was found when S- and M-phase extracts were compared (Fig. 8A, lanes 2 and 3, respectively). The inhibitory activity was heat-sensitive (Fig. 8, lanes 4 and 5), indicating that a protein factor is a likely candidate. As in animal cells, several classes of CDK inhibitors might be present in plant cells, which could explain why it is difficult to detect a difference in CDK inhibitors when crude extracts isolated from S- or M-phase cells are used.

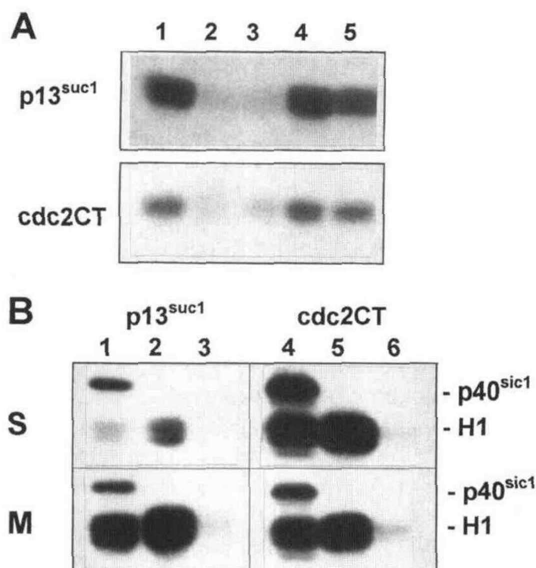

Figure 8. Inhibition of the cdc2 kinase activity by the addition of crude cell extracts and by purified yeast and mouse CDK inhibitors. A, Inhibition of cdc2 kinase activity purified by immunoprecipitation with cdc2CT or by p13^{suc1}-binding. Histone kinase activity was purified from cells in the M phase (lane 1). Histone kinase activity was inhibited by adding 10 μg of protein extract from S-phase cells (lane 2), M-phase cells (lane 3), heat-inactivated (95°C, 5 min) S-phase extract (lane 4), or M-phase extract (lane 5). Following the in vitro kinase reaction, samples were subjected to SDS-PAGE, and phosphorylation of histone H1 was detected by autoradiography. B, Inhibition of cdc2 kinase by the yeast p40^{sic1} and the mouse p27^{kip1} CDK inhibitors. CDK was purified by p13^{suc1}-binding (lanes 1–3) and by immunoprecipitation with cdc2CT (lanes 4–6) from S- and M-phase cells. The histone kinase activity was determined directly (lanes 2 and 5) or after preincubation with the animal CDK inhibitor p27^{kip1} (lanes 3 and 6) or with the *S. cerevisiae* inhibitor p40^{sic1} (lanes 1 and 4). The phosphorylated proteins were separated by SDS-PAGE and detected by autoradiography. The positions of histone H1 and p40^{sic1} are indicated.

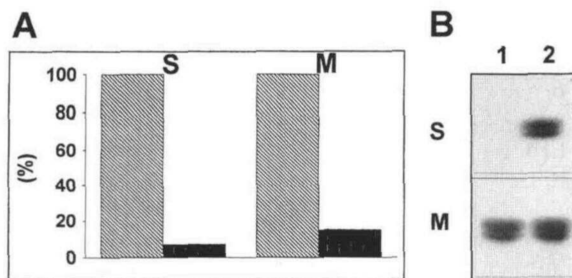

Figure 7. p13^{suc1} binds the same cdc2 that is recognized by cdc2CT, and the p13^{suc1}-bound cdc2 kinase is activated by the addition of cdc2CT. A, Depletion of cdc2 kinase activity from extracts by p13^{suc1} beads. Extracts prepared from S- and M-phase cells were incubated with p13^{suc1} beads, and the cdc2 kinase activity was determined after immunoprecipitation with cdc2CT before (striped bars) or after depletion (black bars). The values are given as a percentage of activity before depletion. B, The p13^{suc1} purified kinase can be activated by the addition of cdc2CT. cdc2 kinase was purified from S- and M-phase cells by p13^{suc1}-binding, and the histone kinase activity was determined before (lane 1) or after (lane 2) incubation of the purified p13^{suc1}-bound kinase with cdc2CT. The phosphorylation of histone H1 is shown after autoradiography.

To examine the effect of known CDK inhibitors, two different classes of these proteins were chosen: the yeast p40^{sic1} is known to inhibit CDKs complexed with B-type cyclins (Schwob et al., 1994), whereas the animal p27^{kip1} inhibits G1 cyclin-CDK complexes as well (Polyák et al., 1994). The effect of these two proteins on the activity of the plant cdc2 kinase was studied. The p27^{kip1} effectively abolished the kinase activity irrespective of whether it was added to p13^{suc1}-bound or immunoprecipitated plant cdc2 kinase isolated from S- or M-phase extracts (Fig. 8B, lanes 3 and 6). In contrast, the yeast p40^{sic1} was most effective on the p13^{suc1}-bound CDK isolated from S-phase extract (Fig.

8B, lanes 1 and 4) and was a good substrate for the plant cdc2 kinase.

DISCUSSION

In several plant species, a pair of *cdc2* homologs has been found with 80 to 90% identity. From sequence comparison, a certain degree of specialization is expected, since the alfalfa *cdc2aMs* is more similar to one of the soybean *cdc2* genes than to the other alfalfa homolog, *cdc2bMs* (Hirt et al., 1993; Miao et al., 1993). The pairwise comparison of the dicotyledonous cdc2 sequences, however, does not hold for the monocotyledonous homologs. If there is a separation in function among the closely related plant *cdc2* genes, this must have happened fairly recently in the evolution of the plants. In Arabidopsis only one *cdc2* homolog has been found (Ferreira et al., 1991; Imajuku et al., 1992). Ectopic overexpression of a dominant negative mutant form of this gene blocked the cell cycle in Arabidopsis and tobacco plants, but had no effect on the distribution of cell cycle phases (Hemerly et al., 1995). These data indicated that the Arabidopsis *cdc2* may be involved in both the G1/S and G2/M transitions or that it has an overlapping function with another closely related *cdc2* gene.

The antibody against the C-terminal 16 amino acids of the alfalfa cdc2aMs protein reacted with both alfalfa cdc2 proteins, and therefore cannot be used to study the function of these proteins separately. To gain further information about how many CDKs are to be found in alfalfa, we compared the proteins detected by the specific alfalfa cdc2 antibody with the number of proteins detected by an antibody raised against the evolutionarily conserved PSTAIRE motif. The PSTAIRE antibody is known to react with several members of the CDK family (Meyerson et al., 1992). We found that the two antibodies recognize the same set of bands even in purified CDK preparations, suggesting that the isolated alfalfa cdc2aMs and cdc2bMs are the only PSTAIRE-containing cdc2 proteins in alfalfa.

Active cdc2 was immunopurified from cells at various cell cycle stages. Based on this activation pattern, we conclude that in plants one cdc2 (or closely related cdc2 proteins) must form complexes with S- and M-phase-specific cyclins and take part in both the G1/S and the G2/M transitions. In this respect the regulation of the cell cycle in plants is similar to that in yeast, in which a single cdc2 is complexed with various cyclin subunits at different points in the cell cycle. In contrast, in animal cells several CDK proteins with distinct functions have evolved (Meyerson et al., 1992; Heuvel and Harlow, 1993). The sequence similarity between the two closest members, CDK1 and CDK2, is only around 60%, and the C-terminal sequence was found to be divergent enough to generate specific antibodies. The purification of CDKs with these antibodies revealed that the regulation and substrate specificity of CDK1 and CDK2 are different. CDK2 is activated earlier in the cell cycle (Pagano et al., 1992), and CDK1 and CDK2 form complexes with different although partially overlapping sets of cyclins (Rosenblatt et al., 1992). A loss-of-function mutation of the *Drosophila* CDK1 homolog is blocked at the G2/M transition without any adverse effects on the S phase (Stern et al., 1993). Dominant negative mutations of the various members of the CDK family also showed specific arrest in G1/S or G2/M transitions (Heuvel and Harlow, 1993).

Although cdc2 is present in the $p13^{suc1}$-bound complex from S-phase extracts, it has a low histone kinase activity. However, in some cases an active kinase activity has been isolated from S-phase cells in plants by $p13^{suc1}$-binding (Magyar et al., 1993). We observed that prolonged storage of the samples results in the activation of the S-phase complex (L. Bögre, unpublished results). We also found that the addition of the alfalfa C-terminal cdc2 antibody to $p13^{suc1}$-purified S-phase kinase resulted in elevated kinase activity. These data suggested to us that an inhibitory protein might be present in the S-phase extract. This inhibitor might bind to the C terminus of the cdc2 protein because binding of the antibody to this part activated the kinase, presumably by competing for the same site or causing a conformational change that displaces the inhibitor. It is interesting to note that a dominant mutation in the *S. pombe cdc2* maps to this conserved region, and it was postulated that this *cdc2* allele abrogated the binding of an unknown inhibitor (Labib et al., 1995). Recently, an inhibitor of a plant cdc2 has also been suggested to be present during endosperm endoreduplication in corn (Grafi and Larkins, 1995). The $p13^{suc1}$-bound kinase activity could be found only during normal cell division in early development of the endosperm and was reduced later in the endoreduplicating tissue. Moreover, when extracts made from endoreduplicating tissue were mixed with extracts taken at an earlier time, the $p13^{suc1}$-bound kinase activity was inhibited, indicating the presence of an inhibitor in the endoreduplicating cells. The identity of this inhibitor is not known (Grafi and Larkins, 1995).

In animal and yeast cells, various classes of CDK inhibitors have been characterized (Sherr and Roberts, 1995). Two of these inhibitors, the *S. cerevisiae* $p40^{sic1}$ (Schwob et al., 1994) and the mouse $p27^{kip1}$ (Polyák et al., 1994), were tested and both were found to reduce the activity of the plant cdc2 kinase. This further demonstrates the conservation of regulatory mechanisms in the eukaryotic cell cycle and predicts that this class of regulatory molecules will also be found in plants. The addition of the $p40^{sic1}$ protein to plant extracts in several instances reconstituted the activity of the hypothesized endogenous plant inhibitor. A reduction in the activity of the $p13^{suc1}$-bound kinase was the most pronounced effect, whereas the cdc2 kinase isolated through the C-terminal cdc2 antibody was almost unaffected. $p40^{sic1}$ is also a preferable substrate for cdc2 kinase in the S phase. An endogenous plant protein with a similar molecular mass co-purified in our experiments with (and was phosphorylated by) CDK when it was isolated by $p13^{suc1}$-binding or immunoprecipitation from S-phase extracts. An S-phase-specific phosphorylation of a 40-kD protein in in vitro kinase reaction was the original observation in yeast, and the purification of this protein led to the discovery of $p40^{sic1}$ (Mendenhall, 1993). These observations predict the existence of a $p40^{sic1}$ homolog in plants, but further work will be required to confirm this.

To understand the function of cdc2, it is not only important to know how the activity of the enzyme is regulated but also where the cdc2 kinase is localized within the cell during the cell cycle. In agreement with the studies on corn (Colosanti et al., 1993), we found a predominant nuclear staining of cdc2 in interphase cells. By using synchronously dividing cells we also observed an increased immunofluorescence signal as cells proceeded to the G2 and M phases. On the other hand, in fractionated cell extracts, cdc2 protein could be detected by western blotting in both the cytoplasm and the nucleus. The mobility of the cdc2 protein in SDS-PAGE is clearly different in the two fractions, indicating that different forms of cdc2 are localized to the cytoplasm and to the nucleus. We suggest that the cdc2 protein in the cytoplasm might be part of a complex that is less accessible to the cdc2CT. In contrast, a readily observable cytoplasmic immunostaining was reported with the PSTAIRE antibody (Mineyuki et al., 1991), which would suggest that the PSTAIRE region is accessible in the cytoplasm. Furthermore, we wanted to relate the localization with the regulation of CDK activity. We found that CDK activity was predominantly nuclear during the S and G2 phases and could be found in the cytoplasm only in the late G2 phase and in mitosis. The binding of an inhibitor to the C-terminal portion of the cytoplasmic CDK during the S phase would also explain why this form was not readily detectable in immunofluorescence. These data suggest a mechanism for storing inactive cdc2 complexes in the cytoplasm during the S phase. Activation of the cdc2 complex would require dissociation of the inhibitor prior to translocation to the nucleus.

It has been shown that the *cdc2* gene is expressed not only in dividing tissues but also in cells that are temporally arrested in the cell cycle during development, such as in the root pericycle (Martinez et al., 1992; Hemerly et al., 1993). Therefore, cdc2 expression was suggested as a molecular marker of competence for cell division in plant cells. One way to keep the cdc2 kinase inactive in these nondividing cells is the restricted expression of cyclins. However, it has been shown recently that some of the cyclins such as *cycMs3* are expressed in nondividing cells (Meskiene et al., 1995), so another mechanism besides cyclin expression might be important to regulate cdc2 activity during plant development. Our data suggest that the inactivation of cdc2 kinase by inhibitory proteins might represent such a mechanism.

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