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Cell division in higher plants: A *cdc2* gene, its 34-kDa product, and histone H1 kinase activity in pea

(mitosis/cell cycle/protein phosphorylation/*Pisum sativum*/polymerase chain reaction)

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ABSTRACT **The mitotic cell cycle of yeast and animal cells is regulated by the *cdc2* gene and its product, the p34 protein kinase, and by other components of the MPF or histone H1 kinase complex. We present evidence that *cdc2*, p34, and a histone H1 kinase also exist in higher plants. Protein extracts from 10 plant species surveyed display a 34-kDa component recognized by a monoclonal antibody directed against an evolutionarily conserved epitope of fission yeast p34. Nondenatured protein extracts of mitotic *Pisum sativum* (garden pea) tissues were fractionated by gel filtration, electrophoretically separated under denaturing conditions, and immunoblotted. p34 crossreactive material was apparent in both low and high molecular mass fractions, indicating that pea p34 occurs as both a monomer and as part of a high molecular mass complex. Histone H1 kinase activity was found predominantly in the higher molecular mass fractions, those to which the least phosphorylated form of pea p34 was confined. We also report the cloning of the pea homologue of *cdc2* by polymerase chain reaction. DNA sequence analysis reveals perfect conservation of the hallmark "PSTAIR" sequence motif found in all *cdc2* gene products analyzed to date.**

Cell division in higher plants is largely confined to perpetually embryonic foci called meristems. Beyond the hypothesis of "principal control points" in the cell cycle (1), little is known about mitotic regulation in plants. On the other hand, cell cycle control in yeasts is now understood in considerable detail at the genetic and biochemical levels (2, 3). Among the cell division cycle (*cdc*) genes analyzed in the fission yeast *Schizosaccharomyces pombe*, the *cdc2* gene appears to be a central positive regulator. At restrictive temperature, recessive temperature-sensitive *cdc2* mutants arrest at "start," in late G_1 and at the G_2/M transition (4), cell cycle points corresponding to the previously identified principal control points of the higher plant cell cycle (1).

The product of the *cdc2* gene, p34, is a protein kinase, homologues of which have been identified in budding yeast (5), human cells (6, 7), mouse (8, 9), rat (10, 11), sea urchin (12), starfish (13, 14), clam (15), chicken (16), and *Xenopus* (17, 18). p34 kinase activity in HeLa cells is positively regulated by its G_2/M-phase-specific association with a 50-kDa protein (19). This protein is homologous to B cyclins, proteins that accumulate until, and are dramatically degraded at, the metaphase/anaphase transition of each mitotic cycle in marine invertebrate oocytes (19). Cyclin and p34 have been identified as components of both the maturation- or M-phase-promoting factor (MPF), a complex required for mitotic induction in *Xenopus* (17, 18, 20), and the M-phase-associated histone H1 kinase (H1K) of starfish (13, 14), sea urchin (12), and clam (15). H1K activity is correlated with phosphorylation of the cyclin and dephosphorylation of the p34 components of the p34/cyclin complex in starfish oocytes (21).

The ubiquity of *cdc2* homologues and the correlation between proposed plant cell cycle controls (1) and the fission yeast *cdc2* mutant phenotype suggest that the higher plant cell cycle may be controlled by a p34-centered regulatory system. We have used immunological, biochemical, and molecular genetic approaches in an initial characterization of this cell cycle regulatory system in a higher plant, the garden pea (*Pisum sativum*).†

MATERIALS AND METHODS

Plant Materials. Plant seed was obtained from the following sources: *Arabidopsis thaliana*, G. Redei (University of Missouri); black bean (*Phaseolus vulgaris*), local market; soybean (*Glycine max* cv. Williams), J. Harper (University of Illinois); *Sorghum bicolor*, M. Schuler (University of Illinois); *Medicago truncatula* cv. Jemalong, R. Dickstein (Harvard University); zucchini (*Cucurbita pepo* cv. Gray), Dessert Seed (El Centro, CA); alfalfa (*Medicago sativa* cv. Iroquois), Allied Seed (Napa, ID); garden pea (*Pisum sativum* cv. Alaska), Atlee Burpee (Warminster, PA). Carrot (*Daucus carota* cv. Danver's Half Long) suspension culture TC-1 was obtained from J. Widholm (University of Illinois).

Protein Extraction. For the species survey, seeds were surface-sterilized in commercial bleach, rinsed in sterile water, imbibed overnight in aerated water, sown in sterile coarse vermiculite, and grown in complete darkness at 23°C. Four-day-old shoots were harvested, weighed, and ground in a prechilled mortar with 5 volumes of extraction buffer (70 mM Tris, pH 8/3 mM EDTA/250 mM sucrose/15 mM 2-mercaptoethanol/5 mM dithiothreitol/2 mM phenylmethylsulfonyl fluoride with leupeptin at 50 μg/ml) per gram fresh weight of tissue. Insoluble material was removed by filtration through Miracloth followed by microcentrifugation. Proteins were precipitated by addition of an equivalent volume of acetone at −20°C, collected by microcentrifugation, resuspended in 1× SDS/PAGE sample buffer (62.5 mM Tris, pH 6.8/10% glycerol/5% 2-mercaptoethanol/2.3% SDS), aliquoted, and stored at −20°C. Protein concentration was assayed by the Bradford method (22). Carrot suspension cultures were maintained in Murashige and Skoog medium supplemented with 2,4-dichlorophenoxyacetic acid (0.4 μg/ml), harvested by centrifugation, ground in a glass homogenizer, and extracted as above.

In all other experiments, 1-cm epicotyledonary hooks were harvested from 10-day-old etiolated pea seedlings and homogenized in a Polytron (Brinkmann) at medium speed in ≈10 volumes of modified extraction buffer (extraction buffer

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Abbreviations: PCR, polymerase chain reaction; H1K, histone H1 kinase.

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†The sequence reported in this paper has been deposited in the GenBank data base (accession no. X53035).

without sucrose or proteinase inhibitors) per gram fresh weight of tissue. Extracts were filtered through one layer of Miracloth and cleared by ultracentrifugation at 150,000 × *g* for 30 min at 4°C. The supernatant was brought to 35% saturation with $(NH_4)_2SO_4$ dissolved in modified extraction buffer and was stirred at 4°C overnight. Precipitated proteins were collected by centrifugation at 16,000 × *g* for 30 min at 4°C. Pellets were resuspended in and dialyzed against dilution buffer (70 mM Tris, pH 7.5/5 mM EGTA/1 mM dithiothreitol/150 mM NaCl/60 mM β-glycerophosphate with leupeptin at 3μg/ml). After dialysis, insoluble particulates were removed by centrifugation at 16,000 × *g* for 30 min at 4°C.

Gel Filtration of Native Proteins. Approximately 5 mg of $(NH_4)_2SO_4$-precipitated and dialyzed protein was loaded onto a low-pressure Sephacryl S-200 HR (Pharmacia) gel filtration column and eluted with dilution buffer at a flow rate of 8 ml/hr at 4°C. Fractions of 530 μl were collected and frozen at −80°C. The column was calibrated by loading equivalent A_{280} units of alcohol dehydrogenase (150 kDa), ovalbumin (45 kDa), and cytochrome *c* (12.4 kDa) (Sigma) dissolved in dilution buffer. A portion of each fraction was precipitated with an equal volume of acetone, resuspended in 1× SDS/PAGE sample buffer, electrophoresed, and transferred to nitrocellulose for immunoblotting. The remainder of each fraction was used for H1K assays.

Immunoblots. Samples were electrophoresed in SDS/15% polyacrylamide gels. Separated proteins were electroblotted to nitrocellulose overnight at 50 V (constant voltage), probed with the anti-cdc2 MAb-J4 mouse monoclonal antibody (7), and detected by secondary goat anti-mouse antibodies conjugated to alkaline phosphatase (Promega). Positive control fission yeast p34 protein was partially purified from isopropyl β-D-thiogalactoside-induced *Escherichia coli* BL21(DE3)-LysS (23), carrying the plasmid pcdc2-T7, a derivative of pRK172 that contains a cDNA copy of *Sch. pombe cdc2* (7). Crude bacterial lysate samples were prepared by resuspending bacterial suspensions in 0.1 volume of 1× SDS sample buffer.

Histone H1K Assays. The assays were performed as described (14), with modifications. Reaction mixtures consisted of 20 μl of fractionated protein, 20 μl of TMDE buffer (70 mM Tris, pH 7.5/0.2% Triton X-100/10 mM $MgCl_2$/1 mM dithiothreitol/5 mM EGTA) containing 40 μg of histone (Sigma type III-S), 1 μM of cAMP-dependent protein kinase inhibitor peptide (Sigma) (24), 20 μM ATP, and 5 μCi of [γ-^{32}P]ATP (3000 Ci/mmol; 1 Ci = 37 GBq) in a total volume of 40 μl. Reactions were begun with the addition of ATP and stopped after 15 min at 30°C with the addition of 20 μl of 3× SDS sample buffer. Products were electrophoresed in SDS/15% polyacrylamide gels and stained with Coomassie blue. Gels were destained, dried, and exposed to Kodak X-Omat film at −80°C.

Polymerase Chain Reaction (PCR). Oligonucleotides were prepared by β-cyanoethylphosphoramidite chemistry on an Applied Biosystems model 380A DNA synthesizer at the University of Illinois Biotechnology Center's Genetic Engineering Facility. Oligonucleotide primers for PCR amplification of a pea *cdc2* gene segment were designed to complement DNA sequences encoding the following oligopeptides of *Sch. pombe* p34 protein (25): Gly-Glu-Gly-Thr-Tyr-Gly (forward primer) and Trp-Tyr-Arg-Ala-Pro-Glu (reverse primer). The former primer incorporated an *Xho* I site and the latter a *Bam*HI site for cloning of PCR products. Total cellular RNA was prepared from etiolated pea epicotyl hooks (26), and cDNA synthesis and PCR amplification were carried out in a single tube (27). Amplification products were phenol-extracted, ethanol-precipitated, digested with *Xho* I and *Bam*HI, and ligated into a plasmid designed to express the peptide product encoded by the amplified segment. Recombinants were screened immunologically. Details of the oligonucleotide design, amplification, cloning, and screening procedures will be reported elsewhere.

DNA Sequence Analysis. All DNA sequencing was performed by the dideoxy chain-termination method using double-stranded plasmid templates prepared by CsCl gradient centrifugation and multiple ethanol precipitations with ammonium acetate. Bacteriophage T7 promoter and PCR primers were used as sequencing primers. A Sequenase Version 2.0 kit (United States Biochemical) and its included protocols were used for all DNA sequence determinations, with minor modifications; namely, primer and template were coprecipitated in sodium acetate following template denaturation. DNA sequences were analyzed by DNAStar software.

RESULTS

p34 Homologues in Plants. Total crude protein extracts from etiolated seedlings of an assortment of higher plant species were electrophoretically separated, blotted to nitrocellulose, and probed with antiserum directed against p34 from the fission yeast *Sch. pombe*. The monoclonal antibody employed, MAb-J4, was selected for its broad crossreactivity against p34 proteins from yeast and HeLa cells (7). A unique or discretely polymorphic signal at 34 kDa was observed for every plant species surveyed (Fig. 1). The positive signal at 34 kDa obtained from aseptically grown carrot cell cultures confirmed that the ubiquitous 34-kDa antigen did not originate in a microbial contaminant common to all of our growth-chamber-grown plant materials (Fig. 2*a*). Similar protein blots probed with normal mouse serum displayed no signals corresponding to those shown (H.S.F., unpublished data).

Anti-p34 crossreactive material identified in some plant extracts comprises a discrete polymorphism around 34 kDa. This is especially apparent in extracts of epicotyledonary hooks of etiolated garden pea (Fig. 2*b*). p34 is a phosphoprotein in all systems studied to date, and such polymorphism in protein extracts from mitotically nonsynchronous cell populations has been attributed to distinct phosphorylation states of the protein (9, 28).

A p34-Containing Complex in Pea. In animal and yeast systems, p34 occurs both as a monomer and as a component of a high molecular mass complex known as MPF or H1K. We asked whether the crossreactive material from pea could be identified as part of a complex of >34 kDa by examining

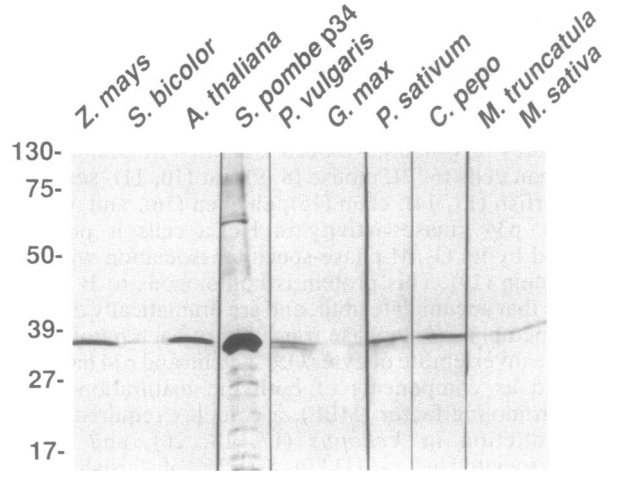

FIG. 1. Protein blot of whole-cell extracts of various angiosperm plant species probed with anti-p34 monoclonal antibody MAb-J4. A crude lysate of *E. coli* carrying a *Sch. pombe* cdc2 cDNA copy is included as a positive control. Molecular mass markers (kDa) are shown at left. See *Materials and Methods* for full names of plant species.

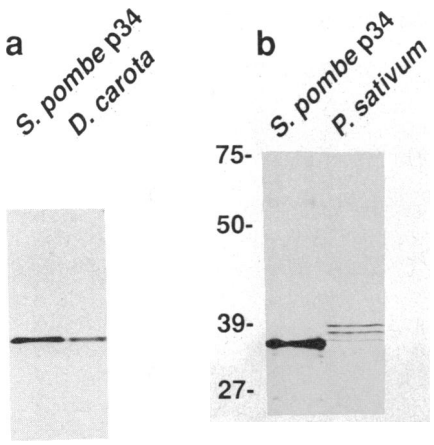

FIG. 2. (*a*) Protein blot of *Sch. pombe* p34 protein and a whole-cell extract of logarithmic-phase cultured carrot cells, probed with monoclonal antibody MAb-J4. (*b*) Polymorphism in immunoblot signal obtained when a whole-cell extract from *P. sativum* was electrophoretically separated under denaturing conditions, transferred to nitrocellulose, and probed with MAb-J4. *Sch. pombe* p34 control antigen was produced in *E. coli* and therefore displays no phosphorylation-mediated polymorphism. Molecular mass markers (kDa) are shown at left in *b*.

fractions from size-separated nondenatured pea protein extracts. An $(NH_4)_2SO_4$ cut of proteins extracted from etiolated pea epicotyledonary hooks was fractionated by Sephacryl S-200 gel filtration chromatography and fractions were concentrated, electrophoretically separated under denaturing conditions, blotted to nitrocellulose, and probed with anti-p34 monoclonal antibody MAb-J4 (Fig. 3). A signal at 34 kDa is apparent in fractions that span the range of molecular size standards with which the column was calibrated, 12.4–150 kDa. This result suggests that p34 is present as a monomer and as a component of one or more higher molecular mass complexes in mitotic pea tissue. The presence of crossreactive material below 34 kDa is probably attributable to "tailing" in the elution of monomeric p34. The presence of crossreactive material across the entire gradient above 34 kDa may reflect both tailing and the occurrence of more than one p34-containing complex in pea. For example, genetic and biochemical data suggest that p34 and p13, the product of the *suc1* gene, are physically associated in *Sch. pombe* (29).

It is also interesting that the distribution of the components of the p34 polymorphism is correlated with specific size ranges in the chromatographic elution profile (Fig. 3). The slowest migrating p34 species is confined to fractions 30–41, while the fastest migrating form is found only in fractions 31–38. The signal of intermediate mobility appears across the entire elution profile.

H1K Activity in Pea. Although the authentic *in vivo* substrate for yeast and animal p34 protein kinase has yet to be identified, casein and histone H1 are effective substrates *in vitro*. Whereas casein is phosphorylated by monomeric or complexed forms of p34, H1K activity is correlated with p34's association with a cyclin-like protein during the G_2 and M phases of the cell cycle (19, 30, 31). We therefore tested the hypothesis that H1K activity, if present in mitotic pea extracts, should be confined to the higher molecular mass fractions from gel filtration. Sephacryl S-200 fractions were assayed for their ability to catalyze the transfer of $[^{32}P]PO_4$ from [γ-^{32}P]ATP to histone H1. As can be seen in Fig. 4, H1K activity was largely confined to the high molecular mass range (fractions 30–36). Two additional observations can be made regarding Fig. 4. First, H1K activity is correlated with the presence of the fastest migrating variant of anti-p34 crossreactive material (compare Figs. 3 and 4), in keeping with findings in other systems that dephosphorylated p34 migrates faster in denaturing polyacrylamide electrophoretic gels than its phosphorylated variants and that p34 dephosphorylation is required for the protein's M-phase functions, including H1K activity (9, 21, 32–35). Second, another phosphorylated protein, of ≈55 kDa, displays differential electrophoretic mobility across the gel filtration elution profile. Cyclin, another component of the H1K and MPF complexes, has been reported at 42–65 kDa in a variety of species and is present in a phosphorylated form as a component of active H1K during metaphase (12, 21, 36). While we have no direct evidence for the existence of cyclin in pea, the ≈55 kDa signal, which shifts to a lower mobility between fractions 35 and 37, is a likely cyclin candidate.

PCR Cloning and DNA Sequence Analysis of the Pea *cdc2* Homologue. The products of yeast and mammalian *cdc2* genes are protein kinases and, as such, contain amino acid sequence motifs common to other protein kinases (37). Among these are the sequences Gly-Xaa-Gly-Xaa-Xaa-Gly and Ala-Pro-Glu (37), which are separated by 158 amino acids in fission yeast p34. In yeast and HeLa p34, these sequences flank the perfectly conserved 16-amino acid domain known as PSTAIR (6). The position of the PSTAIR region between these conserved elements provided an ideal DNA sequence assay for the authenticity of putative *cdc2* clones generated by PCR. High-stringency PCR was used to construct chimeric pea/yeast *cdc2* clones, which were subsequently screened immunologically (unpublished data). In brief, we

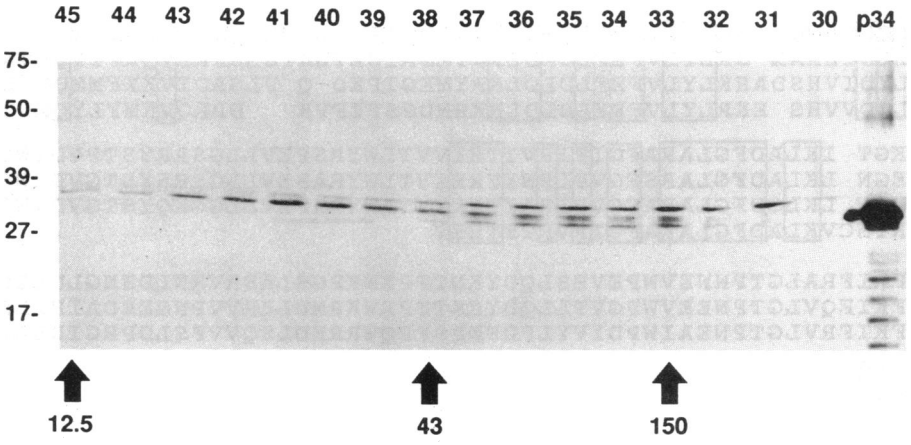

FIG. 3. Blot of Sephacryl S-200 gel filtration chromatographic fractions of pea probed with monoclonal antibody MAb-J4. Fraction numbers, in order of elution from column, are shown across the top, right to left. Column calibration standards (kDa) are shown across the bottom. p34, the product of overexpression in *E. coli* of the *Sch. pombe cdc2* gene, was loaded in far right lane.

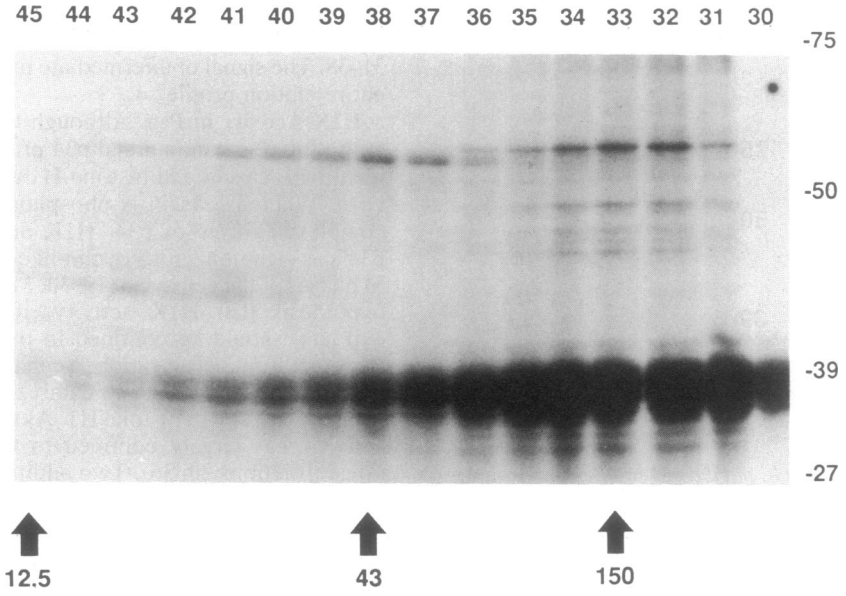

FIG. 4. SDS/PAGE of products from H1K assays of fractions from Sephacryl S-200 chromatographic fractionation of native pea proteins. Fraction numbers are shown across top, molecular mass standards for gel are shown at right, and calibration standards for column are shown across bottom. Histone H1 migrates at ≈35 kDa.

deleted a segment of the fission yeast *cdc2* gene in pcdc2-T7 (7), which was then replaced by PCR products generated with primers based on the above protein kinase consensus motifs and a pea cDNA template. These chimeric genes were then expressed in *E. coli*, and the protein products were separated by SDS/PAGE, blotted, and probed with MAb-J4. Based on DNA sequence analysis, all positive clones identified by this screening procedure proved to be copies of a single pea *cdc2* homologue (Fig. 5). Discrete positive signals in hybridizations of the putative *cdc2* clone with filter-bound mRNA prepared from pea epicotyl hooks confirmed that the template which generated our PCR clones was not a spurious contaminant (H.S.F., unpublished data). Pea *cdc2* carries all 16 amino acids of the PSTAIR domain, as well as another conserved protein kinase motif, Arg-Asp-Leu (37). Overall amino acid sequence identities for the cloned portion with fission yeast, budding yeast, and mammalian cell *cdc2* are 60%, 64%, and 65%, respectively. Only a single base difference was detected in a comparison of the complete nucleotide sequences of the four pea *cdc2* PCR clones analyzed, presumably due to a synthesis error made by *Thermus aquaticus* DNA polymerase (H.S.F., unpublished data).

DISCUSSION

The past 2 years have witnessed a dramatic convergence of biochemical and genetic views of the eukaryotic cell cycle (38). However, our understanding of higher plant cell cycle regulation has yet to contribute to or benefit from this new synthesis. We have exploited some of the tools made available from fission yeast studies to establish the basic outlines of the *cdc2*/p34 cell cycle regulatory system in higher plants. Our finding of the ubiquity of p34 protein among higher plant species is in accord with a recent report of its immunological detection in *Arabidopsis thaliana*, oats, *Chlamydomonas*, and brown and red algae (39) with different immunological

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Hela          MEDYTKIEKIGEGTYGVVYKGRHK  TTGQ VVAMKKIRLESEEEGVPSTAIREISLLKE
S. pombe      MENYQKVEKIGEGTYGVVYKARHK  LSG RIVAMKKIRLEDESEGVPSTAIREISLLKE
S. cerevisiae MSGELANYKRLEGVGEGTYGVVYKALDLRPGQGQRVVALKKIRLESEDEGVPSTAIREISLLKE
P. sativum                    VVYKARD RVTNET I ALKKIRLEQEDEGVPSTAIREISLLKE

LHRP     NIVSLQDVLMQD SRLYLIFEFLSMDLKKYLDSIPP GQYM DSSLVKSYLYQILQGIVFCHSRRVLH
VNDENNRSNCVRLLDILHAE SKLYLVFEFLDMDLKKYMDRISETGATSLDPRLVQKFTYQLVNGVNFCHSRRIIH
LKDD     NIVRLYDIVHSDAHKLYLVFEFLDLDLKRYMEGIPKD Q PLGADIVKKFMMQLCKGIAYCHSHRILH
MQHR     NIVRLQDVVHS EKRLYLVFEYLDLDLKKHMDSSPEFVK   DPRQVKMFLYQMLQGIAYCHSHRVLH

RDLKPQNLLIDDKGT IKLADFGLARAFGIPIRVYTHEVVTLWYRSPEVLLGSARYSTPVDIWSIGTIFAELATKK
RDLKPQNLLIDKEGN LKLADFGLARSFGVPLRNYTHEIVTLWYRAPEVLLGSRHYSTGVDIWSVGCIFAEMIRRS
RDLKPQNLLINKDGN LKLGDFGLARAFGVPLRAYTHEIVTLWYRAPEVLLGGKQYSTGVDTWSIGCIFAEMCNRK
RDLKPQNLLIDRRTNCVKLADFGLARAFGIPVRTFTHEV

PLFHGDSEIDQLFRIFRALGTPNNEVWPEVESLQDYKNTFPKWKPGSLASHVKNLDENGLDLLSKMLIYDPAKRIS
PLFPGDSEIDEIFKIFQVLGTPNEEVWPGVTLLQDYKSTFPRWKRMDLHKVVPNGEEDAIELLSAMLVYDPAHRIS
PIFSGDSEIDQIFKIFRVLGTPNEAIWPDIVYLPDFKPSFPQWRRKDLSQVVPSLDPRGIDLLDKLLAYDPINRIS

GKMALNHPYFNDLDNQIKKM
AKRALQQNYLRDFH
ARRAAIHPYFQES
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FIG. 5. Derived amino acid sequence of pea *cdc2* homologue PCR product, compared with published sequences of gene products of HeLa *cdc2Hs*, *Sch. pombe cdc2*, and *Saccharomyces cerevisiae CDC28* (6). Boxed amino acids represent segments of sequence identity among the four species' p34 proteins. Regions in pea *cdc2* sequence representing PCR primers are underlined.

probes. The interspecies variation in the appearance of polymorphic p34 immunoblot signals (Fig. 1) may result from the variation in abundance of mitotic cells among the species examined (monocot vs. dicot). We assume that this polymorphism reflects differential phosphorylation states of plant p34 proteins, as it does in murine and HeLa cells (9). Direct support for this proposition will require purification of plant p34 by immunoprecipitation or p13-Sepharose affinity chromatography (36). Whereas MAb-J4 has proven inefficient for immunoprecipitation, cloning of the authentic pea *cdc2* will now permit the preparation of antisera specific for pea p34. In accord with reports from other systems (34), we have been unable to dephosphorylate pea p34 with commercial alkaline phosphatase preparations (unpublished data).

In all other systems studied to date, p34 occurs as component of one or more protein complexes during parts of the cell cycle (12–15, 17–21, 30, 31). Our results suggest this to be the case in pea. In the experiments reported above, extracts were prepared from developmentally complex pea tissues, containing both dividing and nondividing cells, the former distributed throughout the stages of the cell cycle. Therefore, we can make no claims as to the cell cycle stage specificity of p34 monomer and aggregates in pea. However, by analogy with yeast and mammalian systems, the complexed forms of p34 would be expected to occur predominantly during G_2 and M phases of the cell cycle (19, 30, 31).

Invertebrate and amphibian p34/cyclin complexes phosphorylate histone H1 *in vitro*. Our results suggest that pea also contains a high molecular mass H1K complex. In animal and yeast systems, the occurrence of a dephosphorylated, complexed p34 is correlated with maximal H1K activity (9, 12, 15, 19, 21, 30, 31–36). Our data are consistent with these findings, in that the kinase activity predominated in fractions in which a high molecular mass, presumably dephosphorylated form of pea p34 was present.

Our cDNA cloning strategy has verified that not only does pea carry a *cdc2* homologue but that its product expresses the unique epitope recognized by MAb-J4 (unpublished data). Proteins displaying striking sequence similarity to p34, yet involved in altogether different cellular functions, have been identified in budding yeast (40, 41). The products of our cloning procedure, being full-length pea/yeast chimeric *cdc2* cDNAs, can now be functionally tested for complementation of yeast *cdc2* mutants. The recovery of a higher plant *cdc2* homologue will now permit us to examine the role of p34 and its associated subunits in the regulation of mitosis in model plant cell cycle systems and in a variety of higher plant developmental processes.

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