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Reconstitution of Arabidopsis Casein Kinase II from Recombinant Subunits and Phosphorylation of Transcription Factor GBF1

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In contrast to the well-defined tetrameric structure of animal and yeast casein kinase II (CKII), plant CKII is found in two forms: a monomeric form and an oligomeric form whose subunit composition is not well defined. The Arabidopsis homologs of the catalytic subunit α (CKA1) and the regulatory subunit β (CKB1) of CKII were expressed in *Escherichia coli* to examine their ability to form complexes, the effect of CKB1 on the catalytic activity, and the relationship of the recombinant enzymes to those isolated from plant material. Both subunits were found mainly in the inclusion body fraction in the bacterial expression strains, and they were solubilized and renatured with the recovery of catalytic (CKA1) and stimulatory (CKB1) activities. The combination of purified CKA1 and CKB1 proteins resulted in up to 100-fold stimulation of casein kinase activity compared with the CKA1 activity alone, showing that CKB1 has biochemical properties similar to those of the β subunit from animals. CKA1 and CKB1 spontaneously assembled into a tetrameric complex, $CKA1_2CKB1_2$, which had properties very similar to those of the oligomeric CKII form isolated from broccoli. However, the properties of the catalytic subunit CKA1 alone differed from those of the broccoli monomeric form of CKII-like activity. Phosphorylation of transcription factor GBF1 with the reconstituted $CKA1_2CKB1_2$ enzyme resulted in stimulation of its DNA binding activity and retardation of the protein–DNA complex; these results are identical to those obtained previously with isolated nuclear CKII from broccoli.

INTRODUCTION

Casein kinase II (CKII) is a multifunctional protein kinase that has been implicated in the regulation of central cellular functions, such as cell division and growth, gene expression, and DNA replication (for review, see Meisner and Czech, 1991). Recently, phosphorylation of the transcription factor c-Jun by CKII was shown to be a part of the signal transduction mechanism regulated by phorbol esters and growth factors (Lin et al., 1992). Phosphorylation by CKII negatively regulated the DNA binding and transactivating activities of c-Jun in resting cells, whereas in phorbol ester-stimulated cells, dephosphorylation of the CKII sites was correlated with induction of AP-1 activity, of which c-Jun is the major component. Thus, an apparently constitutive protein kinase activity, such as CKII, can participate in the control of a signal transduction pathway by counteracting the activity of a regulated, yet-to-be-isolated protein phosphatase (Lin et al., 1992).

Phosphorylation by nuclear CKII from broccoli in vitro was shown to have a stimulatory effect on DNA binding activity of the Arabidopsis transcription factor GBF1 (Klimczak et al., 1992), which interacts with the G-box, a promoter element required for expression of several light-inducible genes and present in many other inducible plant promoters (Giuliano et al., 1988). The majority of extracted plant GBF activity was abolished by phosphatase treatment, and this inactivation was reversed by rephosphorylation with CKII. This regulatory phosphorylation provides an entry point into the mechanisms of regulation of gene expression in plants. The precise physiological function of this GBF1 phosphorylation remains to be elucidated, as does the precise function of plant CKII in gene expression. An essential step in this direction is to characterize better the regulatory properties of CKII, which requires the production of large amounts of purified enzyme for detailed mechanistic studies.

CKII enzymes isolated from animals and yeast possess a characteristic tetrameric structure, $\alpha_2\beta_2$, composed of two catalytic α subunits and two regulatory β subunits (Pinna, 1990; Tuazon and Traugh, 1991). Both subunits are highly conserved throughout evolution: α subunits from Drosophila and humans are 86% identical and β subunits are up to 89% identical (for extensive sequence alignments, for example, see Padmanabha

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et al., 1990; Hu and Rubin, 1991). The α subunit is often encoded by two genes: α and α'. The molecular mass range of the catalytic α subunit is between 37 and 44 kD, and that of the regulatory β subunit is in most cases between 24 and 26 kD. Reconstitution experiments using animal CKII components show that the β subunit has a dramatic effect on the catalytic α subunit by stimulating its activity toward most substrates and enhancing the stimulatory effect of polycations (for more details, see Tuazon and Traugh, 1991; Collinge and Walker, 1994). Consequently, the interaction between the α and β subunits plays a central role in determining the activity and substrate specificity of CKII.

Enzymatic activities with distinct characteristics of CKII have been isolated from plants, but their relationship to the animal and yeast counterparts is not completely resolved. Oligomeric forms of plant CKII react with antibodies against animal CKII, but only individual cross-reacting bands corresponding to the catalytic subunit were observed in maize (single band of 39 kD; Dobrowolska et al., 1992), pea (double band of ~36 kD; Li and Roux, 1992), and broccoli (single band of 40 kD; L.J. Klimczak, unpublished data). No cross-reaction to the putative regulatory subunits was shown even in those sufficiently purified preparations in which noncatalytic components were observed by protein staining (Li and Roux, 1992; Zhang et al., 1993). In addition, monomeric forms fulfilling most of the operational criteria of CKII activity were isolated from several plants, either alone (wheat, Yan and Tao, 1982) or together with oligomeric forms (maize, Dobrowolska et al., 1992; liverwort, Kanekatsu and Ohtsuki, 1993; and broccoli, Klimczak and Cashmore, 1994). A monomeric form of CKII was also isolated from *Dictyostelium* (Kikkawa et al., 1992). This is in contrast to other systems, such as yeast, in which no monomeric forms were identified in spite of specific searching efforts (Bidwai et al., 1992).

Molecular biological studies are helping to clarify outstanding questions about the structure of plant CKII and its relationship to the animal and yeast counterparts. One cDNA encoding a homolog of the catalytic α subunit was cloned from maize (Dobrowolska et al., 1991) and two cDNAs were cloned from Arabidopsis (Mizoguchi et al., 1993). The maize and Arabidopsis α subunits encoded by these clones were 93% identical to each other and showed a high level of homology with yeast and animal α subunits (72 to 76% identity). The encoded proteins had a molecular mass of 39 kD, were catalytically active, and showed the crucial biochemical features of isolated α subunits from animals and yeast. The recombinant maize α homolog was able to assemble with recombinant human regulatory subunit β into a tetrameric complex (Boldyreff et al., 1993). Interestingly, the biochemical properties of the recombinant maize α subunit were distinct from those of the monomeric activity, CKIIB, isolated from maize. CKIIB was also unable to assemble with human β subunit.

Because of the lack of cross-reactivity of the noncatalytic components in plant oligomeric CKII preparations with anti–animal CKII antibodies, it was not clear whether such components represent homologs of the β subunit of animal and yeast CKII. It was recently shown that such homologs do exist in plants when two Arabidopsis genes, *CKB1* and *CKB2*, that show up to 55% identity with β subunits from other species were cloned by complementation of yeast mutations in the CKII catalytic subunit (Collinge and Walker, 1994). This was the first indication that plant CKII may have a composition similar to its yeast and animal counterparts and that it may be regulated in a similar manner. It has remained to be established whether the cloned homologs can perform the biochemical functions of the animal regulatory subunit β and how the enzymatic properties of the recombinant enzymes relate to the CKII forms extracted from plant material.

In this study, we describe the expression in bacteria of Arabidopsis homologs of catalytic subunit α (CKA1) and regulatory subunit β (CKB1) of CKII and demonstrate that their interaction results in significant stimulation of the catalytic activity and formation of a tetrameric complex, $CKA1_2CKB1_2$. The recombinant CKII activities are also compared with those isolated from plant material. We show that the recombinant CKII from Arabidopsis can stimulate the DNA binding activity of Arabidopsis transcription factor GBF1, as did CKII isolated from broccoli (Klimczak et al., 1992). Thus, the regulatory phosphorylation of GBF1 has been reconstituted entirely from homologous bacterially expressed components.

RESULTS

Expression of CKA1 and CKB1

The catalytic subunit of Arabidopsis CKII, CKA1, and the β subunit structural homolog CKB1 were expressed in *Escherichia coli* to characterize their interactions and catalytic properties. Protein kinase activity could be detected in CKA1 expression lysates using standard assays with casein or GBF1 as substrate. This activity showed the crucial features of CKII: it used GTP as a phosphate donor and was strongly inhibited by heparin (data not shown). The catalytic activity of CKA1 was stimulated several-fold when CKA1 extracts were preincubated with extracts from CKB1-expressing bacteria. This stimulatory activity was then used as an assay for CKB1 from various fractions of *E. coli* extracts (Table 1). Low levels of both CKA1 and CKB1 activity could be detected in the soluble fractions (low- and high-salt extracts) of expression lysates, but they did not represent any substantial protein accumulation and could not be easily purified (see Methods). Higher levels of both subunits were found in the insoluble (inclusion body) fraction, and because they could be renatured efficiently (see the following section), the renatured fractions were used for large-scale purification.

Table 1. Distribution of Catalytic and Stimulatory Activities in Fractions of Bacterial Lysates and Their Purification from Inclusion Bodies on Phosphocellulose[a]

Extract	Subunit	Total Activity[b] (nmol/min)	Total Protein (mg)	Specific Activity (nmol/min/mg)
Low salt (soluble)	CKA1	9.6	11	0.87
	CKA1 + CKB1	284	18.2	15.6
High salt (soluble)	CKA1	2.2	6.9	0.31
	CKA1 + CKB1	38.7	12.4	3.1
Solubilized/renatured inclusion bodies	CKA1	37	37.5	1
	CKA1 + CKB1	2222	78	28.5
Phosphocellulose-purified fraction	CKA1	7.2	1.35	5.4
	CKA1 + CKB1	377	3	125

[a] The values correspond to yields from 1 liter of bacterial cultures.
[b] Activity was determined using 0.5 μg of GBF1 per assay as described in Methods.

Preparative Renaturation and Purification of CKA1 and CKB1 from Solubilized Inclusion Bodies

High levels of catalytic (CKA1) and stimulatory (CKB1) activities were detected after the renaturation procedure in the insoluble inclusion body fraction (about 10 times more than in the soluble fractions, as seen in Table 1). This corresponded to an accumulation of significant amounts of isopropyl β-D-thiogalactopyranoside (IPTG)–inducible proteins of 40.7 kD (CKA1) and 36.9 kD (CKB1) in the inclusion body pellet, as identified by SDS-PAGE (Figure 1, lanes 2 and 4).

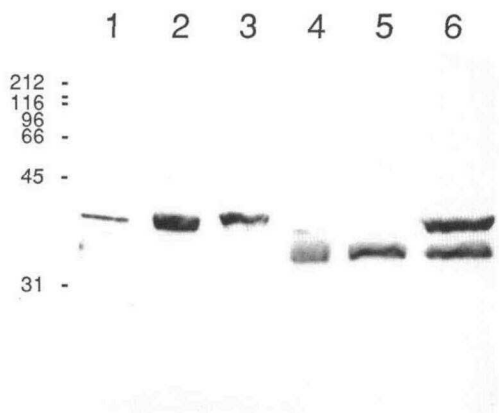

Figure 1. *Expression and Purification of CKA1 and CKB1.*

Shown is a Coomassie blue–stained SDS–polyacrylamide gel of extracts from: inclusion bodies of the CKA1-expressing *E. coli* strain without IPTG induction (lane 1); inclusion bodies of the CKA1-expressing *E. coli* strain after IPTG induction (lane 2); phosphocellulose-purified CKA1 (lane 3); inclusion bodies of the CKB1-expressing *E. coli* strain after IPTG induction (lane 4); phosphocellulose-purified CKB1 (lane 5); phosphocellulose-purified renatured CKA1–CKB1 mixture (lane 6). The amounts of inclusion bodies loaded in lanes 1, 2, and 4 correspond to 50-μL culture volume; purified proteins in lanes 3, 5, and 6 correspond to 1- to 2-mL culture volumes. The positions of molecular mass markers are shown at left in kilodaltons.

Two alternative approaches could be used for reconstitution and purification of expressed CKA1 and CKB1, as described in Methods. The subunits could be reconstituted and purified individually to obtain separate subunits to study their assembly or to generate specific antibodies. Alternatively, they could be combined and renatured together, as described for Drosophila CKII (Lin and Traugh, 1993), to produce large amounts of highly active enzyme rapidly. All three renatured protein products, CKA1, CKB1, and the CKA1–CKB1 complex, were purified after renaturation by one-step procedures to near homogeneity (Figure 1, lanes 3, 5, and 6, respectively).

The optimal level of stimulation was obtained when CKA1 and CKB1 were reconstituted in a 1:1 molar ratio (for details, see Methods). The specific activity of the catalytic subunit alone was 5.4 nmol/min/mg toward GBF1 and 41 nmol/min/mg toward casein, and that of the optimally reconstituted holoenzyme was 388 and 210 nmol/min/mg toward GBF1 and casein, respectively. The same specific activities were obtained when individually purified subunits were mixed in a 1:1 molar ratio. Because the specific activities of the renatured enzymes were similar to other recombinant CKII preparations (for example, see Lin and Traugh, 1993) and were the same as those of the recombinant Arabidopsis preparations obtained from the soluble fractions (data not shown), the renaturation treatment appeared not to have any adverse effect on the activity of the enzyme.

The stimulatory effect of CKB1 addition on casein kinase activity of CKA1 resulted in up to 30-fold enhancement of casein phosphorylation. An even stronger (~100-fold) stimulation of the catalytic activity of CKA1 by CKB1 was observed with transcription factor GBF1 as substrate (Figure 2). These high stimulation levels were observed with diluted enzyme preparations and longer (20 to 30 min) incubation times. They were

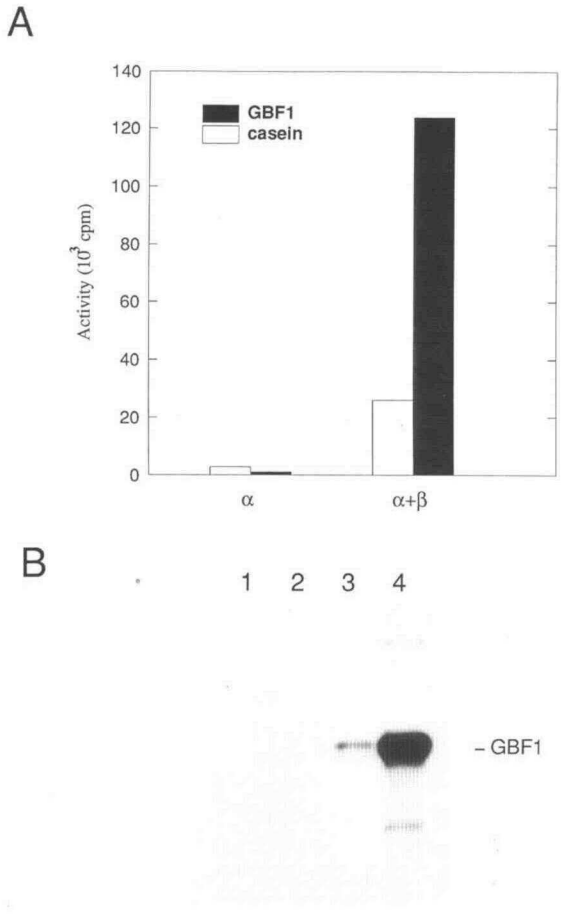

Figure 2. Stimulation of Protein Kinase Activity of CKA1 by Reconstitution with CKB1.

(A) Phosphorylation of casein and CBF1 as determined by total incorporation of radiolabeled phosphate.

(B) SDS–PAGE analysis of phosphorylated protein products: Lanes 1 and 2, CKA1 alone; lanes 3 and 4, CKA1 preincubated with CKB1; lanes 1 and 3, no substrate added; lanes 2 and 4, with 0.5 μg of GBF1 added.

slightly lower when purified and concentrated fractions were assayed for 5 min (20- to 60-fold for GBF1 and four- to sixfold for casein); this suggests an additional effect of CKB1 on enzyme stability. The same levels of stimulation were observed when subunits from the renatured preparation were reconstituted with those from the soluble extract (data not shown). As shown in Figure 2B (compare lanes 3 and 4), this stimulation represents a genuine increase of substrate phosphorylation and not additional phosphorylation of the added CKB1. Phosphorylation of very minor endogenous protein bands (such as the one in Figure 2B, lane 3) was observed in the absence of exogenous substrates, and it most likely represents the autophosphorylation of the kinase subunits (both subunits migrate very closely to GBF1 [39 kD]).

Formation of a Tetrameric Complex by CKA1 and CKB1

The interaction of CKA1 and CKB1 was accompanied by the formation of an oligomeric complex. The reconstituted mixture of CKA1 and CKB1 (molar ratio of 1:1; see Methods) eluted from gel filtration at a position distinct from that of CKA1 alone—it was shifted toward higher molecular sizes (Figure 3). A similar shift was observed in glycerol gradient sedimentation (data not shown). The CKA1–CKB1 complex migrated in gel filtration at higher molecular sizes and in gradient sedimentation at lower molecular sizes than its closest molecular marker, aldolase. Such migration indicates an asymmetric (ellipsoid rather than globular) shape of the molecule and does not allow the direct calculation of molecular masses. Therefore, native molecular parameters for the oligomeric form were determined as a Stokes radius value of 50.6 Å for gel filtration and a sedimentation coefficient of 6.2 for gradient sedimentation. These values correspond to a native molecular mass of 154 ± 20 kD calculated according to Siegel and Monty (1966) and indicate a tetrameric form. Because

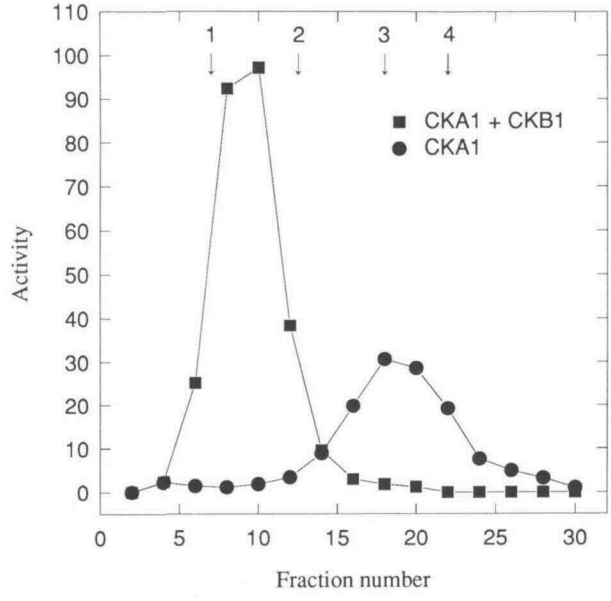

Figure 3. Formation of a Tetrameric Complex of CKA1 with CKB1.

The separation of reconstituted CKA1–CKB1 and CKA1 alone by gel filtration through Sephacryl S-200 at 400 mM KCl is as described in Methods. Numbered arrows indicate the positions of molecular size markers: arrow 1, catalase (52 Å); arrow 2, aldolase (46 Å); arrow 3, BSA (35 Å); arrow 4, ovalbumin (27 Å).

CKA1 and CKB1 are present in the complex at the molar ratio of 1:1, its composition corresponds to $CKA1_2CKB1_2$.

Biochemical Similarities and Differences between Plant-Extracted and Recombinant Forms of CKII

We compared several biochemical properties of the recombinant subunits expressed in bacteria (catalytic subunit CKA1 alone and the reconstituted $CKA1_2CKB1_2$ complex) with the CKII forms purified from plant material. We have previously identified a 150 kD-oligomeric form of CKII in broccoli nuclei (Klimczak et al., 1992), and we recently described a monomeric CKII form in broccoli cytosolic extracts (Klimczak and Cashmore, 1994). We will refer to these two distinct forms as oCKII and mCKII, respectively.

Because mCKII from broccoli does not adsorb to phenyl–Sepharose at moderate salt concentrations (Klimczak and Cashmore, 1993), whereas oCKII does, this property provided a useful distinguishing characteristic and was systematically investigated for all four forms. We found that only mCKII isolated from plants did not adsorb to phenyl–Sepharose at 300 mM potassium phosphate or 500 mM KCl (Figures 4A and 4B, filled squares), whereas both the recombinant enzymes (CKA1 catalytic subunit [Figures 4A and 4C, open circles] and the $CKA1_2CKB1_2$ holoenzyme [data not shown]) and oCKII from broccoli (Figures 4A and 4C, filled circles) adsorbed under

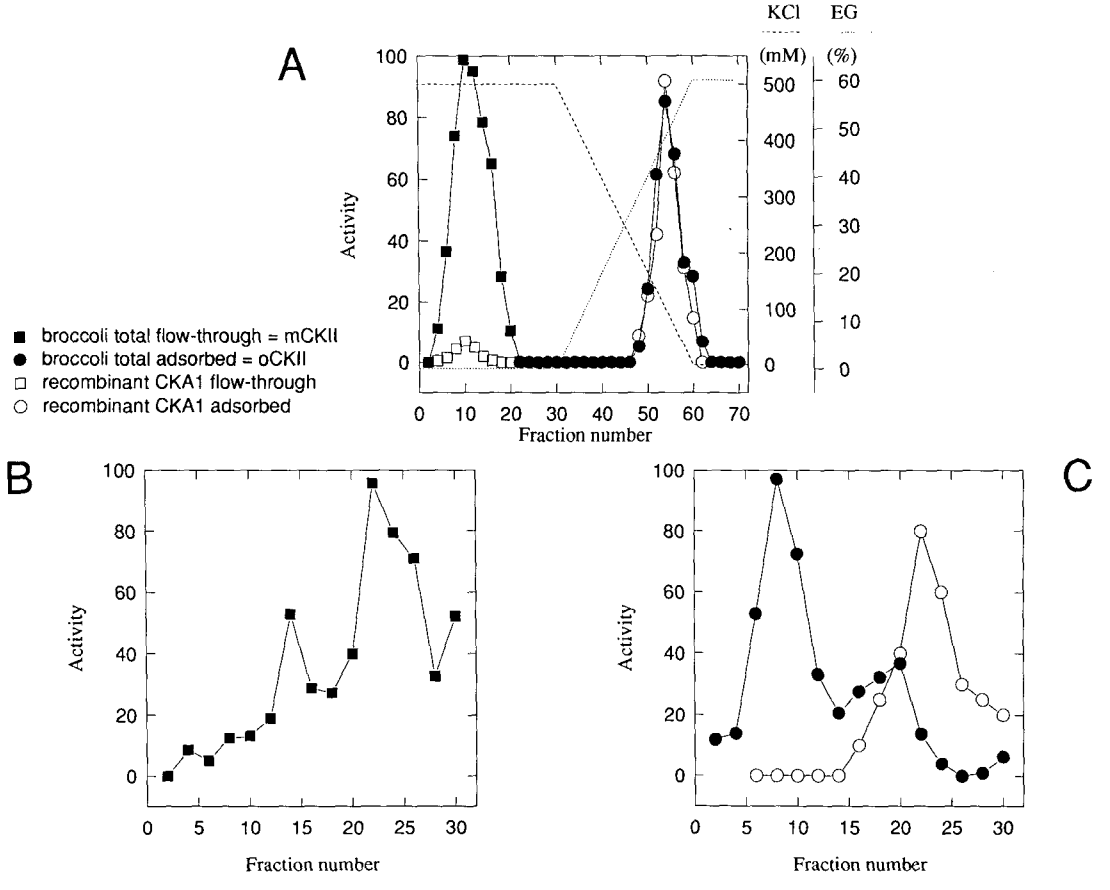

Figure 4. Different Characteristics of Adsorption to Phenyl–Sepharose of Recombinant and Plant CKII Forms.

(A) Total CKII activity extracted from broccoli (filled symbols) was separated on phenyl–Sepharose at 500 mM KCl into the flow-through (filled squares, left peak) and adsorbed fractions (filled circles, right peak). Recombinant CKA1 was predominantly adsorbed under these conditions (open circles, right peak), and only traces of protein kinase activity were found in the flow-through (open squares). EG, ethylene glycol.

(B) The phenyl–Sepharose flow-through fraction of broccoli extract (left peak from **[A]**, filled squares) was separated by glycerol gradient sedimentation and revealed a predominant monomeric peak (mCKII). Fractions are numbered from bottom (high molecular mass) to top (low molecular mass).

(C) The phenyl–Sepharose adsorbed peaks (right peaks from **[A]**) of broccoli total extract (filled circles) and recombinant CKA1 (open circles) were separated by glycerol gradient sedimentation as is shown in **(B)**. The activity from broccoli (oCKII) corresponds to an oligomeric peak, and recombinant CKA1 activity corresponds to a monomeric peak in the same position as mCKII in **(B)**.

these conditions and could be eluted with a gradient of ethylene glycol. It appears that mCKII is much more hydrophilic than the other forms, or it is in a conformational state in which the hydrophobic areas are significantly less exposed to the surface of the protein.

With regard to substrate specificity, we observed that the reconstituted holoenzyme $CKA1_2CKB1_2$ and broccoli oCKII showed very similar patterns of substrate phosphorylation and a similar response to polylysine, which stimulated the phosphorylation of casein 10- to 20-fold (Figures 5B and 5D). Broccoli mCKII phosphorylated these substrates with a specificity similar to that of oCKII, but was less sensitive to polylysine; the level of stimulation was in the three- to fivefold range (Figure 5A). The level of GBF1 phosphorylation by mCKII, oCKII, and $CKA1_2CKB1_2$ was comparable to that of casein in the presence of polylysine, as was already observed for the oligomeric activity from isolated broccoli nuclei (Klimczak et al., 1992). A substantially distinct pattern of phosphorylation was observed with the recombinant CKA1 alone: polylysine had no stimulatory effect, but rather had a weak inhibitory effect, and GBF1 was phosphorylated much less than casein (Figure 5C).

Phosphorylation by Reconstituted CKA1–CKB1 Stimulates DNA Binding Activity of Transcription Factor GBF1

Phosphorylation by CKII isolated from broccoli nuclei was shown to stimulate DNA binding activity of Arabidopsis transcription factor GBF1 (Klimczak et al., 1992). When reconstituted Arabidopsis $CKA1_2CKB1_2$ was used to phosphorylate GBF1, an identical pattern of stimulation of GBF1 binding to the specific DNA probe was observed (Figure 6). In addition to an ~20-fold enhancement of formation of the protein–DNA complex by the phosphorylated GBF1, the complex was slightly retarded in the mobility shift assay in relation to the complex formed by nonphosphorylated GBF1. This stimulatory effect was observed only when ATP and GTP, which are known to function as a phosphate donor for CKII, were used in the phosphorylation assay, but not when CTP was used.

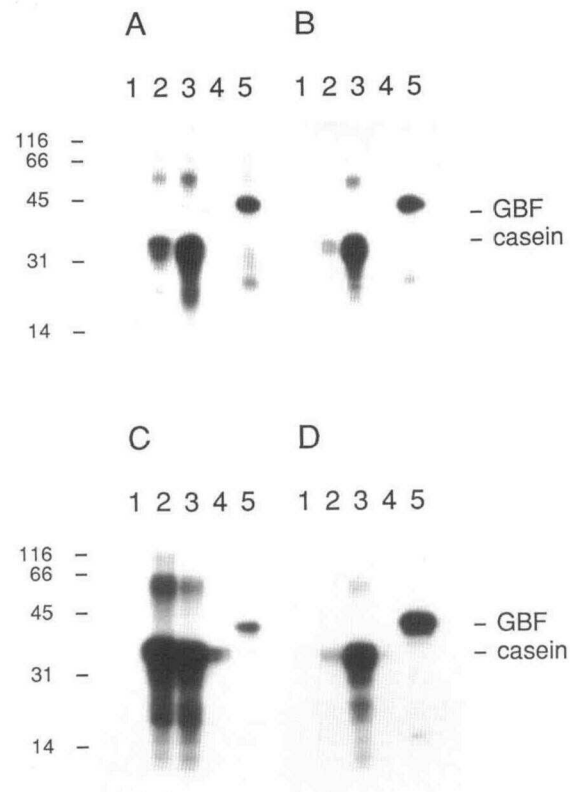

Figure 5. Substrate Specificity of Recombinant and Plant CKII Preparations.

(A) mCKII isolated from broccoli.
(B) oCKII isolated from broccoli.
(C) Recombinant CKA1 alone.
(D) Recombinant CKA1 reconstituted with CKB1.
Radiolabeled phosphorylated substrates were separated by SDS–PAGE and autoradiographed. Lanes 1, no substrate added; lanes 2, 0.25 mg/mL dephosphorylated casein; lanes 3, 0.25 mg/mL dephosphorylated casein and 0.2 mg/mL polylysine; lanes 4, 0.25 mg/mL histone IIIS; lanes 5, 0.025 mg/mL GBF1. The positions of the molecular mass markers are shown at left in kilodaltons.

DISCUSSION

Although much attention has been devoted to animal and yeast CKIIs as potential regulators of nuclear functions, plant CKII has been less extensively studied, mostly due to the paucity of identified substrates and difficulties of purification. The identification of the regulatory phosphorylation of plant transcription factor GBF1 by CKII (Klimczak et al., 1992) provided a case with significant potential for studying the role of CKII in the mechanisms of regulation of gene expression in plants. The elucidation of such mechanisms requires better understanding of the regulatory properties of plant CKII. A prerequisite for detailed analysis of plant CKII is the availability of large amounts of the enzyme for functional characterization and preparative production of phosphorylated products. This is particularly important because there are apparently two forms of plant CKII that may have different functions. Both forms have unique properties and cannot therefore be considered exact equivalents of the animal and yeast enzymes.

Using expression in bacteria of cloned homologs of the catalytic and regulatory subunits of CKII isolated from Arabidopsis, we obtained large amounts of both subunits. CKA1, a homolog of the animal/yeast catalytic subunit, showed low levels of protein kinase activity directed toward casein, as described previously (Boldyreff et al., 1993; Mizoguchi et al., 1993). The reconstitution of CKA1 with one of the recently isolated homologs of the regulatory β subunit, CKB1 (Collinge and Walker, 1994), resulted in pronounced stimulation of the catalytic activity

not only with casein, but also with GBF1 as substrates. This demonstrates that plant CKII activity can be significantly modulated by interactions between the catalytic and regulatory subunits and that CKB1 is not merely a structural homolog, but is also a functional homolog of a CKII β subunit.

The interaction between Arabidopsis CKA1 and CKB1 is of particular interest in view of the presence of monomeric CKII-like activities in plants. Such forms were described as early as 1982 (Yan and Tao, 1982), and it was later demonstrated that the monomeric activity coexisted in maize with an oligomer of unclear composition (Dobrowolska et al., 1992). Similar distribution was observed in the bryophyte liverwort (Kanekatsu and Ohtsuki, 1993) and in broccoli (Klimczak and Cashmore, 1994). In broccoli, mCKII was found in the cytosolic fraction (Klimczak and Cashmore, 1994), whereas oCKII was detected predominantly in the nucleus (Klimczak et al., 1992; Klimczak and Cashmore, 1994).

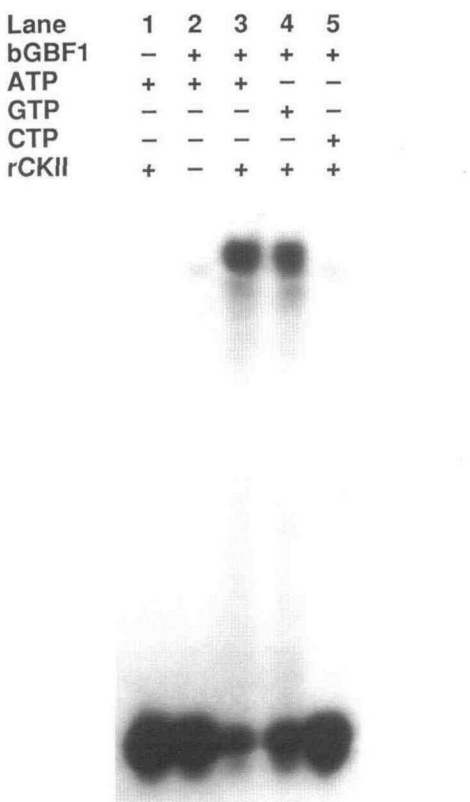

Figure 6. Effect of Phosphorylation by Reconstituted $CKA1_2CKB1_2$ on DNA Binding Activity of Transcription Factor GBF1.

After preincubation with different components of the phosphorylation reaction, a mobility shift assay was performed with a specific G-box oligonucleotide probe. Lane 1, kinase and ATP; lane 2, ATP and GBF1; lane 3, kinase, ATP, and GBF1; lane 4, kinase, GTP, and GBF1; lane 5, kinase, CTP, and GBF1. bGBF1 and rCKII indicate bacterially expressed GBF1 and reconstituted CKII, respectively; (+) and (–) indicate presence or absence, respectively, of a reaction component.

The properties of mCKII are clearly distinct from those of recombinant CKA1. Unlike mCKII, CKA1 is not stimulated by polylysine, and it shows much higher hydrophobic character in hydrophobic interaction chromatography. Accordingly, the mCKII activity cannot result only from the unmodified product of the *CKA1* gene. mCKII could result from some modifications of the CKA1 protein (sufficiently extensive to lower its hydrophobicity), or it could be the product of yet another, possibly related gene. Such a gene would likely be different from the other CKII α homolog isolated from Arabidopsis, *CKA2*, which is 94% identical to *CKA1* (Mizoguchi et al., 1993) and therefore would not be likely to account for the distinctiveness of mCKII. It is necessary to obtain the partial amino acid sequence of the mCKII protein to resolve this question conclusively. If mCKII proves to be encoded by *CKA1*, the recombinant protein could be used to assay for those modifications that are responsible for the changes in biochemical properties. Either case will mean yet a further novelty of plant CKII.

mCKII appears to represent an alternative pathway of formation of CKII-like activity. Although it is a monomer, its properties resemble those of oCKII: it is stimulated by polylysine and phosphorylates GBF1 much more efficiently than does CKA1. Their distinct structures suggest that, in spite of their similarities, they could be subject to distinct regulatory controls. Their different subcellular distributions could also reflect functional specialization of these two forms.

Because the properties of the $CKA1_2CKB1_2$ complex are so similar to those of oCKII isolated from plant material, it appears likely that the oCKII activity results from the association of these two subunits. However, our understanding of plant oCKII is still rather fragmentary. So far, only Roux et al. have reported successful purifications to near homogeneity of two oligomeric CKII forms from pea plumules: a nuclear form (Li and Roux, 1992) and a cytosolic form (Zhang et al., 1993), which are quite distinct from each other. These forms differ in their associated noncatalytic components, but their identity and relationship (both to each other and to animal β subunits) were not defined. Because no monomeric forms were detected in those studies, it is possible that the composition and distribution of CKII forms are subject to some developmental control. In those cases in which a mixture of oligomeric and monomeric forms was observed in plant extracts, the monomeric form appeared to be easier to purify. For instance, Dobrowolska et al. (1992) purified a 39-kD monomer of CKII from maize (CKIIB), whereas the oligomeric form observed in the same preparation (CKIIA) was not purified. Our studies of broccoli CKII indicated that the amount of mCKII did not decrease substantially during purification and that a single protein of 40 kD could be purified (L.J. Klimczak, unpublished data). On the other hand, the amount of oCKII tended to diminish very rapidly if purification of a partially enriched preparation was continued toward homogeneity. Some of these problems may result from a significantly higher hydrophobicity of oCKII, but they may also reflect additional components affecting the activity. For instance, partially pure preparations of broccoli oCKII contain many efficient endogenous substrates (Klimczak and

Cashmore, 1994). These substrates could form stable complexes with the catalytic subunits alone or with the complexes that they form with β subunits, as was described for several animal substrates of CKII (Miyata and Yahara, 1992; Bojanowski et al., 1993; Cardenas et al., 1993; Ludemann et al., 1993). Using the expressed CKA1 and CKB1, it will be possible now to generate antibodies specific for each subunit and to address in greater detail the nature of endogenous oCKII forms by studying their interaction in vivo and during purification of plant extracts.

The question about the composition of endogenous plant CKII forms also encompasses the roles of the dual subunits identified in Arabidopsis: CKA1 and CKA2 as well as CKB1 and CKB2 (Mizoguchi et al., 1993; Collinge and Walker, 1994). Double α subunits (α and α′) were identified in many animal and yeast systems and were shown to be encoded by two separate, but highly homologous genes (compiled, for example, in Padmanabha et al., 1990). The α and α′ subunits are believed to be functionally equivalent: in yeast, disruptions of individual subunits show no detectable phenotype, but disruption of both subunits is lethal (Padmanabha et al., 1990). In addition, no significant biochemical differences were detected between the human α and α′ subunits expressed in *E. coli*, in particular with regard to their abilities to interact with the β subunit (Bodenbach et al., 1994). Human α and α′ differ from each other much more than Arabidopsis CKA1 and CKA2: α′ is 40 amino acids shorter than α. Considering the high level of homology between Arabidopsis CKA1 and CKA2 (Mizoguchi et al., 1993), it does not seem likely that plant α subunits would be clearly distinguishable from each other in vitro. Indeed, maize α subunit, which is more closely related to Arabidopsis CKA2 than to CKA1 (Dobrowolska et al., 1991) and which was expressed in *E. coli*, showed properties very similar (Boldyreff et al., 1993) to those described here for Arabidopsis CKA1. The existence of double β subunits is less common. In addition to Arabidopsis, they have been found only in yeast (Padmanabha and Glover, 1987; Bidwai et al., 1994; Collinge and Walker, 1994). However, in contrast to the yeast β subunits, which are quite different from each other (Bidwai et al., 1994), Arabidopsis CKB1 and CKB2 are very closely related. They show 80% overall identity and 93% identity in the area of the protein that is common with other β subunits (Collinge and Walker, 1994). Such a high level of identity again suggests possible functional equivalence. In spite of such high identity within catalytic and regulatory subunits of CKII and the possibility to compensate for a loss of a single subunit, it is still possible that individual subunits may perform slightly different functions when a double set of active subunits is present in vivo. The levels of expression of members of both CKA1/2 and CKB1/2 pairs were shown to be different in various plant tissues (Mizoguchi et al., 1993; Collinge and Walker, 1994), and it remains to be evaluated how different the corresponding levels of proteins are. Such an evaluation will help to understand the contribution of individual CKA1/2 and CKB1/2 subunits to the formation of oCKII in vivo.

The findings described in this study acquire particular biological relevance with the demonstration that the reconstituted $CKA1_2CKB1_2$ enzyme can exert the same stimulatory effect on the DNA binding activity of GBF1 as nuclear oCKII from broccoli. The availability of large amounts of these components should be useful in helping to address both the biological aspects of this regulatory interaction in vivo (mapping of phosphorylation sites and specific detection of phosphorylated forms) and the mechanistic aspects in the reconstituted system in vitro (structure–function relationships and isolation of dephosphorylation activities).

METHODS

Chemicals

Isopropyl β-D-thiogalactopyranoside (IPTG) was purchased from Gold Biotechnology, St. Louis, MO; γ-^{32}P-ATP (specific activity 3000 Ci/mmol) was obtained from Du Pont-New England Nuclear; phosphocellulose P11 came from Whatman; Sephacryl S-200, phenyl-Sepharose, and DEAE-Sephacel came from Pharmacia Biotech Inc. (Piscataway, NJ); hydroxylapatite (HA Ultrogel) came from IBF Biotechnics (Savage, MD); partially dephosphorylated casein, histone IIIS, and molecular mass markers came from Sigma Chemical Co.; other chemicals were analytical grade.

Gene Clones

CKA1, the Arabidopsis homolog of the CKII catalytic α subunit, was reisolated from a cDNA library in λZAP (Stratagene) from RNA of dark-adapted *Arabidopsis thaliana* ecotype Columbia (kindly provided by J. Ecker, University of Pennsylvania, Philadelphia) using sequence information published by Mizoguchi et al. (1993). It corresponded to the clone ATCKA1 described therein. The reading frame was inserted fusionless into the T7 expression vector pET11d and transformed in the BL21(DE3)(pLysS) strain of *Escherichia coli* (Studier et al., 1990).

CKB1, the regulatory β subunit of CKII, was isolated as recently described (Collinge and Walker, 1994), and the reading frame was inserted fusionless into the expression vector pT7-7 and transformed into strain BL21(DE3) of *E. coli* (Studier et al., 1990).

Growth, Induction, and Lysis of Bacteria

Expression strains were grown in Luria-Bertani medium at 37°C to an OD of 0.6 to 0.8, IPTG was added to the culture to the final concentration of 0.4 mM, and incubation was continued for 3 hr. Cells were collected by centrifugation, washed in 0.9% (w/v) NaCl, and lysed in a 1/50th culture volume of TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) (the CKB1-expressing strain was incubated for 10 min on ice in the presence of 20 μg/mL lysozyme; for the CKA1 strain, the addition of exogenous lysozyme was not necessary). After lysis by sonication, the lysates were centrifuged for 30 min at 100,000*g*, and the supernatant was assayed for protein kinase activity. This lysate is referred to as the low-salt extract. The pellet of the 100,000*g* centrifugation was reextracted with 1 M KCl in TE buffer and was centrifuged for 15 min at 20,000*g*–this step yielded the high-salt extract and the inclusion body pellet.

Protein Kinase Assays

Protein kinase assays were performed as described by Klimczak and Cashmore (1993) using 0.5 mg/mL casein or 0.025 mg/mL recombinant G-box binding factor 1 (GBF1) as a substrate. One unit of catalytic activity is defined as the amount of enzyme incorporating 1 nmol of phosphate into the corresponding substrate per 1-min incubation time and is referred to as 1 nmol/min. The stimulatory activity was measured by preincubation of small amounts of catalyic activity (diluted to near-background levels) with the assayed fractions for 5 min on ice (both components could be mixed in their respective buffers); this was followed by a protein kinase assay with casein or GBF1. These assays determined the catalytic activity directly and the stimulatory activity indirectly by enhancement of the catalytic activity. The stimulatory effect was saturable with regard to the amount of both CKA1 and CKB1 extracts added. When equal volumes of extracts equivalent to equal culture volumes of CKA1 and CKB1 were mixed, the assay conditions were two to three times below saturation with CKB1. Accordingly, CKA1 was provided in excess, and the assay represented a measurement proportional to the amount of CKB1. Thus, the values in Table 1 are intended as measures of the yields of both subunits, and they reflect the activities of equal culture volumes of CKA1 and CKB1 but not the optimal yield of reconstituted activity, which could be obtained by adding a three- to fivefold excess of CKB1.

Expression and Purification of Soluble CKA1 and CKB1

The presence of casein kinase II (CKII) subunits was determined in the CKA1 and CKB1 expression strains by two activity assays. For CKA1, the standard protein kinase assay was performed as previously described with casein or GBF1 as substrates. For CKB1, stimulation of catalytic activity of CKA1 was measured by preincubation of small amounts of CKA1-containing extracts (diluted to near-background levels) with the assayed CKB1 fractions for 5 min on ice. This was followed by protein kinase assay with casein or GBF1. Native CKA1 and CKB1 were purified from low-salt extracts by a procedure based on that of Grankowski et al. (1991). Sequential chromatography on phosphocellulose, hydroxylapatite, and phenyl-Sepharose was performed as described previously (Klimczak and Cashmore, 1993). The levels of expression were low, and no substantial accumulation of IPTG-induced polypeptides was observed in crude lysates in SDS-polyacrylamide gels stained with Coomassie blue R 250, but proteins of 41 kD (CKA1) and 37 kD (CKB1) were enriched when purified. However, the final preparations were not yet completely homogeneous in spite of the three chromatographic steps performed.

Solubilization and Renaturation of Insoluble CKII Subunits

The protocols for solubilization and renaturation of proteins were based on those published by Lin and Traugh (1993). Inclusion bodies were solubilized for 2 hr at room temperature in 1/66th culture volume of 8 M urea, 0.1 M DTT. Renaturation was performed by diluting the solubilized protein 66-fold in a renaturation buffer composed of 100 mM Tris-HCl, pH 8.0, 0.4 M arginine, 2 mM EDTA, 4.5 mM DTT, and 1 mM oxidized glutathione and by incubating overnight at 4°C.

Purification of Renatured CKII Subunits

Renatured CKA1, CKB1, and the CKA1–CKB1 mixture were dialyzed in two changes of 10 volumes of dialysis buffer containing 50 mM Tris-HCl, pH 7.0, 15% glycerol, and 5 mM 2-mercaptoethanol and loaded on a phosphocellulose column (1/66th culture volume). The column was eluted with a linear gradient of 8 column volumes of 0 to 1 M KCl (CKA1 and CKA1–CKB1) or 0 to 0.4 M KCl (CKB1) in dialysis buffer. The proteins eluted as single peaks: CKA1 at 700 mM KCl, CKB1 at 200 mM KCl, and CKA1–CKB1 at 400 mM KCl.

Higher yields of activity were obtained when CKA1 and CKB1 were mixed and reconstituted together than when they were reconstituted and purified individually. In particular, the yield of individually purified CKB1 was quite low, and it was sensitive to proteolysis.

The described procedure of renaturation and purification of CKA1 and CKB1 had to be followed exactly to obtain high specific activities of both subunits. Preparations of low specific activities that did not form the tetrameric complex were obtained, for instance, when the concentration of the diluted, renatured solutions was attempted by precipitation or ultrafiltration.

Reconstitution of the CKA1–CKB1 Complex

For purified CKA1 and CKB1, the maximal yield of activity was obtained when they were mixed in a 1:1 molar ratio. Although the subunits appeared to be present in equimolar amounts in the crude renatured fractions (Figure 1, lanes 2 and 4), the yield of activity was suboptimal when they were renatured together after being mixed in the 1:1 ratio on the basis of the observed protein amounts, and the purified complex showed a two- to threefold molar excess of CKA1. The optimal yield of activity was obtained when the CKB1 extract was added to CKA1 in a two to three volume excess. The active complex purified after such reconstitution showed CKA1 and CKB1 in equimolar amounts (Figure 1, lane 6). This suggests that CKB1 renatures less efficiently than CKA1, which results in a shortage of active CKB1 when both subunits are provided in equimolar amounts as denatured proteins.

Purification of Recombinant GBF1

Recombinant Arabidopsis GBF1 was expressed in *E. coli* and purified by native chromatography on heparin–Sepharose as described by Klimczak et al. (1992).

Purification of Monomeric and Oligomeric CKII from Broccoli

Broccoli CKII was purified from total extracts as described recently (Klimczak and Cashmore, 1994) by chromatography on phosphocellulose and hydroxylapatite. Monomeric CKII and oligomeric CKII were separated as described by Klimczak and Cashmore (1994) by gel filtration or by hydrophobic interaction chromatography on phenyl–Sepharose.

Determination of Native Molecular Parameters

Phosphocellulose-purified renatured CKA1 and the renatured CKA1-CKB1 mixture were concentrated 10-fold using Amicon microconcentrators (Beverly, MA). Gel filtration through Sephacryl S-200 was performed in a buffer containing 0.1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 50 mM Tris-HCl, pH 7.0, 400 mM KCl, 15% glycerol, and 5 mM 2-mercaptoethanol as described by Klimczak and Cashmore (1993). Glycerol gradient sedimentation was performed at 55,000 rpm for 24 hr in a Beckman SW60 rotor (Palo Alto, CA) in a linear gradient of 10 to 30% glycerol in 400 mM KCl,

50 mM Tris-HCl, pH 7.0, 5 mM 2-mercaptoethanol. The calculations of native molecular masses and frictional ratios were performed as described by Siegel and Monty (1966). Partial specific volumes were calculated from amino acid sequences of CKA1 and CKB1 as described by Klimczak and Cashmore (1993).

Other Methods

The electrophoretic mobility shift assay was performed as previously described (Klimczak et al., 1992), as was protein electrophoresis (Klimczak and Cashmore, 1993). For analysis of phosphorylated products, proteins were precipitated by 20% trichloroacetic acid. Protein gels were stained for 15 min with Coomassie blue (0.005% dye in 10% acetic acid, 10% isopropanol) and destained with two changes: overnight and for 4 hr in 10% acetic acid, 5% methanol.

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