cdc2MsB, a cognate cdc2 gene from alfalfa, complements the G1/S and 
 budding yeast cdc28 mutants

Heribert Hirt*, Aniko Páy, Laszlo Bögre, Iruke Meskiene and Erwin Heberle-Bors
Institute of Microbiology and Genetics, University of Vienna, Vienna Biocenter, Dr. Bohugasse 9, 1030 Vienna, Austria

Summary

The product of the cdc2 gene encodes the p34cdc2 protein kinase that controls entry of yeast cells into S phase and mitosis. In higher eukaryotes, at least two cdc2-like genes appear to be involved in these processes. A cdc2 homologous gene has previously been isolated from alfalfa and shown to complement a fission yeast cdc21 strain. Here the isolation of cdc2MsB, a cotate cdc2 gene from alfalfa (Medicago sativa) is reported. Southern blot analysis shows that cdc2MsA and cdc2MsB are present as single copy genes in different tetraploid Medicago species. cdc2MsB encodes a slightly larger mRNAS (1.5 kb) than cdc2MsA (1.4 kb). Both genes were found to be expressed at similar steady state levels in different alfalfa organs. Expression levels of both cdc2Ms genes correlate with the proliferative state of the organs. Complementation studies revealed that in contrast to cdc2MsA, cdc2MsB was not able to rescue a cdc21 strain. cdc2MsB was also unable to rescue a G2M arrested cdc28 budding yeast strain which could be rescued by expression of the cdc2MsA gene. Conversely, cdc2MsB but not cdc2MsA was found to complement the G1/S block of another cdc28 budding yeast mutant. These results suggest that cdc2MsA and cdc2MsB function at different control points in the cell cycle.

Introduction

All eukaryotes have been shown to contain a 34 kDa protein kinase which plays a central role in the regulation of cell division. The kinase has been shown to be the product of the cdc2 CDC28 genes in yeasts and animals (for review, see Nurse, 1990) and in various plant species (for review, see Doonan, 1991) and is denoted p34cdc2.

The p34cdc2 kinase is part of the mitosis promoting factor (MPF). MPF activation of the p34cdc2 with cyclin protein kinase activity is required for onset of mitosis (Boehmer and Beach, 1990; Murray and Kirschner, 1989). At metaphase, cyclins are degraded and protein kinase activity vanishes (Murray et al., 1989; Pelham et al., 1989). In contrast to yeast, several cdc2-related genes have been identified in higher eukaryotes (Walther et al., 1989; Elledge and Spottswood, 1991; Lehner and O’Farrell, 1991).

We have previously reported (Colasanti et al., 1991; Ferreira et al., 1991; Hirt et al., 1991) that we have isolated a homologue of the cdc2 gene from alfalfa (Hirt et al., 1991). In the present paper, we describe the isolation of cdc2MsB, a cotate cdc2 gene from alfalfa (Medicago sativa). In transcript analysis of alfalfa, we have found both cdc2MsA and cdc2MsB transcripts. While cdc2MsA was shown to complement a fission yeast mutant (Hirt et al., 1991), it was not able to perform the same function. In contrast, cdc2MsB was able to complement a G2M arrested cdc28 budding yeast strain which could be rescued by expression of the cdc2MsA gene. Conversely, cdc2MsB but not cdc2MsA was able to complement the G1/S block of another cdc28 budding yeast mutant. These results suggest that cdc2MsA and cdc2MsB function at different control points in the cell cycle.

Results

Isolation and sequencing of cdc2MsB cDNA from Alfalfa

We isolated a 2.5 kb cDNA clone from an alfalfa cDNA library (for review, see Nurse, 1990) and in various plant species (for review, see Doonan, 1991) and is denoted p34cdc2.

The p34cdc2 kinase is part of the mitosis promoting

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*For correspondence (fax +43 1 7986524).
ted to cause an amino acid exchange at position 203 (see Figure 2). This resulted in the exchange of a serine in cdc2MsA by a phenylalanine which is the commonly found amino acid in most cdc2 proteins. Since the 3' nontranslated nucleotide sequence is also highly similar to cdc2MsA, this clone is assumed to be an allele of cdc2MsA.

One clone showed marked differences in the restriction pattern and the subsequently determined nucleotide sequence. This clone was denoted cdc2MsB. As shown in Figure 1, the cDNA sequence shows a nontranslated region of 69 nucleotides before the first ATG. An ORF of 879 nucleotides putatively encodes a protein of 293 amino acids. The cDNA has a 345 nucleotide long 3' end and contains a poly(A)⁺ tail of 12 nucleotides. Interestingly, all cdc2MsA clones were truncated at both the 5' and 3' ends (data not shown). This is surprising since four clones were found of the cdc2MsA type and only one for cdc2MsB. This possibly reflects different mRNA stabilities of the two cdc2Ms transcripts.

Sequence comparison of alfalfa cdc2MsA and cdc2MsB

Alignment of the predicted alfalfa cdc2MsA and cdc2MsB protein sequences as shown in Figure 2 revealed 89% identity. The amino acid exchanges did not appear to be particularly clustered in any one region, except possibly in the carboxy-terminal domain. cdc2MsB contains all the motifs of a functional protein kinase and has a perfect copy of the PSTAIR sequence, which is a trademark of the cdc2 proteins. Comparison of cdc2MsB with the predicted protein sequences of the recently reported cdc2 cDNAs from Arabidopsis thaliana, maize and pea (Colasanti et al., 1991; Feiler and Jacobs, 1991; Ferreira et al., 1991) resulted in sequence similarity to cdc2MsA. Alignment with these were analysed by Southern blotting. The genomic DNAs were digested with HindIII and hybridized with 3'-specific probes of the cdc2MsA and cdc2MsB genes (Figure 3b and a, respectively). The restriction patterns for cdc2MsA and cdc2MsB were very different. All Medicago varia lines tested (Figure 3a and b, lanes 1–3) and also M. sativa (Figure 3a and b, lane 4) contained the cdc2MsA and the cdc2MsB genes. Since none of the probes contained HindIII sites within their nucleotide sequences, this indicates that four copies of the cdc2MsA, as well as of the cdc2MsB gene, are present per genome. In conclusion, cdc2MsA and cdc2MsB represent different genes and are present as single copy genes per haploid set of chromosomes.

Expression of cdc2MsA and cdc2MsB in alfalfa suspension-cultured cells and organs

To determine the transcript sizes of the cdc2MsA and cdc2MsB genes, total RNA was isolated from M. varia suspension-cultured cells and separated on a 1% agarose gel containing 1% formaldehyde. Hybridization with the gene-specific 3' probes revealed mRNA sizes of 1.5 kb for cdc2MsB and 1.4 kb for cdc2MsA (data not shown).

To investigate whether cdc2MsA and cdc2MsB are expressed in an organ-specific manner, we analysed alfalfa root, young leaf (3 mm long), adult leaf, stem, vegetative meristem, flower bud and suspension-cultured cells for steady state transcript levels of the two genes as shown in Figure 4, lanes 1–7, respectively. Since the use of total RNA resulted in hardly detectable hybridization signals, poly(A)⁺ RNA was isolated from each organ and analysed by Northern hybridization with cdc2MsA- and cdc2MsB-specific probes (Figures 5a and b, respectively).
Figure 1. Nucleotide sequence of the alfalfa cdc2MsB cDNA and its predicted amino acid sequence.
Figure 2. Sequence alignment of the predicted alfalfa cdc2MsB and cdc2MsA proteins. Identical amino acid residues are indicated by dots. Differences are shown by single letter code.

Figure 3. Southern blot analysis of cdc2MsA and cdc2MsB of M. varia × M. sativa hybrid (lane 5) and a M. varia line (lanes 1-3). M. sativa (lane 4) and a M. sativa hybrid (lane 5) were hybridized with gene-specific 3' cdc2MsA and cdc2MsB (a) and with cdc2MsA (b).

We have previously shown that cdc2MsA was able to complement a cdc2 MsA yeast strain at the restrictive temperature (Hirt et al., 1991). To test whether the same is true for the cdc2MsB gene, we cloned the cdc2MsB gene into the yeast expression vector pSM-1. This plasmid contains the SV-40 promoter which was used efficiently in Saccharomyces cerevisiae (Jones et al., 1988). After transformation of the cdc2MsB containing yeast strain with either pSM-1/cdc2MsA, pSM-1/cdc2MsB or the empty vector pSM-1, cells were selected for leucine prototrophy at the permissive temperature of 25°C for 3 days. Transformants were plated on to fresh plates lacking leucine and grown at 25, 28, 30, 33, 35 and 37°C for 2 days. Fission yeast cells transformed with pSM-1, pSM-1/cdc2MsA and pSM-1/cdc2MsB were shown to grow at 35°C.
found to grow on plates at 28°C as shown in Figure 5 (a–c), respectively. However, at temperatures above 30°C, only cdc2MsA/pSM-1-transformed cells were able to form colonies (Figure 5c). We conclude that cdc2MsB is not able to complement the fission yeast cdc2-33 mutant.

cdc2MsB can complement the G1/S transition but not the G2/M transition of cdc28 budding yeast mutants

To test whether cdc2MsB can complement the CDC28 function in budding yeast, we cloned cdc2MsB into the yeast expression vector pYES2 to give pYES2/cdc2MsB. Also, the cdc2MsA gene was cloned into the same vector to give pYES2/cdc2MsA. In the pYES2 plasmid, expression of the cdc2Ms genes can be induced by the GAL1 promoter. Two different cdc28 budding yeast mutants were transformed with pYES2/cdc2MsA, pYES2/cdc2MsB, and the empty pYES2 plasmid as a control. The one Saccharomyces cerevisiae strain was the cdc28-4ts mutant which is known to block the cells in "START", i.e., when cells are about to enter the S phase. The other S. cerevisiae strain was the cdc28-1Nts mutant, which was shown to block cells in G2/M (Surana et al., 1991).

Transformants were first selected for uracil prototrophy. Then for induction of the GAL1 promoter, cells were plated on to fresh medium plates containing galactose and incubated for 2 days at 23, 25, 28, 30, 33, 35 and 37°C. cdc28-1N cells which were transformed with either the pYES2/cdc2MsB construct (Figure 6c) or with pYES2 alone (Figure 6a) were able to grow only at 23, 25 and 28°C (Figure 6, 28°C) but not at higher temperatures (Figure 6, 36°C). In contrast, pYES2/cdc2MsA-transformed cdc28-1Nts cells were able to grow at 25, 28, 30, 33 and 36°C (Figure 6b). This shows that alfalfa cdc2MsA but not cdc2MsB is able to substitute for the mutated CDC28 protein at the G2/M transition.

When cdc28-4ts budding yeast cells were transformed with pYES2/cdc2MsB (Figure 6f), they were able to grow at 23, 25, 28, 30, 33 and 36°C. In contrast, cdc28-4ts cells which were transformed with pYES2/cdc2MsA (Figure 6e) or pYES2 (Figure 6d) did not grow at temperatures above 28°C. This shows that the alfalfa cdc2MsB is able to substitute for the CDC28 function in G1/S.

To analyse the complementing effect of the alfalfa cdc2 genes on cell division activity of budding yeast cells in a different way, the cell numbers of S. cerevisiae cdc28-4 and S. cerevisiae cdc28-1N cells transformed with pYES2, pYES2/cdc2MsA or pYES2/cdc2MsB, were determined at 28°C and 33°C as shown in Figure 7 (a and b, respectively). At the permissive temperature of 28°C, S. cerevisiae cdc28-1N cells which were transformed with pYES2, pYES2/ cdc2MsA or pYES2/cdc2MsB grew equally well (Figure 7a). Under the restrictive conditions at 33°C, only pYES2/cdc2MsA-transformed cells showed an increase in cell number (Figure 7a). When the same analysis was performed with S. cerevisiae cdc28-4 cells that were transformed with all three constructs, incubation at the restrictive temperature resulted in a cell number increase only in those that expressed the cdc2MsB gene (Figure 7b).

Discussion

In budding and fission yeasts, the induction of both replication and mitosis requires the action of the p34cdc2 gene, several cdc2-like kinases have been cl...
Since alfalfa is an autotetraploid plant, the possibility existed that the two cdc2Ms genes might be alleles. Considerable evidence argues against this possibility. First, we found a cDNA clone which is nearly identical to cdc2MsA. Although several nucleotide differences were observed, only one produced an amino acid substitution. Furthermore, the 3' noncoding region was also highly similar to cdc2MsA. Therefore, this clone probably represents a true allele. In contrast, the cdc2MsB gene is only 89% identical to cdc2MsA at the amino acid level, and the 3' nontranslated sequences of cdc2MsA and cdc2MsB differ considerably. Secondly, Southern blot analysis with the cdc2MsA and cdc2MsB genes showed that each of the two genes is present in four copies, i.e., one copy per haploid genome. Two different alfalfa species, *M. varia* and *M. sativa*, both contained cdc2MsA as well as cdc2MsB, therefore, we consider cdc2MsA and cdc2MsB to be different genes.

The presence of two different cdc2-like genes implies that the encoded kinases should perform different functions. A crucial test for the function of a cdc2 homologous gene is the complementation of temperature-sensitive yeast mutants that are defective in the function of the cdc2 kinase and cannot pass through the cell cycle at the restrictive temperature. When the two alfalfa cdc2 genes were tested for complementation of a fission yeast cdc2ts mutant, only the cdc2MsA gene was able to substitute for the fission yeast cdc2 gene, suggesting that the cdc2MsB might have a different function in the cell cycle. When the same genes were tested in two budding yeast mutants, one of which arrests at the G1/S, the other at the G2/M transition point (Surana et al., 1991), cdc2MsB could only rescue the G1/S arrest but not the G2/M block while cdc2MsA was found to complement the G2/M but not the G1/S transition. This is the first time that cdc2 cognates of higher eukaryotes have been shown to alternatively complement yeast mutants which are blocked at the two major control points of the eukaryotic cell cycle. These data indicate that cdc2MsA and cdc2MsB perform different functions in the cell cycle of yeast cells and suggest that they might have analogous functions in plant cells. Although complementation in yeast is an indication, it is clearly not sufficient proof for such a role in plants.

Accumulating evidence implies the animal cdc2-like kinases to be involved in the transition from G1 to S phase (Dunphy and Newport, 1989; Fang and Newport, 1991; Pines and Hunter, 1990). Also in higher plants, several cdc2-like genes have been reported (Colasanti et al., 1991; Feiler and Jacobs, 1991; Ferreira et al., 1991; Hata, 1991; Hirayama et al., 1991), but their functions are unclear. We have previously isolated a homologue of the fission yeast cdc2 gene from alfalfa (Hirt et al., 1991). Here, we report the isolation and characterization of cdc2MsB, a cognate cdc2 gene from *M. sativa*.

Figure 7. Complementation studies of budding yeast cdc28ts mutants with alfalfa cdc2MsA and cdc2MsB.

(cdc28-1N) and cdc28-4N budding yeast mutants were transformed with pYES2, pYES2/cdc2MsA and pYES2/cdc2MsB. Ura3 prototrophic transformants were grown in galactose-containing medium at 28°C or at 33°C. (a) cdc28-1N cells transformed with pYES2 at 28°C (□) and 33°C (○), or with pYES2/cdc2MsA at 28°C (●) and 33°C (▲) or with pYES2/cdc2MsB at 28°C (▲) and 33°C (●). (b) cdc28-4N cells transformed with pYES2 at 28°C (□) and 33°C (○) or with pYES2/cdc2MsA at 28°C (●) and 33°C (▲) or with pYES2/cdc2MsB at 28°C (▲) and 33°C (●).
primes. Therefore, it appears possible that the two alfalfa cdc2Ms genes which we have identified are the only cdc2-like genes in this species. The high similarity of the predicted amino acid sequences appears to be puzzling on first sight. However, it should be noted that yeast cells only possess one cdc2 kinase which performs both the G1/S and the G2/M transition steps in the cell cycle. Inspection of the amino acid differences in the cdc2Ms kinases with the mapped sites of S. pombe cdc2 mutants (Ayscough et al., 1992; Carr et al., 1989), revealed two sites where amino acid changes could contribute to functional differences of the encoded kinases. In one case the S. pombe cdc2wee mutant tw2w maps to a region where both cdc2Ms kinases show several differences to each other (position 138–142 in Figure 2). In the other case, the S. pombe cdc2 mutant L210 has an exchange from a phenylalanine to a leucine. Interestingly, the cdc2MsB sequence has the wild-type phenylalanine at this position whereas the cdc2MsA has a serine (position 203 in Figure 2). Experiments to verify the different roles of the cdc2Ms kinases face the problem of the identification and quantification of the different protein kinase activities. The presently used p13E-1 affinity columns are known to bind a multitude of proteins, including at least some of the cdc2-like kinases (Hunter, personal communication). Antibodies raised against the cdc2 proteins or the highly conserved PSTAIR peptide are commonly used to identify these kinases on Western blots. Owing to the high similarity of the cdc2 kinases, these antibodies cross-react with the different cdc2-like kinases (Fang and Newport, 1991).

Apart from different roles during the cell cycle, some of the cdc2-like genes appear to be expressed in a tissuespecific manner as reported for the human subtypes (Meyerson et al., 1992). In Northern blots of various alfalfa organs, we found only minor differences in expression of the two genes. However, comparison of young and adult leaf showed a marked change in transcript levels of both cdc2Ms genes. This suggests a correlation of cdc2Ms transcripts with the proliferative state of the organs. Flow cytometric analysis of these organs (data not shown) and cdc2 in situ hybridization data in Arabidopsis (Martinez et al., 1992) corroborate this finding. To determine more precisely which cells in the different organs express cdc2MsA or cdc2MsB, in situ hybridization with genespecific probes will be necessary. On the other hand, the cdc2 protein kinase is known to be subjected to transcriptional, translational and post-translational controls in animals and yeast (for review, see Nurse, 1990). Therefore, the finding of similar steady state transcript levels of the cdc2Ms genes in different vegetative organs does not preclude that the encoded kinases have different activities and perform distinct roles in particular tissues or specialized cells.

**Experimental procedures**

**Plant tissue culture**

A suspension culture of M. varia (line A2) was used (Bögre et al., 1988). The suspension culture was derived from callus tissue which originated from stem segments and was propagated in Murashige and Skoog medium (Murashige and Skoog, 1962) supplemented with 1 mg l^{-1} 2,4 dichlorophenoxyacetic acid and 0.2 mg l^{-1} kinetin.

**Library screening and sequence analysis**

Poly(A)^+ RNA was isolated from somatic embryos from induced alfalfa suspension-cultured cells (Györgyey et al., 1991). A λZAP cDNA library was prepared according to the manufacturer (Stratagene, California, USA). Approximately 300 000 colonies were hybridized with a ^32P-labeled alfalfa 0.75 kb Pst1 cdc2MsA gene fragment (Hirt et al., 1991). Five positive clones were isolated and sequenced in both directions with a T7 polymerase sequencing kit (Pharmacia LKB, Uppsala, Sweden). Three clones were found to be identical to the cdc2MsA gene described earlier (Hirt et al., 1991). One clone was found to be an allelic form of cdc2MsA. The fifth was found to encode cdc2MsB.

**Southern blot analysis**

DNA (10 μg) from different lines of M. varia cv. Rumbler and M. sativa cv. RA3 was digested with HindIII and separated on 0.7% agarose gels. After blotting, the nylon filters were hybridized to cdc2MsA - or cdc2MsB-specific probes. To generate cdc2MsA - or cdc2MsB-specific probes, primers specific to the 3' nontranslated regions of the two alfalfa cdc2 genes were synthesized: GGGTGAGCAGGAGGTTTCCT and GGAAGCTTTAAGGAGTTGTG for cdc2MsA: ATGTGAGTATGATCACCTCA and GAAAGCTTGGGTTCAGGAAGAG for cdc2MsB. Amplification of gene-specific fragments of the two genes was carried out by PCR according to the conditions as described in the section on RNA extraction and PCR quantification of transcript levels. The 226 and 170 bp fragments were digested with HindIII and PstI and cloned into Bluescript vectors pBlue II KS+ (Stratagene). Gene-specific probes were obtained by isolating the digested cdc2MsA and cdc2MsB fragments from agarose gels and ^32P-random primed labelling according to standard protocols.

**Complementation studies**

For complementation in fission yeast, the cdc2MsA and cdc2MsB cDNAs were inserted into the yeast/E. coli shuttle vector pSM-1. In this vector, the cdc2Ms genes are expressed by the SV-40 promoter which is a strong constitutive promoter in fission yeast (Jones et al., 1988). The cdc2MsA cDNA was amplified by PCR with the following primers: TCG TTG GAA
The plasmid pT219U, the cDNA was ligated into the E. coli yeast shuttle vector pSM-1. The cdc22M5 CDNA was cloned directly into pSM-1. S. pombe cdc2-33, leu- cells (Nurse et al., 1976) were transformed by the LiCl method (Ito et al., 1983) and were selected for leucine prototrophy. Transfomers were selected on medium plates and grown at 25, 28, 30, 33, and 36°C for 2 days.

For complementation in budding yeast, the cdc22MA and cdc22MS6 CDNAs described above were inserted into pYES2 (Invitrogen Inc., USA). In this shuttle vector, the cdc22M5 genes are expressed by the galactose-inducible GAL1 promoter. S. cerevisiae W303 strains, carrying either the cdc28-4D or the cdc28-1N6 mutation (Surana et al., 1991), were transformed with pYES2/cdc22M5A, pYES2/cdc22MS6 or pYES2 as a control (Ito et al., 1983). Transformants were first selected for uracil prototrophy at 25°C, then colonies were plated on galactose-containing medium plates and grown at 23, 25, 28, 30, 33, and 36°C.

Determination of cell numbers per well was performed by growing the cells under permissive temperature conditions for 10 hr. A cell mass of 1 x 10^6 cells mL^-1 was used. Cells were then grown in galactose media either at 23°C or 30°C for a total of 12 hr. At 2 hours intervals, aliquots were taken and cell numbers were counted under the microscope.

RNA extraction and quantification of transcript levels

Suspension cultured cells or plant tissue was frozen in liquid nitrogen and total RNA extracted by grinding in a mortar as described by Cathala et al. (1983). Poly(A)^+ RNA was isolated from 100 μg total RNA with Dynabeads according to the instructions of the manufacturer (Dynal, Oslo, Norway). RNA was separated on 1% agarose gels containing 1% formaldehyde, blotted to nylon membranes and hybridized to 32P-labeled cDNA probe (A. thaliana DNA fragment (Unfried and Gründler, 1990).

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References


EMBL Data Library accession number X70707 (M. sativa CD2 kinase mRNA).