Plant Physiol. (1995) 109: 687–696

Multiple Isoforms of Arabidopsis Casein Kinase I Combine Conserved Catalytic Domains with Variable Carboxyl-Terminal Extensions[1]

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Three cDNA clones encoding isoforms of casein kinase I (CKI) were isolated from *Arabidopsis thaliana*. One full-length clone, designated *CKI1*, contained an open reading frame of 1371 bp encoding a protein of 51,949 D with an isoelectric point of 9.7. In addition to the highly conserved catalytic domain (of about 300 amino acids), the Arabidopsis CKI isoforms contain 150 to 180 amino acid carboxyl-terminal extensions, which show among themselves a lower level of sequence conservation. These extensions do not show any sequence similarity to nonplant CKI isoforms, such as rat testis CKIδ, which is their closest isolated homolog, or to yeast CKI isoforms. Three additional isoforms of Arabidopsis CKI were found in the data bases of expressed sequence tags and/or were isolated serendipitously in nonspecific screening procedures by others. One of them also shows a carboxyl-terminal extension, but of only 80 amino acids. Casein kinase activity was detected in the soluble fraction of *Escherichia coli* strains expressing the CKI1 protein. This activity showed the crucial properties of CKI, including the ability to phosphorylate the D4 peptide, a specific substrate of CKI, and inhibition by *N*-(2-aminoethyl)-5-chloroisoquinoline-8-sulfonamide, a specific CKI inhibitor. Like several recombinant CKI isoforms from yeast, CKI1 was able to phosphorylate tyrosine-containing acidic polymers.

CKI is a multifunctional protein kinase found in most eukaryotic cells and associated with soluble, membrane, and nuclear fractions (for review, see Tuazon and Traugh, 1991). CKI is one of a small number of protein kinases that preferentially phosphorylate Ser's and Thr's located in acidic regions and are isolated in a constitutively active form. CKI is distinguished in this group (in particular from casein kinase II, an entirely distinct activity) by its monomeric structure, exclusive utilization of ATP as a phosphate donor, high pI, and moderate sensitivity to heparin.

CKI substrates include metabolic enzymes (glycogen synthase), cytoskeletal proteins (myosin), and nuclear proteins (p53, simian virus 40 large T antigen, DNA topoisomerases, and RNA polymerases) (reviewed by Tuazon and Traugh, 1991; Cegielska and Virshup, 1993; Hoekstra et al., 1994). This wide substrate specificity suggests a central function for CKI in cellular regulation. Because CKI can phosphorylate targets located in the proximity of previously phosphorylated Ser's/Thr's (Flotow et al., 1990), it could be involved in hierarchical protein phosphorylation and could modulate signal transduction operated by second-messenger-responsive protein kinases (Roach, 1991). The newly discovered dual specificity of yeast CKI isoforms (phosphorylation of Tyr in addition to Ser/Thr) (Hoekstra et al., 1994) suggests further intriguing implications about the regulatory functions and autoregulation of CKI.

CKI has typically been purified as an approximately 30- to 40-kD monomer, including a 36/38-kD doublet purified from broccoli (Klimczak and Cashmore, 1993). Although proteins in the range of 25 to 55 kD have also been isolated (reviewed by Graves et al., 1993), the biochemical properties have not indicated any significant heterogeneity of this enzyme. However, multiple sequences of cDNA clones encoding CKI isoforms revealed that CKI is indeed a family of quite diverse forms. Four distinct isoforms have been identified so far in mammalian cells, of which α and β encode proteins of approximately 38 kD (Rowles et al., 1991), whereas the isoform δ encodes a protein with a molecular mass of 49 kD (Graves et al., 1993). The γ isoform comprises at least three distinct members of 43, 45.5, and 49.7 kD (Zhai et al., 1995). In the yeast *Saccharomyces cerevisiae*, three members of the CKI subfamily have been isolated: *YCK1* and *YCK2* genes encode proteins of approximately 62 kD (Robinson et al., 1992), and the *HRR25* gene, identified originally as a mutant sensitive to DNA strand

[1] This work was supported by grant DCB-9105415 from the National Science Foundation to A.R.C. and L.J.K. and grant GM38409 from the National Institutes of Health to A.R.C., as well as grants from the Italian Research Council (Special Project RAISA, publication No. 2172) and the European Community (Project BIOTECH) to G.G. C.L. and D.P. were supported by fellowships from the National Institutes of Health and the Italian Research Council, respectively. This is publication No. 115 of the Agency for New Technologies, Energy, and the Environment, Casaccia Research Center, Biotechnology and Agriculture Sector.

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Abbreviations: CKI, casein kinase I; CKI-7, *N*-(2-aminoethyl)-5-chloroisoquinoline-8-sulfonamide; IPTG, isopropylthio-β-galactoside.

interruptions (Hoekstra et al., 1991), encodes a 55-kD protein (DeMaggio et al., 1992). The *YCK1* and *YCK2* genes contain C-terminal prenylation sites, and their products have been shown to be membrane bound (Wang et al., 1992; Vancura et al., 1994).

Additional CKI genes have been isolated from other organisms, including the yeasts *Schizosaccharomyces pombe* (Kearney et al., 1994; Wang et al., 1994) and *Klyuveromyces lactis* (M. Wesolowski-Louvel, unpublished data), *Caenorhabditis elegans* (Wilson et al., 1994), and humans (Tapia et al., 1994).

The diversity of CKI isoforms raises questions concerning the organization of this subfamily of protein kinases in plants. Certain plant protein kinases, such as the calcium-dependent protein kinase (Harper et al., 1991), have been found to have quite unique structural features, so that the isolation of plant CKI forms will allow further evaluation of the evolutionary and functional diversity of the CKI subfamily. Not less importantly, the availability of cloned plant CKIs will provide the material for more extensive studies of the biological and biochemical functions of this protein kinase in plants.

In this paper we describe the cloning and characterization of three cDNAs from the plant *Arabidopsis thaliana* that are homologous to CKI from yeast and mammals. All three contain variable C-terminal extensions of 15 to 18 kD that are attached to the protein kinase domains, which in the case of the full-length clone, *CKI1*, brings the mass of the encoded protein to 52 kD. These extensions show no similarity to previously isolated CKI isoforms. We have expressed the CKI1 protein in *Escherichia coli* and demonstrated that it shows protein kinase activity with properties characteristic of CKI.

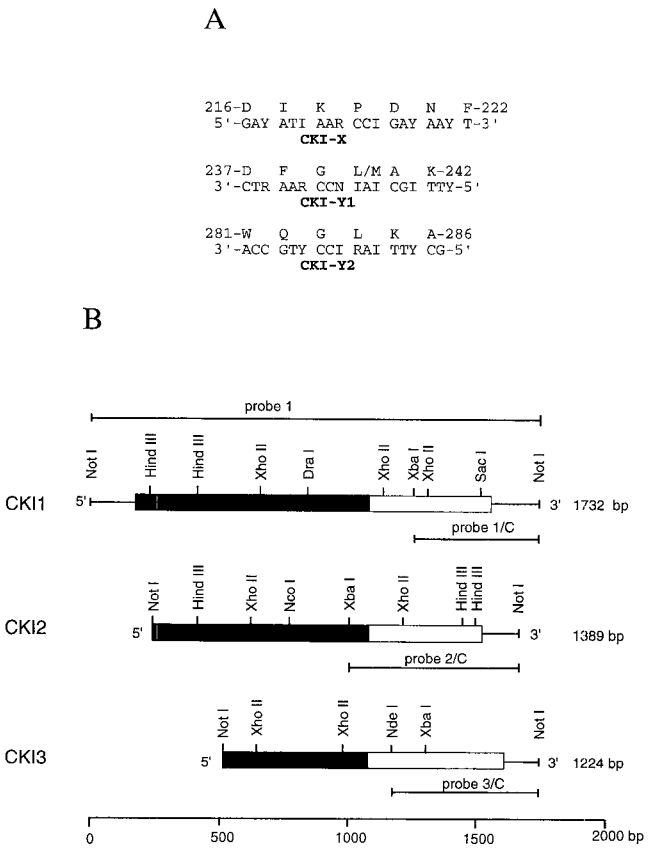

Figure 1. Cloning of Arabidopsis cDNAs homologous to mammalian and yeast CKI. A, Degenerate oligonucleotides used for amplification of CKI cDNA. Above each oligonucleotide, the conserved amino acids used to design the oligonucleotides are shown together with their position in the bovine CKIα (Rowles et al., 1991). Nonstandard bases are: R, A+G; I, inosine; Y, C+T; N, A+C+G+T. B, Physical maps of the *CKI* cDNA clones. Lines indicate the 5′ and 3′ nontranslated regions, black rectangles are conserved catalytic domains, and white rectangles are C-terminal extensions. The extent of the probes used for Southern and northern analyses (Figs. 2 and 3, respectively) is also shown.

MATERIALS AND METHODS

Preparation of the Probe for Screening

Oligonucleotides were synthesized with an Applied Biosystems oligonucleotide synthesizer and purified through Applied Biosystems OPC cartridges according to the manufacturer's instructions. Total RNA was isolated from *Arabidopsis thaliana* plants (ecotype Columbia) according to a published protocol (Giuliano et al., 1993). Degenerate upstream (CKI-X) and downstream (CKI-Y1 and CKI-Y2) oligonucleotides (Fig. 1A) were deduced from conserved amino acid regions found in the catalytic domain of CKI enzymes. To prepare a CKI probe from Arabidopsis mRNA, we used these oligonucleotides and the technique of mixed oligonucleotide-primed amplification of cDNA (Lee and Caskey, 1990). Two different reverse transcription reactions containing 250 ng of the CKI-Y1 and CKI-Y2 oligonucleotides as primers were set up in the volume of 10 μL with the Perkin-Elmer RNA PCR kit (Giuliano et al., 1993). After reverse transcription and addition of the upstream (CKI-X) primer, 35 cycles of amplification were performed in a volume of 50 μL, using the following steps: denaturation, 1 min at 93°C; annealing, 1 min at 40°C; extension, 1 min at 65°C. Amplified bands were purified through a 1.5% low-melting-point-agarose gel cast in 0.5× Tris-borate-EDTA buffer (Sambrook et al., 1989), excised, and labeled with Klenow polymerase using the oligonucleotides used for the PCR reaction as primers.

Screening of the cDNA Library

A cDNA library in λZAP (Stratagene) from dark-adapted *A. thaliana* (ecotype Columbia) was kindly provided by Dr. J. Ecker (University of Pennsylvania, Philadelphia). The library was screened by hybridizing replicate filters at 50°C in 5× Denhardt's solution, 0.5% SDS, 5× SSPE, 20 μg/mL denatured salmon sperm DNA. Filters were then washed two times for 30 min at 50°C in 1× SSPE, 0.1% SDS (Sambrook et al., 1989), dried briefly, and exposed overnight on Kodak XAR film with one intensifying screen. Clones showing positive hybridization on both filters were subjected to a second cycle of screening. The cDNA inserts from positive clones were recovered into plasmids by the automatic excision process according to Stratagene's in-

structions. Both strands of each insert were sequenced using custom-made oligonucleotides and the Sequenase (United States Biochemical) kit.

Southern Blot Analysis

Genomic DNA of Arabidopsis (2 μg/lane) was digested overnight with *Bam*HI or *Nco*I at 37°C, size-fractionated in 0.8% agarose gels, and alkali-blotted onto Hybond N^+ (Amersham) using the manufacturer's instructions. The filters were hybridized overnight at 55°C in 5× Denhardt's solution, 0.5% SDS, 5× SSPE, 20 μg/mL denatured salmon sperm DNA and washed two times for 30 min in 1× SSPE, 0.1% SDS at 55°C (medium stringency) or 0.1× SSPE, 0.1% SDS at 60°C (high stringency).

Northern Blot Analysis

Total RNA (20 μg) and poly(A)$^+$ RNA (2 μg) were isolated from Arabidopsis rosettes, prestained with ethidium bromide, size-fractionated on 1% agarose-formaldehyde gels, and blotted overnight onto Hybond N^+ membrane. The blotting was controlled by illuminating the membrane with UV light, and the RNA was immobilized by baking the membrane in a vacuum oven at 80°C for 2 h. Two sets of conditions were used: high stringency was used with the gene-specific C-terminal probe of *CKI1* and low stringency was used with the N-terminal catalytic domain probe of *CKI1*. The membrane was hybridized overnight at 42°C in 50% formamide, 1× Denhardt's solution, 0.5% SDS, 5× SSPE, 5% dextran sulfate, 100 μg/mL denatured salmon sperm DNA (high stringency) or 25% formamide, 1× Denhardt's solution, 0.5% SDS, 5× SSPE, 10% dextran sulfate, 100 μg/mL denatured salmon sperm DNA (low stringency). The membrane was washed at room temperature for 30 min in 0.2× SSPE, 0.1% SDS (high stringency) or in 2× SSPE, 0.1% SDS (low stringency).

Sequence Data Retrieval

Several sequences analyzed in this paper were retrieved from electronic data bases. Partial cDNA sequences of *Arabidopsis* CKI isoforms were from the Database of Expressed Sequence Tags at the National Center for Biotechnology Information (available on the World Wide Web at http://ncbi.nlm.nih.gov:2555/r_dbest.html): CKI4 corresponds to the clone of GenBank accession No. Z17920 (Y. Parmentier, M.C. Criqui, A. Durr, and J. Fleck, unpublished data), and CKI5 corresponds to accession No. T13780 (Newman et al., 1994). The *Kluyveromyces lactis* CKI isoform (gene RAG8) was retrieved from the EMBL data base (available on the World Wide Web at http://www.ebi.ac.uk/srs/wgetz) with accession No. X79679 (M. Wesolowski-Louvel, unpublished data).

Sequence Analysis

Sequence analysis was done with the GCG software package, version 7 (Genetics Computer Group, 1991). Sequence alignments were performed using the program PILEUP (gapweight = 3.0, lengthweight = 0.1). Similarity consensus was identified using the program PRETTY (-CASE) (threshold = 1.0, plurality = 2.0) with the default symbol comparison table based on the Dayhoff PAM-250 matrix. In both cases, default program settings were used. Highlighting of sequence similarity/identity was performed using Microsoft Excel macros (Haygood, 1993). The macros were reprogrammed to convert the lowercase output of PRETTY (nonsimilar) to normal uppercase and the uppercase output (similar) to uppercase on gray background and then to identify identical residues by a reverse font (black background/white letters) (L.J. Klimczak, unpublished data).

Construction of the *E. coli* Expression Clone

The *CKI1* clone was amplified for 15 cycles using oligonucleotides CCATGGATCGGAATCAAA (upstream) and AGAATTCAGAGGAGAGAG (downstream), which create *Nco*I and *Eco*RI sites upstream and downstream of the coding region, respectively. The PCR product was cloned in the *Eco*RV site of pBluescript SK+ and sequenced. The fragment was then excised with *Nco*I and *Bam*HI, subcloned into the T7 expression vector pET11d, and transformed into the BL21(DE3) strain of *E. coli* (Studier et al., 1990). The deduced amino acid sequence is identical to that of the original *CKI1* clone.

Growth and Lysis of the Expression Strain

Bacteria were inoculated from a fresh colony and were grown for various times (1–24 h) in Luria broth in the presence or absence of 0.4 mM IPTG at room temperature or at 37°C. Cells were collected by centrifugation, washed in 0.9% NaCl, and incubated for 10 min on ice in the presence of 20 μg/mL lysozyme in 1/50 culture volume of 10 mM Tris-HCl, pH 8.0. After lysis by sonication, the lysate was centrifuged for 30 min at 100,000*g* and the supernatant was assayed for protein kinase activity.

Although maximal induction of casein kinase activity with IPTG occurred at 37°C (see "Results"), the optimal yield (three to five times more activity) was obtained at room temperature even though the activity was induced only 50% by IPTG.

Protein Kinase Assay

Protein kinase assays were performed as described by Klimczak and Cashmore (1993) using 0.5 mg/mL casein or 0.5 mM D4 peptide (DDDDVASLPGLRR) (Flotow and Roach, 1991) as a substrate. D4 peptide and rat testis CKIδ were kindly provided by Dr. Paul Graves (Indiana University School of Medicine, Indianapolis) (Graves et al., 1993).

RESULTS

Cloning of Arabidopsis CKI Isoforms

Several recently published protein sequences of CKI show regions of high conservation between animal and yeast enzymes (for a compilation, see Graves et al., 1993). We designed degenerate oligonucleotides based on some

of these regions (shown in Fig. 1A) and used them for PCR amplification of Arabidopsis CKI cDNAs. One of the two combinations of oligonucleotides (CKI-X and CKI-Y2) gave an amplified band of the expected molecular size (approximately 0.2 kb; data not shown). This band was purified from an agarose gel, labeled, and used to screen an Arabidopsis cDNA library in λZAP made from dark-adapted plants. Five positive clones were isolated by screening 400,000 plaques. The inserts were recovered by automatic excision into plasmids and sequenced. The isolated clones represent three distinct cDNAs that encode open reading frames with strong sequence similarities to animal and yeast CKIs. We will call these cDNAs *CKI1*, *CKI2*, and *CKI3*. Three of these clones represent the same gene, *CKI2*, whereas the other two represent distinct genes, *CKI1* and *CKI3* (Fig. 1B). The *CKI1* cDNA is 1732 bp long and contains a 1371-bp open reading frame, whereas cDNAs representing *CKI2* and *CKI3* are 5′-truncated clones containing incomplete open reading frames of 1251 and 1092 bp, respectively. The region of sequence similarity to CKI constitutes a portion of about two-thirds of the length of the three clones and corresponds to the catalytic domain of protein kinase. The clones are highly similar to each other in the region of the catalytic domain, whereas the C-terminal one-third of the clones is much less conserved.

Arabidopsis genomic DNA was analyzed by Southern blot analysis using the divergent C-terminal fragments of the clones as gene-specific probes and the full-length *CKI1* clone as a generic probe for the CKI subfamily (Fig. 1B). Under high-stringency conditions, each of the three C-terminal probes hybridized to single genomic bands of different sizes (in the *Nco*I digest, the bands recognized by the probes migrated at close, but not identical, positions) (Fig. 2, panels 1/C, 2/C, and 3/C). It appears that each of the isolated clones is encoded by a single-copy gene. Under medium stringency, the full-length *CKI1* probe hybridized to several genomic bands, some of which corresponded to

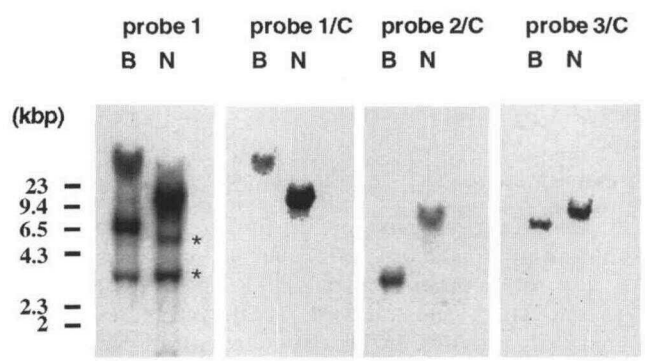

Figure 2. Southern hybridization analysis of genomic sequences homologous to *CKI* clones. Hybridization was performed on Arabidopsis genomic DNA cut with *Bam*HI (B) and *Nco*I (N). In the first panel to the left, the full-length *CKI1* probe was used at medium stringency. In the three remaining panels, the nonconserved C-terminal probes of *CKI1*, *CKI2*, and *CKI3* were used at high stringency to identify the bands corresponding to each gene. The extent of the probes is shown in Figure 1B. Molecular weight markers are shown on the left. In the first panel, asterisks mark the bands detected by the full-length probes but not by the gene-specific probes.

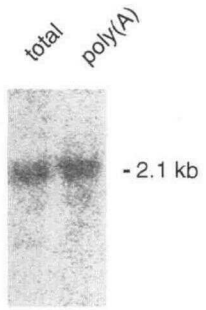

Figure 3. Northern hybridization analysis of *CKI1* mRNA using a gene-specific C-terminal probe (Fig. 1B) under high stringency. Hybridization and washing conditions are described in "Materials and Methods." The radioactive image was exposed for 24 h and was detected using the PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

those recognized by the C-terminal probes. However, there were also additional bands (marked by asterisks in the panel "probe 1" of Fig. 2) that were not recognized by the C-terminal probes. These bands are consistent with the presence of additional *CKI* genes in the *Arabidopsis* genome, although some of them might also originate from unmapped N-terminal fragments of our clones.

Because the C-terminal regions of the CKI clones represent a novel and unique feature of CKI (see below), it was necessary to demonstrate that they are actively transcribed and do not represent pseudogenes or cloning artifacts. For this purpose, the C-terminal fragment of *CKI1* was used in northern blot analysis under high-stringency conditions. A single band of mRNA of approximately 2.1 kb was found to hybridize to this probe (Fig. 3), showing that *CKI1* is actively transcribed and that its C-terminal fragment is indeed represented in the poly(A)$^+$ fraction of mRNA. Hybridization with the catalytic-domain probe of CKI1 (filled box in Fig. 1B) under low stringency revealed an mRNA band migrating at the same position (data not shown). In the context of the Southern hybridization data, which indicate that a CKI1-based probe has enough homology to detect other Arabidopsis CKI mRNAs, this result suggests that the CKI genes may be transcribed into mRNAs of very similar lengths or that the other genes are not expressed very efficiently in rosettes. Preliminary experiments indicate that the *CKI1* mRNA is expressed in most Arabidopsis organs with little variation in the abundance of the mRNA (data not shown). Because of the growing number of Arabidopsis CKI isoforms, more extensive systematic studies on the expression of each isoform with the use of gene-specific probes will have to be performed.

Sequence Alignment and Structural Features of CKI Isoforms

The deduced amino acid sequences of the isolated CKI cDNAs are shown in Figure 4. The complete cDNA *CKI1* contains a 171-bp 5′ untranslated region with several stop codons in front of the first initiation codon of the open reading frame. A second initiation codon is located six

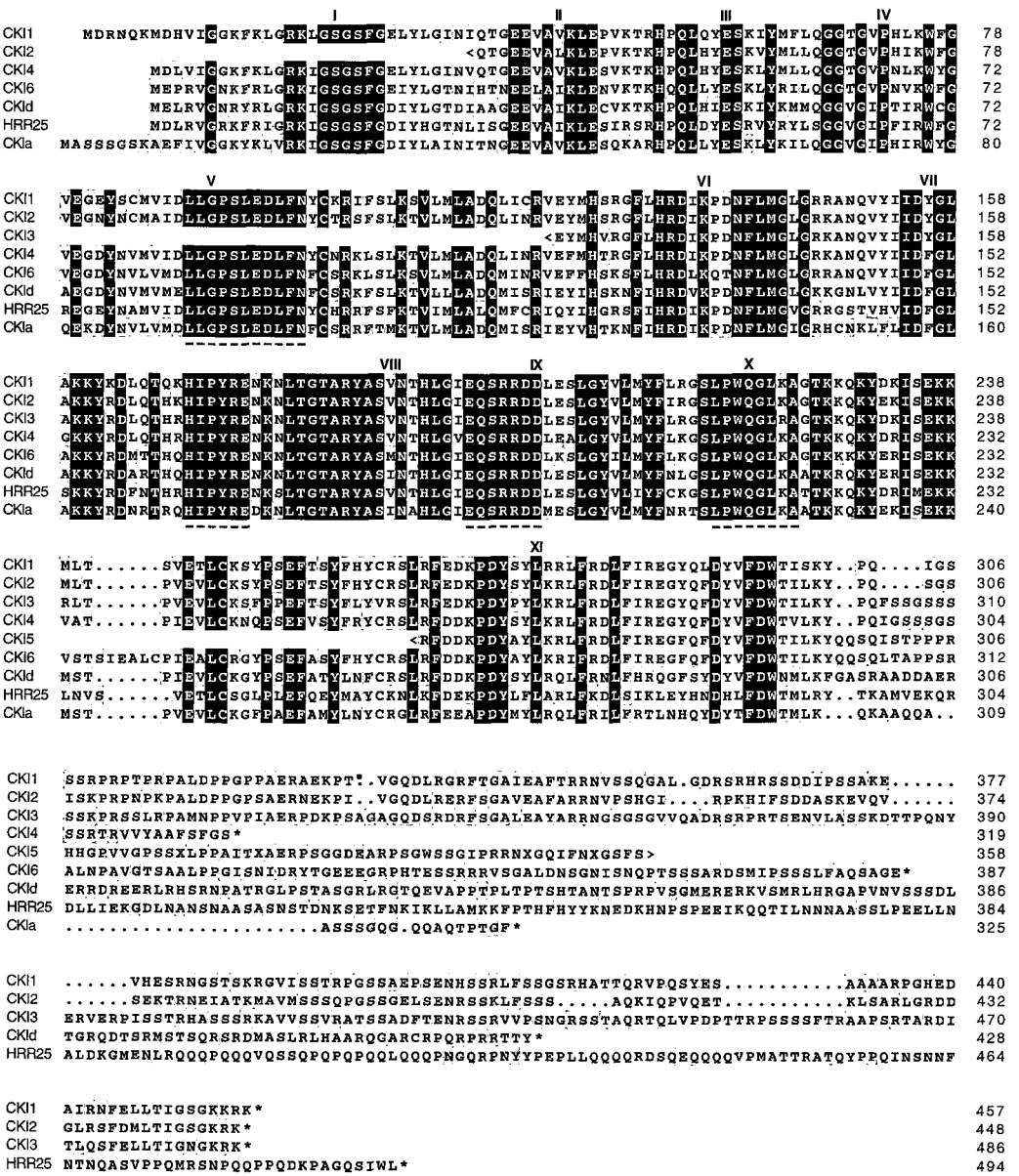

Figure 4. Alignment of amino acid sequences of various CKI isoforms. Arabidopsis isoforms: CKI1, CKI2, and CKI3 (this work); CKI4 (Y. Parmentier, M.C. Criqui, A. Durr, and J. Fleck, unpublished data, and Ali et al., 1994); CKI5 (Newman et al., 1994); CKI6 (Mindrinos et al., 1994). CKId = CKIδ, rat testis-specific δ isoform (Graves et al., 1993); HRR25, yeast CKI isoform (Hoekstra et al., 1991); CKIa = CKIα, bovine α isoform (Rowles et al., 1991). Roman numerals refer to protein kinase motifs I through XI (Hanks et al., 1988). CKI signature sequences are underlined (Graves et al., 1993). Dots denote gaps introduced to optimize the alignment (see "Materials and Methods"). Stop codons are marked by asterisks. Arabic numerals identify the positions of the right-most amino acid in each row. (For incomplete sequences, marked by "<" and ">," the numbers are based on the homologous amino acids of CKI1.)

amino acids downstream, but there are no other initiation codons before protein kinase domain I (Hanks et al., 1988). The cloned Arabidopsis CKI isoforms are aligned with some characteristic CKI isoforms of animal and yeast origin and several additional CKI homologs from Arabidopsis (Fig. 4), which were isolated by other approaches while this work was in progress. The partial clones CKI4 and CKI5 were first isolated in two independent systematic sequencing efforts of Arabidopsis cDNAs (Y. Parmentier, M.C. Criqui, A. Durr, and J. Fleck, unpublished data, and Newman et al., 1994, respectively). The complete clone CKI6 was isolated in map-based cloning as a neighbor to the disease-resistance gene *RPS2* (Mindrinos et al., 1994). Most recently, a full-length clone of CKI4 was isolated in a screen of an Arabidopsis expression library with anti-phosphotyrosine antibodies (Ali et al., 1994).

The Arabidopsis CKI open reading frames have a high content of basic amino acids (the pI of CKI1 is 9.7), in agreement with a pronounced basic character of purified CKI proteins, including that from broccoli (Klimczak and Cashmore, 1993). The N-terminal domains of all of the encoded isoforms contain the protein kinase domains I through XI identified by Hanks et al. (1988) (Fig. 4) and the four short sequences identified by Graves et al. (1993) as signatures of the CKI subfamily (Fig. 4, underlined). Like other members of the CKI subfamily, Arabidopsis CKI forms lack the APE motif in domain VIII. A unique sequence motif that distinguishes Arabidopsis CKI1, CKI2, and CKI3 (but not CKI4 and CKI6) from animal and yeast forms, as well as the majority of protein kinases, is the unusual motif DYG instead of the highly conserved DFG in domain VII (Hanks et al., 1988).

All of the Arabidopsis CKI isoforms are highly similar to each other and to other members of the CKI subfamily in the catalytic domain. Only isoform CKI4 consists of the catalytic domain alone, which comprises about 300 amino acids. The other isoforms possess C-terminal extensions of about 80 (CKI6) or 150 to 180 (CKI1, CKI2, and CKI3) amino acids, which show a lower level of conservation. These extensions show no sequence similarity to other CKI subfamily members (Fig. 4), including CKIδ, which has the highest homology to the Arabidopsis CKIs in the catalytic domain (Fig. 5) and also stands out among animal CKIs as the isoform containing a C-terminal extension.

Sequence similarity in the area of the catalytic domain has been used as a measure of relatedness in the protein kinase family (Hanks et al., 1988). Figure 5 presents a similarity tree constructed on the basis of pairwise similarities by the program PILEUP (Genetics Computer Group, 1991). Arabidopsis CKI isoforms represent a separate branch of the CKI subfamily, distinct from the three branches identified by Kearney et al. (1994) and represented by (a) yeast YCK/CKI, (b) mammalian CKIα and CKIβ, and (c) yeast HRR25. The HRR25 branch is the most similar to the Arabidopsis branch. Mammalian CKIδ was originally grouped within the HRR25 branch, apart from the other mammalian isoforms (Kearney et al., 1994). It appears now that CKIδ has an even greater similarity to the Arabidopsis branch, with which it groups in our alignment (Fig. 5).

Several characteristic sequence motifs are found at the ends of the C-terminal extensions. The TILKY motif at the boundary with the catalytic domain is the last highly conserved region in all Arabidopsis isoforms. Isoforms CKI1, CKI2, and CKI3 also contain the motif FELLTIGXGKRK, which is located directly at their C termini (Fig. 4). Two regions of the C-terminal extensions in CKI1, CKI2, and CKI3 stand out by virtue of their amino acid composition: amino acids 310 to 323 of CKI1 are Pro rich and amino acids 393 to 414 of CKI1 are Ser rich. These are the only regions of the C-terminal extensions that align with other proteins in a similarity search using the BLAST protocol (Altschul et al., 1990). However, the alignment can be made only in other Pro-rich (collagen, extensin) or Ser-rich (phosvitin) areas of those proteins and, although it may pinpoint regions that are functionally relevant on the basis of the composition, it does not represent actual sequence conservation (data not shown).

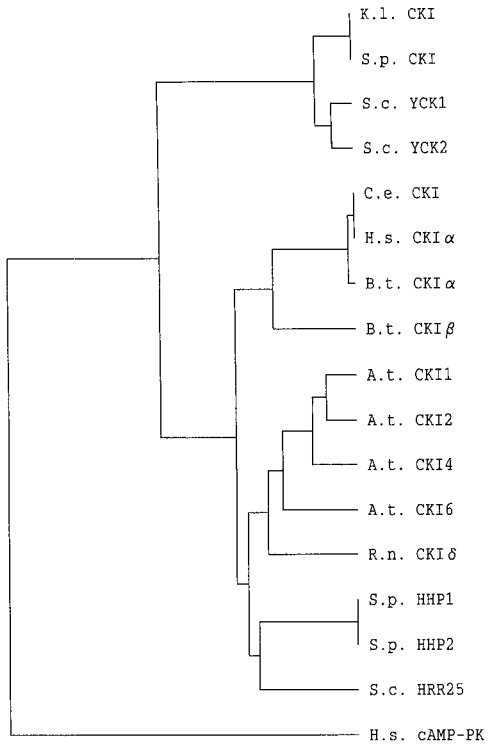

Figure 5. Similarity tree of the CKI subfamily. The tree was constructed using the program PILEUP with the sequences and settings from Figure 4, as well as the sequence of mammalian cAMP-dependent protein kinase (cAMP-PK) for comparison. Only the protein kinase domain fragments (domains I through XI), corresponding to areas homologous to amino acids 41 to 315 of cAMP-PK, were used for the alignment. A.t., *Arabidopsis thaliana*; B.t., *Bos tauros* (cow); C.e., *Caenorhabditis elegans*; H.s., *Homo sapiens* (human); K.l., *Kluyveromyces lactis*; R.n., *Rattus norvegicus* (rat); S.c., *Saccharomyces cerevisiae* (bakers' yeast); S.p., *Schisosaccharomyces pombe* (fission yeast).

Expression of CKI1 in *E. coli*

Because biochemical studies in plant systems have so far revealed the presence of only a 36-kD isoform (Klimczak and Cashmore, 1993), it is of particular interest whether the novel structure of the C-terminally extended Arabidopsis isoforms CKI1, CKI2, and CKI3 correspond to unique biochemical characteristics. Therefore, we expressed the full-length CKI1 in *E. coli* using the pET11d expression vector (Studier et al., 1990). To avoid any possibility that protein fusions may produce artificial changes in the enzyme's properties, we used an expression vector that does not add any external tags to the expressed protein.

To assay for CKI activity, we used the peptide D4. This peptide was developed for specific detection of CKI in unpurified extracts from mammalian cells in the presence of many other protein kinases. It is efficiently phosphorylated only by CKI; all major protein kinases phosphorylate

D4 poorly (Flotow and Roach, 1991). Because no Ser/Thr protein kinase activities are present in *E. coli* extracts, this assay can demonstrate not only the expression of plant CKI but also the substantial similarity of its substrate recognition to that of mammalian CKI.

D4 kinase activity was detected in the soluble fraction of lysates from the bacterial strain expressing CKI1 but not in control lysates from the nonexpressing, vector-transformed host strain BL21(DE3) (Fig. 6A). The activity was inducible with IPTG, but the expression was moderately "leaky": some activity was present even in the absence of IPTG, which enhanced its level 2- to 3-fold (Fig. 6A). In the T7 expression system, some transcription is often observed in the uninduced cell due to the basal level of T7 RNA polymerase (Studier et al., 1990). Such a leakage can be reduced by expression of T7 lysozyme, with the goal of increasing expression, but it was not useful in our case because the presence of T7 lysozyme did not increase the level of overall CKI1 expression (data not shown).

At the maximum induction (about 12 h at room temperature after addition of IPTG), casein kinase was measured with a specific activity of about 0.3 nmol min^{-1} mg^{-1} protein. This value is more than 10 times higher than the level (0.028 nmol min^{-1} mg^{-1}) of total casein kinase activity (all isoforms of CKI and CKII, plus any other protein kinases able to phosphorylate casein) present in broccoli extracts. In addition, crude bacterial extracts appeared to contain inhibitors of the activity, because a 5- to 10-fold increase in the yield of casein kinase activity was observed during purification. However, the total level of recombinant casein kinase activity was still far below the specific activity of highly enriched preparations from plant extracts (Klimczak and Cashmore, 1993). Indeed, no accumulation of protein bands was observed by SDS-PAGE analysis of soluble and 100,000*g* pellet fractions (data not shown), which indicates expression of low amounts of protein with high specific activity. No significant protein accumulation was observed for rat CKIδ, which was also better expressed at room temperature than at 37°C (Graves et al., 1993). It appears that the CKI enzymes may strongly interfere with cellular functions, possibly with the translational apparatus, which would prevent higher levels of accumulation. Low protein expression is generally common for protein kinases present in the soluble fraction of bacterial cells (due to their toxic effects), and higher amounts are usually observed only when inactive proteins are sequestered in inclusion bodies (Russo et al., 1992; Klimczak et al., 1995).

Enzymatic Characterization of CKI1

The protein kinase activity from CKI1-expressing bacteria was partially purified by sequential chromatography on phenyl-Sepharose, DEAE-Sephacel, and phosphocellulose as described by Klimczak and Cashmore (1993). Preliminary examination of this preparation with the activity gel technique revealed a single catalytic band of 55 kD, indicating that the expressed enzyme was not pronouncedly affected by proteolysis (data not shown). Expressed CKI1 was examined with regard to essential enzymatic properties of CKI. In addition to the ability to phosphorylate the CKI-specific peptide D4, the enzymatic activity of CKI1 was inhibited by the CKI-specific inhibitor CKI-7 (Chijiwa et al., 1989), although less efficiently than that of mammalian CKIδ (Fig. 6B). The activity showed a 50% inhibitory concentration of 45 μM, higher than about 8 to 9 μM for the animal enzymes or 21 μM for isolated broccoli CKI (Klimczak and Cashmore, 1993).

In keeping with these highly specific characteristics, CKI1 showed many other enzymatic properties that define the CKI activity. Indeed, the major enzymatic properties of CKI1 are very similar to those of purified approximately 36-kD CKI from broccoli (Table I). Two independent broccoli preparations and three independent recombinant preparations were tested, and the results of a representative set of parallel determinations are presented in Table I. Although some variation (not greater than 30%) was observed in the absolute levels of substrate phosphorylation and inhibitor sensitivity between various preparations, we observed complete reproducibility of the basic CKI characteristics, such as preferential phosphorylation of acidic protein substrates, utilization of ATP alone as a phosphate donor, and inhibition by polylysine and heparin. The most distinct difference, clearly observed in five independent determinations, was that CKI1 phosphorylates the poly-

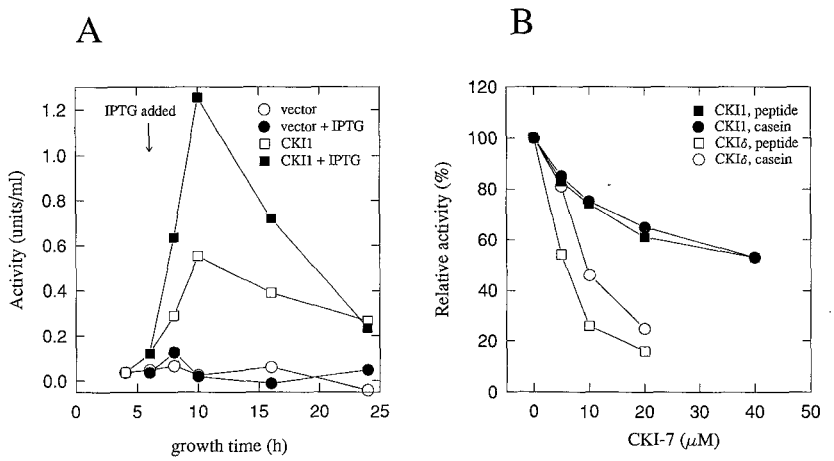

Figure 6. Expression of Arabidopsis CKI1 in *E. coli*. A, Kinetics of the accumulation of D4 peptide-phosphorylating activity during growth at 37°C in expression strains containing the CKI1 construct (squares) and the expression vector alone (circles), in the presence (filled symbols) and absence (open symbols) of IPTG. The activity is expressed in units per 1 mL of culture. One unit is defined as the amount of enzyme that catalyzes the incorporation of 1 nmol of phosphate per minute. B, Phosphorylation of casein (circles) and D4 peptide (squares) by partially purified Arabidopsis CKI1 (filled symbols) and rat testis CKIδ (open symbols).

Table I. *Recombinant CKI1 has enzymatic properties similar to those of purified 36-kD CKI from broccoli*

Assay Conditions	Percent Activity	
	Recombinant CKI1	Broccoli CKI
Standard assay (casein)	100	100
Protein substrates[a]		
Phosvitin	76	96
Myosin light chains	44	45
Myelin basic protein	1.3	4
Histone IIIS	0	0.4
Poly(Glu,Tyr) 4:1	21	0.6
Phosphate donor		
Unlabeled ATP added (1 mM)[b]	2	2
Unlabeled GTP added (1 mM)[b]	94	92
Inhibitor		
Heparin (4 μg/mL)	62	59
Polylysine (0.2 mg/mL)	9	7
CKI-7 (10 μM)	72	63
Salt effect (KCl added)		
50 mM	44	75
100 mM	22	36
200 mM	7	10

[a] Dephosphorylated casein was replaced by the named proteins at a final concentration of 500 μg/mL. [b] To determine the degree of competition, only incorporation of radiolabeled ATP was measured in this experiment.

(Glu,Tyr) substrate more efficiently than does broccoli CKI (background levels). This demonstrates the ability of CKI1 to phosphorylate Tyr as well. The presence of phosphotyrosine was directly confirmed in the phosphorylated product by thin-layer electrophoresis (data not shown).

DISCUSSION

Recent progress in the study of CKI in several mammalian and yeast systems is generating significant interest in this protein kinase as a potential regulator of diverse cellular functions, including signal transduction (Roach, 1991). The ability of CKI to phosphorylate previously phosphorylated regions of proteins makes it particularly suitable to function as a modifier of hierarchical signal transduction cascades and to enhance/attenuate the effects of those protein kinases that are directly controlled by intracellular stimuli. Various isoforms of CKI possess very similar enzymatic properties, including substrate specificities, yet their variable C-terminal domains differentiate them from each other and provide the means for specific interactions. For instance, the C-terminal areas of yeast isoforms YCK1 and YCK2 contain terminal Cys-Cys motifs that are responsible for their membrane attachment (Wang et al., 1992; Vancura et al., 1994), whereas the isoform HRR25 does not have these motifs and is found in the nucleus (Vancura et al., 1994). The YCK1 and YCK2 versus HRR25 isoforms are functionally specialized, because neither YCK1 nor YCK2 can complement the *HRR25* mutations. Thus, the variable C-terminal extensions can be responsible for localization to different cellular compartments and/or interactions with different effectors.

Considering the emerging significance of CKI as a cellular regulator, it is important to analyze the equivalent plant enzyme, both to learn about its specific functions in the plant cell and to evaluate the evolution of CKI as a regulatory activity. These goals require the intensification of complementary biochemical and molecular biological studies.

Although several reports on plant CKI-like activities appeared in the literature (summarized by Tuazon and Traugh, 1991), they described preparations of rather low purity with tentative identification of catalytic subunits not greater than 30 kD. Recently, we reported the purification of homogeneous CKI from cytosolic extracts of broccoli (Klimczak and Cashmore, 1993). The enzyme was isolated as a doublet of monomeric subunits with a molecular mass of 36 and 38 kD. This molecular mass corresponds to that of the commonly isolated species of animal CKI. It is interesting that three Arabidopsis CKI isoforms isolated in this study (CKI1, CKI2, and CKI3) and one isoform isolated independently (CKI6) (Mindrinos et al., 1994) are significantly larger than the purified 36/38-kD broccoli species (or the catalytic domain of CKI to which it corresponds). The isoforms CKI1, CKI2, and CKI3 contain C-terminal extensions that are between 150 and 180 amino acids long, whereas the extension of CKI6 is slightly shorter (80 amino acids).

These isoforms resemble mammalian CKIδ, which contains a C-terminal extension as well. There are very few biochemical data about identification of mammalian CKI enzymes that could correspond to CKIδ, a 49-kD protein that migrates in SDS-PAGE at 55 kD; only two studies have described nuclear CKI forms of 55 kD (reviewed by Graves et al., 1993). CKIδ is most similar to the yeast HRR25 protein, which is implicated in meiosis and DNA repair (Hoekstra et al., 1991) and is localized predominantly in the nucleus (Vancura et al., 1994). Although CKIδ appears a likely candidate for a nuclear isoform of CKI (Graves et al., 1993), there is still no direct confirmation of such a hypothesis. By analogy, some of the extended Arabidopsis isoforms could represent nuclear forms, in contrast to the 36/38-kD species, which appears to be predominantly cytosolic (Klimczak and Cashmore, 1993).

The only Arabidopsis CKI isoform that corresponds in molecular mass to the protein species identified biochemically in plants (Klimczak and Cashmore, 1993) is CKI4, with its predicted mass of 36,716 D (Ali et al., 1994). The full-length isolate was given a distinct name, ADK1, although it clearly shows all of the four short sequences identified as signatures of the CKI subfamily (Graves et al., 1993) (Fig. 4, underlined) and the expressed protein can phosphorylate casein (Ali et al., 1994). Unfortunately, none of the more specific CKI assays has been performed in that study. CKI4 appears to be an equivalent (although not the closest homolog; see Fig. 4) of CKIα and CKIβ, mammalian CKI isoforms that encode only the catalytic domain and correspond in molecular mass to the approximately 36-kD

protein species that is typically isolated through biochemical purification. It is very likely that CKI4 is the homolog of the broccoli CKI gene that encodes the purified 36/38-kD form; this could be confirmed by direct protein sequencing. It is still not certain whether there are any more CKI4-type genes (namely those encoding only the 36-kD catalytic domain) in Arabidopsis or in broccoli. The additional approximately 36-kD isoforms are suggested by the isolation of a doublet of 36 and 38-kD subunits from broccoli (Klimczak and Cashmore, 1993), although posttranslational modifications could be responsible for the doublet as well. This question can also be resolved by direct protein sequencing.

Because of the lack of previous biochemical evidence for any extended CKI proteins in plants, we have focused on the CKI1 isoform, which encodes a protein of 457 amino acid residues with a calculated molecular mass of 52 kD. This isoform appears to be representative of the group of extended Arabidopsis CKI isoforms (CKI2, CKI3, and, to a lesser degree, CKI6). Using a gene-specific C-terminal probe for CKI1, we showed that this extended isoform is indeed expressed. We also showed that CKI1 is catalytically active (in spite of an unusual motif, DYG, in the highly conserved domain VII) and that it shows the characteristic features of a CKI activity. The most unequivocal of them is the ability to phosphorylate the D4 peptide (DDDDVASLPGLRR). This peptide is based on the consensus of CKI phosphorylation sites: a Ser/Thr located behind a stretch of acidic amino acids (Flotow and Roach, 1991). This assay is very specific because the majority of protein kinases recognize basic, rather than acidic, target amino acid sequences and the few other protein kinases that prefer acidic determinants phosphorylate Ser's/Thr's located in front of those stretches (Kemp and Pearson, 1991). CKI1 is also inhibited by CKI-7, a specific inhibitor of CKI (Chijiwa et al., 1989).

Our characterization of the general assay conditions (Table I) has further shown that the enzymatic properties of CKI1 match the defining properties of a CKI-type activity and has revealed that they do not differ significantly from those of the purified approximately 36-kD CKI from broccoli. It is possible, though, that more subtle differences do exist between these two enzymes, and more detailed characterization of the enzymatic parameters, such as K_m or K_i values, may be required to demonstrate them. However, CKI1 does show a much higher ability than broccoli CKI to phosphorylate Tyr-containing acidic polymers. The ability of CKI1 to phosphorylate Tyr residues cannot be a consequence of the C-terminal extension, because it is also the property of CKI4 (ADK1), a 37-kD isoform (Ali et al., 1994). Therefore, at this time we cannot ascribe any specific features of the catalytic activity to the presence of the C-terminal extension in CKI1. This question will be better addressed by comparing the full-length recombinant CKI1 enzyme to its C-terminally truncated fragments; the integrity of the full-length CKI1 will have to be rigorously investigated to eliminate the possibility of proteolyzed contaminants.

Considering the role of phosphorylation of proteins on Tyr residues in signal transduction, the ability of CKI1 to phosphorylate poly(Glu,Tyr) has some intriguing implications. It is interesting that broccoli 36/38-kD CKI does not show any substantial phosphorylation of this polymer and does not autophosphorylate at significant levels on Tyr residues (Klimczak and Cashmore, 1993); in this regard it is similar to the purified mammalian approximately 36-kD species (e.g. Dahmus, 1981). So far, autophosphorylation on Tyr and/or phosphorylation of Tyr's in acidic polymers has been observed only in recombinant CKI isoforms (Ali et al., 1994; Hoekstra et al., 1994; this work) and may be suppressed in vivo, for instance by posttranslational modifications. Tyr phosphorylation/autophosphorylation may represent a mechanism of formation or maturation of CKI activity following the translation of the nascent protein. It is not certain whether the Tyr-phosphorylating activity of CKI would have any physiological relevance in the phosphorylation of physiological substrates. The acidic Tyr polymers are artificial substrates and their level of phosphorylation is significantly lower than that of the standard CKI substrates, the acidic proteins casein and phosvitin (Hoekstra et al., 1994; this work), and it is most likely still lower than that of CKI's physiological substrates. This question will be clarified only when in vivo substrates of CKI that are phosphorylated on Tyr residues become isolated.

So far there is no evidence for the presence in plant extracts of CKI isoforms larger than 38 kD. It is possible that the C-terminal extensions may be proteolytically cleaved in vivo, generating about 36- to 40-kD fragments that consist of the catalytic domain. In such a case, however, the existence of so many isoforms that end up being processed to a highly conserved domain would be rather puzzling. A more attractive hypothesis would be that the variable C-terminal extensions function as specificity determinants for the individual isoforms and confer upon them some unique characteristics of regulatory responsiveness, intracellular localization, and/or substrate recognition, a situation similar to that described in yeast (Vancura et al., 1994). In any of these cases, specific protein-protein interactions could play a role. Antibodies directed specifically against the C-terminal extensions should be particularly useful as tools to address this question.

The combination of the data obtained in this study with those from several independent approaches brings the number of identified Arabidopsis CKI isoforms to six. This large, possibly not yet complete, number raises questions about the distribution, biochemical identity, functional specialization, and physiological relevance of these multiple forms. These questions could be addressed by detailed studies on tissue-specific expression of individual isoforms, generation of isoform-specific antibodies and their use for intracellular localization, and detailed side-by-side comparisons of the regulatory properties of several expressed isoforms. When combined with the identification of physiological substrates of CKI and the biological effects of over-expression of individual isoforms, the studies of plant CKI should be very helpful in revealing its role in the mechanisms of cellular regulation.

ACKNOWLEDGMENTS

We thank Dr. Joseph Ecker for the gift of Arabidopsis cDNA library and the use of the GCG program package, and Drs. Paul Graves and Peter Roach for the gift of the D4 peptide and rat CKIδ.

Received May 1, 1995; accepted July 13, 1995.

The EMBL/GenBank accession numbers for the sequences reported in this article are X78818, X78819, and X78820.

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