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DNA Binding Activity of the Arabidopsis G-Box Binding Factor GBF1 Is Stimulated by Phosphorylation by Casein Kinase II from Broccoli

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To study the phosphorylation of one of the G-box binding factors from Arabidopsis (GBF1), we have obtained large amounts of this protein by expression in *Escherichia coli*. Bacterial GBF1 was shown to be phosphorylated very efficiently by nuclear extracts from broccoli. The phosphorylating activity was partially purified by chromatography on heparin-Sepharose and DEAE-cellulose and was characterized. It showed the essential features of casein kinase II activity: utilization of GTP in addition to ATP as a phosphate donor, strong inhibition by heparin, preference for acidic protein substrates, salt-induced binding to phosphocellulose, and salt-dependent deaggregation. The very low K_m value for GBF1 (220 nM compared to ~10 μM for casein) was in the range observed for identified physiological substrates of casein kinase II. Phosphorylation of GBF1 resulted in stimulation of the G-box binding activity and formation of a slower migrating protein-DNA complex. The conditions of this stimulatory reaction fully corresponded to the properties of casein kinase II, in particular its dependence on the known phosphate donors. The DNA binding activity of the endogenous plant GBF was shown to be reduced by treatment with calf alkaline phosphatase; this reduction was diminished by addition of fluoride and phosphate or incubation in the presence of casein kinase II and ATP.

INTRODUCTION

Phosphorylation of proteins that constitute parts of the transcriptional machinery has emerged as an important mechanism of regulation of gene expression in animal systems. A growing number of examples demonstrate that phosphorylation can affect the DNA binding properties of transcription factors (Prywes et al., 1988; Yamamoto et al., 1988), their ability to activate transcription (Cherry et al., 1988; Tanaka and Herr, 1990), the mobility of DNA-bound protein complexes (Sorger et al., 1987), or the mobility of the factor itself (Jackson et al., 1990).

Because plant nuclear factors have been isolated only recently in a pure form, it has been difficult to obtain direct evidence concerning their phosphorylation and its role. However, some indirect data were collected by subjecting crude factor preparations to biochemical treatments that affect the state of phosphorylation; these results indicated that phosphorylation/dephosphorylation could influence some properties of plant nuclear factors (Datta and Cashmore, 1989; Harrison et al., 1991).

The expansion of our understanding of the role of factor phosphorylation in plants will require the study of better-defined systems comprising highly purified factors and protein kinases. Another useful tool in this research will be highly specific antibodies that allow the analysis of factor phosphorylation in vivo.

We have recently reported the cloning of a plant leucine zipper protein, G-box binding factor 1 (GBF1) (Schindler et al., 1992a), that binds to the G-box promoter sequence characteristic of and required for the expression of a variety of plant genes (Giuliano et al., 1988; Donald and Cashmore, 1990). In this article, we describe experiments in which we have used this protein as a substrate to identify a protein kinase activity that is able to phosphorylate GBF1 very efficiently and to high stoichiometry. We show that this protein kinase has the properties of casein kinase II. Further, we demonstrate that the phosphorylation of GBF1 by casein kinase II stimulates its binding to the specific DNA site.

RESULTS

Purification of Recombinant GBF1

We used the cloned cDNA encoding the nuclear factor GBF1 from Arabidopsis (Schindler et al., 1992a) to obtain large amounts of the pure protein. For this purpose, the cDNA was inserted as a protein fusion into a derivative of an *Escherichia coli* expression vector, pDS56, which contains six histidine

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residues following the initiation codon. We refer to this fusion protein as "bGBF1." Fusion proteins resulting from in vivo expression of such constructs can be rapidly purified to >99% homogeneity in a single-step procedure involving immobilized metal affinity chromatography. This approach has been successfully employed for the study of several low-abundance nuclear factors (e.g., Abate et al., 1990).

We studied the expression of bGBF1 in *E. coli* and found that following induction with isopropylthiogalactoside (IPTG), this fusion protein comprised between 5 and 10% of total cellular protein, as shown in Figure 1. The molecular mass of the fusion protein calculated from the amino acid sequence was 35,799 D, and it migrated in SDS-PAGE gels corresponding to a slightly higher apparent mass of 39 kD. bGBF1 did not form insoluble inclusion bodies, but its N-terminal cluster of histidines appeared not to be exposed to the surface of the protein because the native protein was not retained on metal affinity columns. To be able to take advantage of the rapid purification protocol by immobilized metal chromatography, the cells were lysed in guanidine hydrochloride; after this treatment, the fusion protein was efficiently retained on the column in a fully unfolded state. The subsequent low pH eluate was >99% pure bGBF1. When guanidine hydrochloride was removed by dialysis, the protein tended to precipitate unless diluted. Although low amounts of bGBF1 could be recovered from such precipitates by extraction with 0.5 M KCl, the majority of the precipitate was insoluble. However, all bGBF1 was recovered in soluble form when protein concentration during dialysis was lowered to ~0.1 mg/mL. The qualitative DNA binding properties of this fusion protein were not distinct from those of plant GBF as judged by DNA competition assays and methylation interference studies (Schindler et al., 1992b).

To provide a control for possible artifacts that could result from the unfolding/refolding treatment, we isolated a native bGBF1 using another approach that did not include guanidine hydrochloride and metal affinity chromatography but used heparin-Sepharose chromatography of *E. coli* extracts instead. This native procedure resulted in 60 to 80% overall purity of bGBF1 in one step, whereas peak fractions showed an even greater purity, of ~95% (Figure 1).

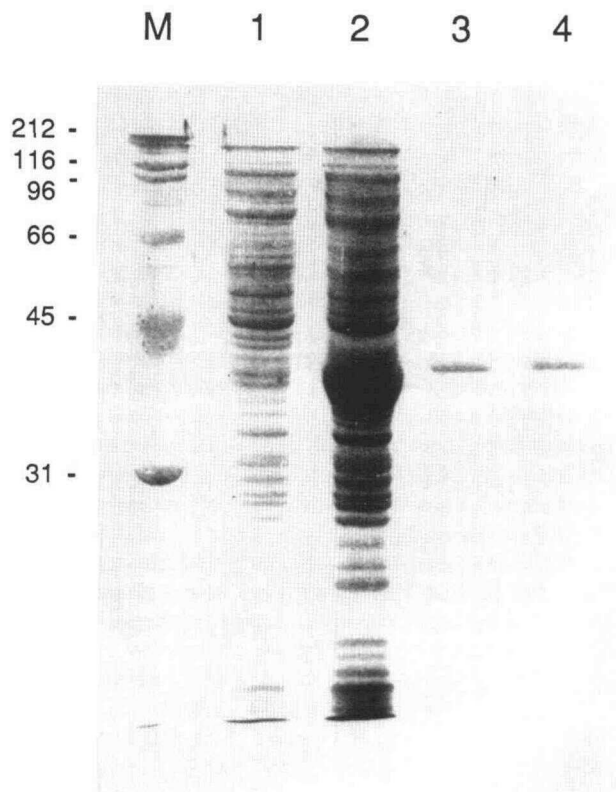

Figure 1. Expression and Purification of bGBF1.

bGBF1 fractions were subjected to SDS-PAGE and staining with Coomassie blue. Lane 1, total protein of noninduced *E. coli* cells; lane 2, total protein of *E. coli* cells after 2-hr induction with IPTG; lane 3, 0.5 μg of native bGBF1 purified by heparin-Sepharose chromatography; lane 4, 0.5 μg of renatured bGBF1 purified by nickel affinity chromatography. M, molecular mass markers, given in kD.

Phosphorylation of GBF1 in Nuclear Extracts

The purified bGBF1 was used as substrate for phosphorylation in the presence of γ-^{32}P-ATP, Mg^{2+}, and crude nuclear extracts from broccoli. Broccoli was selected for these studies because it is closely related to Arabidopsis, from which *GBF1* was cloned, and offers the advantage of providing large amounts of material for biochemical studies. To aid us in the identification of possible phosphorylating activities, we also used a partially purified calcium-dependent protein kinase from maize (Klimczak and Hind, 1990a, 1990b), an enzyme localized also in the nucleus (Li et al., 1991; L.J. Klimczak, unpublished data).

Whereas no phosphorylation was observed with the purified calcium-dependent protein kinase (data not shown), bGBF1 became significantly labeled when incubated with the crude nuclear extract from broccoli, as shown in Figure 2B (lanes 2 and 3). Although bGBF1 was not provided in great excess over the endogenous proteins of the nuclear extract (Figure 2A, arrow), it was the major labeled protein, and it competed successfully with endogenous substrates for phosphorylation and caused their decreased phosphorylation (Figure 2B, compare lanes 1 and 2 or 1 and 3). Accordingly, bGBF1 appeared to have a relatively high affinity for an endogenous nuclear protein kinase. No difference was observed in the level of phosphate incorporation between bGBF1 renatured after purification by metal affinity chromatography in the presence of guanidine hydrochloride and the native bGBF1 purified by chromatography on heparin-Sepharose, which suggests that the phosphorylation site(s) did not arise artificially by the unfolding treatment with guanidine hydrochloride (e.g., by exposing otherwise buried residues) but are present in the natively folded bGBF1.

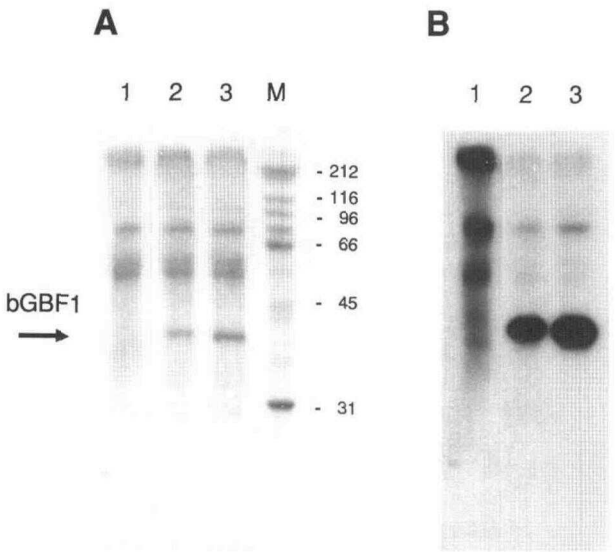

Figure 2. Phosphorylation of *E. coli*-Expressed bGBF1.

Phosphorylated proteins were separated by SDS-PAGE. Lanes 1, nuclear extract alone; lanes 2, nuclear extract with 0.25 μg of native bGBF1 purified by heparin-Sepharose; lanes 3, nuclear extract with 0.5 μg of bGBF1 purified by Ni^{2+}–iminodiacetic acid–agarose chromatography. M, molecular mass markers, given in kD. The arrow indicates the position of bGBF1 migration.
(A) Coomassie blue-stained gel.
(B) Autoradiogram.

Partial Purification of GBF1 Kinase

To characterize and possibly identify the protein kinase activity that phosphorylates GBF1, we developed a purification procedure for the kinase using standard chromatographic techniques. Because it was easy to detect bGBF1 phosphorylation already in crude nuclear extracts, the adsorption properties of GBF1 kinase on various chromatographic materials were determined in microscale batch adsorption assays. It was observed that the bGBF1 kinase activity was adsorbed on phosphocellulose in a salt-dependent manner, which is a characteristic property of casein kinase II (reviewed in Tuazon and Traugh, 1991). Similarly, the bGBF1 kinase was strongly retained on heparin-Sepharose even in the presence of moderate salt concentrations; accordingly, heparin-Sepharose was selected as the first chromatographic step. The redissolved ammonium sulfate precipitate was directly loaded onto the first chromatographic column in a batch procedure without prior dialysis. The activity was eluted as a single peak between 400 and 750 mM KCl, as shown in Figures 3A and 3B. Following dialysis of the pooled active fractions, the enzyme was loaded on a DEAE-cellulose column, from which it was eluted again as a single peak centered around 300 mM KCl (Figures 3C and 3D). Little phosphorylation of bGBF1 was observed in the DEAE-cellulose flow-through fractions, which contained the bulk of casein kinase I activity. The purification procedure of GBF1 kinase is summarized in Table 1, starting with crude nuclear extract. Because isolation of nuclei represents in itself approximately 20- to 50-fold enrichment, total purification of the enzyme was approximately 1000-fold relative to the total broccoli extract. The pooled active DEAE-cellulose fractions were used in all subsequent experiments.

GBF1 Kinase Is a Casein Kinase II-Type Enzyme

Salt dependence of the binding of GBF1 kinase to phosphocellulose indicated that the phosphorylating activity may be casein kinase II. Therefore, we investigated the enzymatic properties characteristic of casein kinase II: utilization of GTP as a phosphate donor, inhibition by heparin, phosphorylation of acidic proteins (casein, phosvitin) but not basic proteins (histones), and stimulation by polycations.

As shown in Table 2, the kinase utilized GTP for bGBF1 phosphorylation at the rate of ~40% of that of ATP. Consistent with this observation, the addition of 40 μM unlabeled GTP to the assay with 2 μM radioactive ATP reduced the incorporation of the latter into bGBF1 to 20%. No such competition was observed when unlabeled CTP or UTP was added (data not shown). In agreement with the data of Baydoun et al. (1981), 40 μM dATP also competed with the incorporation of ATP and resulted in its inhibition to 5% (data not shown).

The enzyme was also strongly inhibited by low concentrations of heparin. The kinase efficiently phosphorylated casein and phosvitin, whereas no phosphorylation of histone IIIS was observed (the addition of histone to the assay mixture only stimulated the phosphorylation of endogenous substrates, not shown). Whereas no pronounced stimulation of the enzyme with polycations was observed with bGBF1 as substrate, approximately sevenfold stimulation of activity was detected when the kinase was assayed with casein (Table 2).

Table 1. Summary of the Purification Scheme of GBF1 Kinase

Step	Volume (mL)	Total Protein (mg)	Total Activity (units)	Specific Activity (units/mL)	Yield, %
Ammonium sulfate	100	450	89,000	0.2	100
Heparin-Sepharose	20	9.2	42,000	4.6	48
DEAE-cellulose	2	2.0	13,200	6.6	15

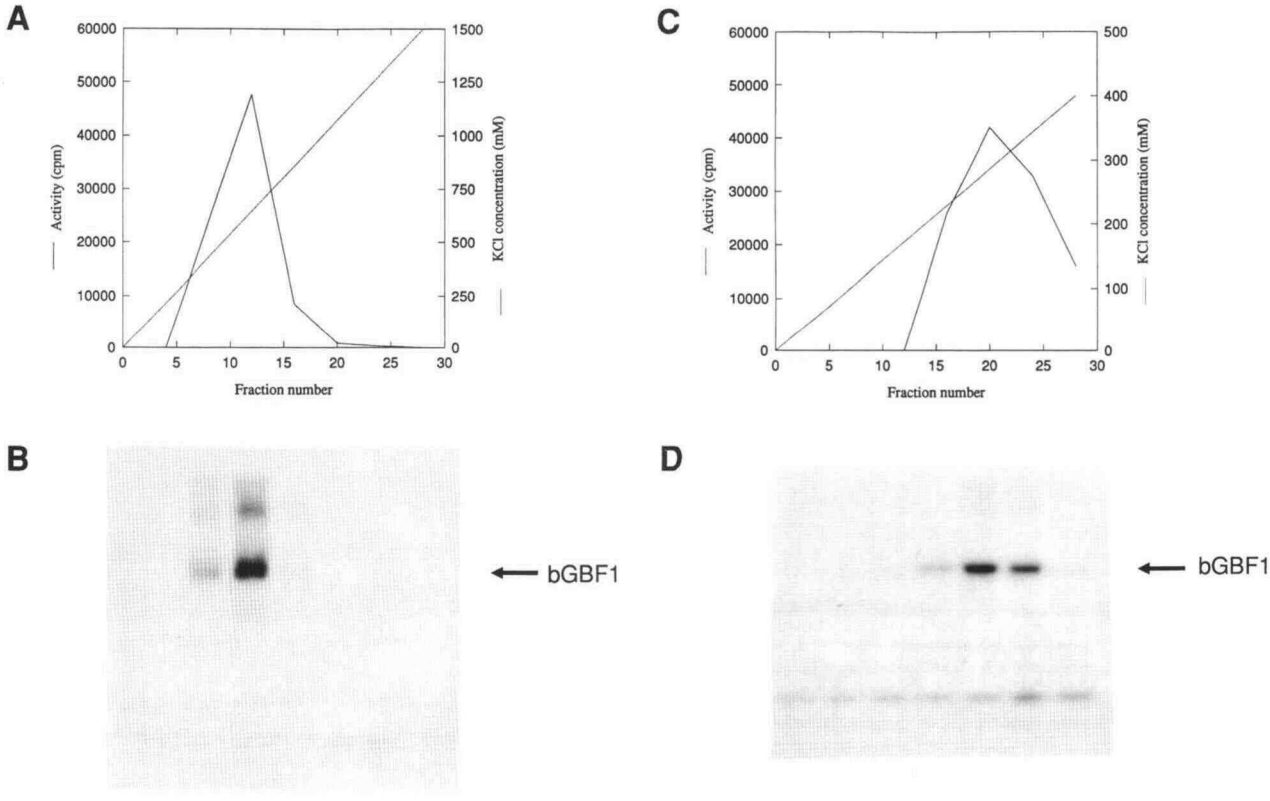

Figure 3. Elution Pattern of bGBF1 Kinase from Heparin-Sepharose and DEAE-Cellulose.

(A) Heparin-Sepharose chromatography, filter paper assay.
(B) Heparin-Sepharose chromatography, autoradiogram of SDS-PAGE polyacrylamide gel.
(C) DEAE-cellulose chromatography, filter paper assay.
(D) DEAE-cellulose chromatography, autoradiogram of SDS-PAGE polyacrylamide gel.
(A) and **(C)** show elution profiles as determined by a filter paper assay of fractions eluted from the columns and assayed for phosphorylation of bGBF1 in the presence of γ-^{32}P-ATP and $MgCl_2$; **(B)** and **(D)** show the reaction products separated by SDS-PAGE and autoradiographed, and their lanes correspond to pooled fractions in the graphs in **(A)** and **(C)**. The arrows indicate the position of bGBF1 migration.

Because the salt dependence of casein kinase II adsorption on phosphocellulose is caused by aggregation, we analyzed the native molecular mass of the GBF1-phosphorylating activity. When gel filtration through Sephacryl S-200 was performed at low salt concentrations (100 mM KCl), the GBF1 phosphorylating activity eluted in the void volume. However, upon gel filtration at moderate salt concentrations (400 mM KCl), it eluted in the included volume corresponding to a molecular mass of ~128 kD (data not shown).

Casein kinase II is known to be present in both the nucleus and the cytoplasm, and we also found that a similar protein kinase activity able to phosphorylate bGBF1 was present in broccoli in the cytosolic fraction (data not shown).

Further Enzymatic Properties of GBF Phosphorylation by Casein Kinase II

The extent of bGBF1 phosphorylation with the partially purified preparation was proportional to the amounts of the substrate and enzyme added and was saturable with regard to both. The kinase phosphorylated bGBF1 at a level more than 10-fold higher than casein when both substrates were provided at the same low concentration of protein (0.01 mg/mL), as shown in Table 2. The very high affinity of the enzyme for bGBF1 was reflected in a low K_m value, determined to be 220 nM (8 μg/mL). At bGBF1 concentrations of >1 μM, substrate inhibition was observed.

Table 2. Effect of Assay Conditions on bGBF1 Kinase Activity

Conditions	Activity, %
Standard assay	100
KCl added (mM)	
50	65
100	54
200	36
400	5
EGTA added, 2 mM	100
N-ethylmaleimide added (mM)	
2	79
10	33
Heparin added (1 μg/mL)	27
Polylysine added (40 μg/mL)	67
bGBF1 replaced by:	
Casein (0.01 mg/mL)	8
Casein (0.2 mg/mL)	21
Casein (0.2 mg/mL) with polylysine (40 μg/mL)	152
Phosvitin (0.2 mg/mL)	37
Histone IIIS	2
ATP replaced by GTP	40

The enzyme possessed an absolute requirement for Mg^{2+} ions for catalytic activity. It showed a broad optimum for this ion between 4 and 20 mM. Mg^{2+} could be efficiently substituted by Mn^{2+}, resulting in an optimum between 0.1 and 0.3 mM at the level of bGBF1 phosphorylation of up to 60% of that achieved with Mg^{2+}, as shown in Figure 4.

GBF1 kinase was inhibited by high concentrations of monovalent salts, but it was still very active at the physiological concentration of 100 to 200 mM KCl; it was sensitive to inhibition with *N*-ethylmaleimide only at high concentrations. EGTA had no effect on the activity (Table 2).

Saturation phosphorylation studies of bGBF1 indicated that up to 3 mol of phosphate could be incorporated per 1 mol of protein, as shown in Figure 5. Whereas incorporation of 2 mol of phosphate occurred at low kinase concentrations, the additional 1 mol required a large excess of the enzyme. Therefore, we conclude that bGBF1 contains two or three (depending on the molar saturation of individual sites) high-affinity phosphorylation sites and some low-affinity sites.

The analysis of hydrolyzed phosphorylated bGBF1 revealed that the product of phosphorylation was 90 to 95% phosphoserine and minor amounts of phosphothreonine, as shown in Figure 6.

Effect of GBF1 Phosphorylation on DNA Binding

We sought to confirm that the product of the phosphorylation reaction was in fact bGBF1. As shown in Figure 7, the radiolabeled protein product formed a complex with the unlabeled G-box–containing probe. The diffuse band of γ-^{32}P-ATP-phosphorylated bGBF1 visible in Figure 7, lane 1, disappeared upon addition of poly(dI:dC) due to binding to a population of DNA molecules of heterogeneous size and migration as a wide smear of bGBF1–DNA complexes (Figure 7, lane 2). Upon addition of increasing amounts of unlabeled G-box DNA, it competed with poly(dI:dC) for the phosphorylated bGBF1 and formed a complex that migrated as a narrow band (Figure 7, lanes 3 to 5). The position of the migration of this band was slightly above the position of the complex of bGBF1 with a radiolabeled G-box probe and corresponded to the position of such complex with casein kinase II–phosphorylated bGBF1 (see arrows in Figure 7; these positions were determined as shown in Figure 8 below). Because bGBF1 was the only protein in the *E. coli* extract that bound to the G-box in the presence of poly(dI:dC) (Schindler et al., 1992a), this experiment unequivocally demonstrated that the major phosphoprotein in the reaction mixture is in fact bGBF1, and not for instance an endogenous protein of the same molecular mass, whose phosphorylation would only be stimulated by bGBF1.

Because bGBF1 appeared to have retained its DNA binding activity, the effect of phosphorylation on G-box binding was quantitatively determined, as shown in Figure 8. After preincubation of native bGBF1 with the kinase preparation in the presence of $MgCl_2$ and ATP, formation of a slower migrating DNA–protein complex was observed together with a significant increase of intensity of the retarded band (Figure

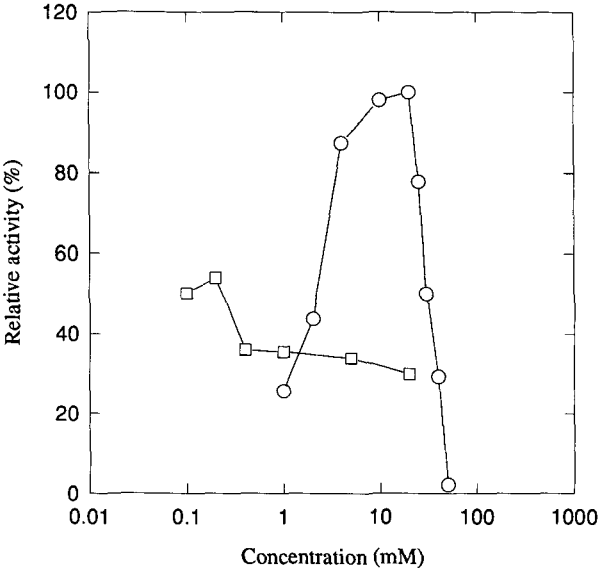

Figure 4. Effect of Mg^{2+} and Mn^{2+} Ions on GBF1 Kinase Activity.

The phosphorylation assay was performed as described in Methods, with the exception of 10 mM $MgCl_2$, which was replaced by varying concentrations of $MgCl_2$ (○) or $MnCl_2$ (□).

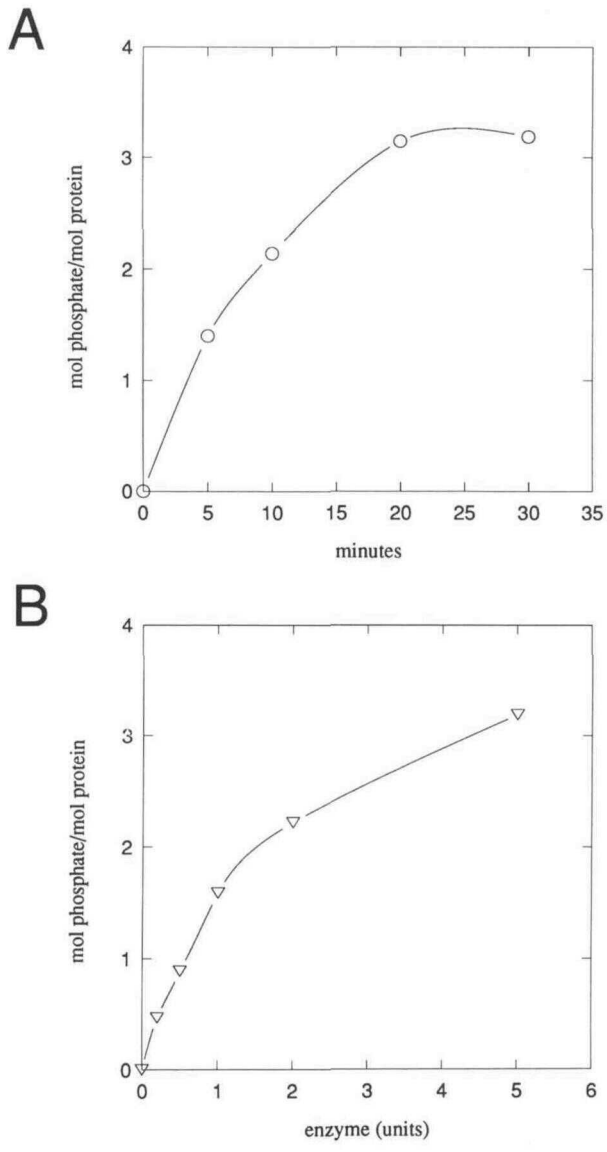

Figure 5. Phosphorylation of bGBF1 to Saturation.

(A) Time course of phosphate incorporation with an excess (20 units) of enzyme.

(B) Phosphorylation with variable amounts of enzyme for 30 min.

8). Because bGBF1 preparation showed some microheterogeneity in the form of minor faster migrating bands, these bands became more visible after the stimulatory phosphorylation. The stimulatory reaction was proportional to the amount of the kinase added (Figure 8, compare lanes 2 to 4 with lane 1) and incubation time (compare panels A and B in Figure 8) and was also saturable with respect to the amount of the kinase added (Figure 8, lanes 7 and 8). This effect was also observed when ATP was replaced with GTP or dATP but not CTP or UTP (Figure 8, lanes 9 to 13).

Endogenous Plant GBF Is a Functional Phosphoprotein

Because GBF1 was demonstrated to be a good protein kinase substrate in vitro, and phosphorylation stimulated its DNA binding activity, we investigated the effect of phosphatase treatment on the endogenous plant GBF to obtain some indirect data on its in vivo status of phosphorylation. As shown in Figure 9, incubation of desalted nuclear extract from broccoli with high dilutions (1:320) of commercial alkaline phosphatase strongly reduced the binding to the G-box oligonucleotide as measured by the abundance of the retarded DNA–protein complexes, which migrated as a spectrum of close bands (Figure 9, lanes 2 and 3). This effect was diminished by the addition of phosphatase inhibitors (10 mM NaF

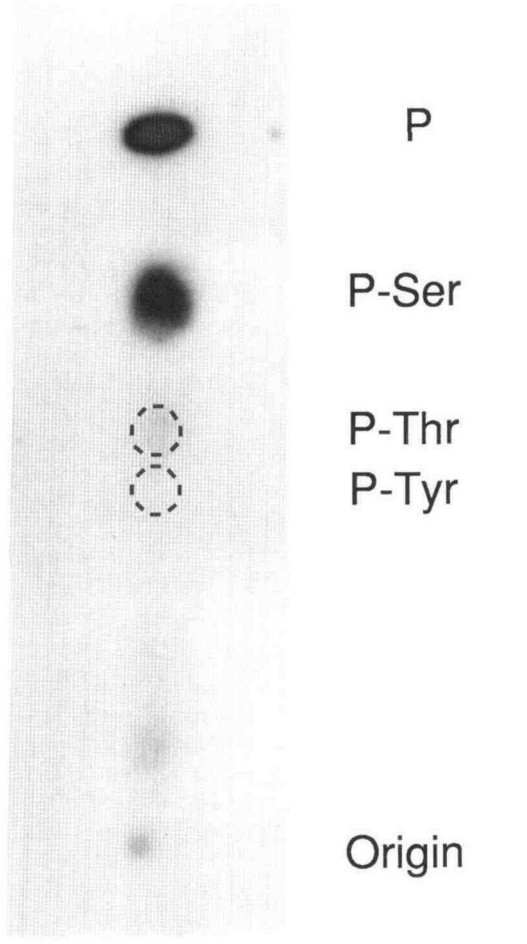

Figure 6. Identification of Phosphorylated Amino Acids in bGBF1.

Hydrolysis products of phosphorylated bGBF1 were separated by thin-layer electrophoresis at pH 3.5 and subjected to autoradiography, as described in Methods. P, inorganic phosphate; P-Ser, phosphoserine; P-Thr, phosphothreonine; P-Tyr, phosphotyrosine.

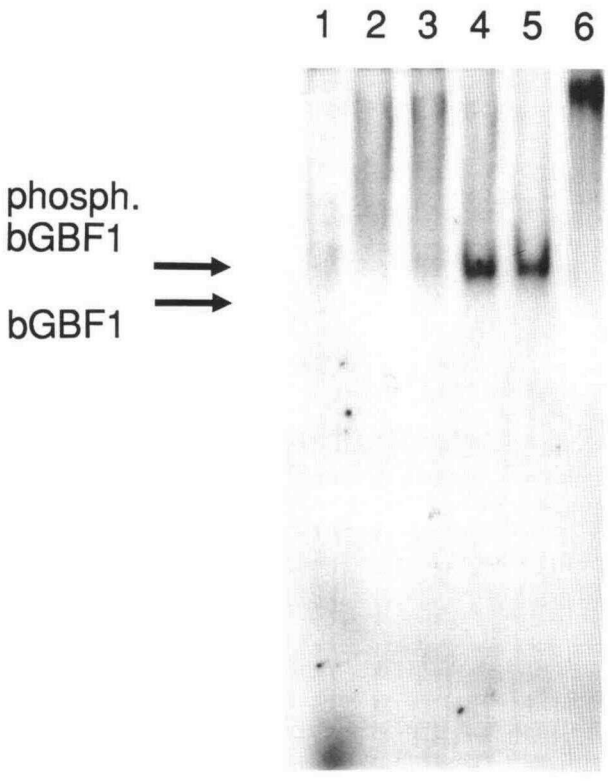

Figure 7. DNA Binding of Phosphorylated GBF1.

Mobility shift assay of phosphorylated bGBF1 was performed with a G-box containing DNA probe. bGBF1 was phosphorylated in the presence of γ-^{32}P-ATP, and 0.05 μg of the phosphorylated protein was incubated in the gel retardation buffer with varying amounts of G-box DNA: lanes 1 and 2, 0 ng; lane 3, 5 ng; lane 4, 10 ng; lane 5, 20 ng; lane 6, 0.25 μg of sonicated salmon sperm DNA. Lanes 2 to 6 contained 2 μg each of poly(dI:dC). Arrows show the positions of migration of the complexes of phosphorylated (phosph. bGBF1) and nonphosphorylated bGBF1 (bGBF1) with a radiolabeled G-box probe (*calibrated from Figure 8*).

and 0.5 mM KPO_4; Figure 9, lane 4) and was not observed at all in the nuclear extract that had not been desalted. The phosphatase effect was also reduced by incubation with the preparation of nuclear casein kinase II from broccoli and ATP (Figure 9, lane 5)—none of which alone had any effect on GBF binding. It is noteworthy that the treatment with phosphatase did not completely abolish the binding because there always remained a less pronounced retarded band (~10% of untreated control, as determined by densitometry of the autoradiogram), which migrated at the front of the group of DNA–protein complexes visible in untreated samples (see arrow in Figure 9). In addition, certain preparations of broccoli GBF showed different properties in that they did not migrate as several bands, but as just one band of a slightly higher mobility, closely corresponding to that of a phosphatase-treated complex (Figure 9, lane 7, slightly overloaded for better resolution). These preparations were not sensitive to the treatment with alkaline phosphatase (data not shown), which suggested that they contained GBF in a dephosphorylated state. These results also showed that the alkaline phosphatase used does not contain some unspecific inhibitors of GBF binding.

DISCUSSION

In this article, we have demonstrated that the nuclear factor GBF1 could be phosphorylated by a protein kinase activity present in plant nuclear extracts. The properties of this protein kinase corresponded to the major operational criteria of identification as casein kinase II: utilization of GTP as a phosphate donor, inhibition by low concentrations of heparin, and phosphorylation of acidic proteins (casein, phosvitin) but not basic proteins (histones) (for a review, see Pinna, 1990; Tuazon and Traugh, 1991). The GBF1-phosphorylating activity displayed salt-dependent deaggregation typical for casein kinase II: at low salt concentrations, it did not adsorb to phosphocellulose and eluted as a high molecular mass complex upon gel filtration. The native molecular mass of the GBF1 kinase, determined to be ~128 kD at moderate salt concentrations, falls within the range observed for casein kinase II. Phosphorylation of bGBF1 was not stimulated by polycations, but stimulation was observed when casein was used as a substrate in place of bGBF1. This characteristic stimulation by polycations of casein kinase II is not observed with all its substrates, and GBF1 appears to be one of such exceptions. We speculate that one of the basic regions of GBF1 may interact with the activation site of casein kinase II and cause direct activation of the enzyme. The presence of both acidic (phosphorylation site[s]) and basic stretches of amino acids in GBF1 could be the reason for its strong affinity toward casein kinase II, because it may be able to interact with at least two different sites of the kinase molecule.

Casein kinase II is one of few plant protein kinases known to represent close biochemical counterparts of animal kinases (the others being casein kinase I and p34^{cdc2} protein kinase). The high degree of protein sequence conservation among the cloned casein kinases II (human, Drosophila, and yeast) suggests that some fundamental function of the enzyme must have been conserved throughout evolution. In the phylogenetic tree constructed by Hanks et al. (1988), casein kinase II shows the closest relationship to the cell cycle-related class of protein kinases, which suggests its likely involvement in general regulatory functions in the cell. Although the enzyme was known to exist in plants for many years (Yan and Tao, 1982a), very little progress has been reported in the identification of its biological substrates. Yan and Tao (1982a) reported purifying from wheat germ a 36-kD protein with casein kinase II activity; they also isolated a 48-kD endogenous protein that was phosphorylated with a

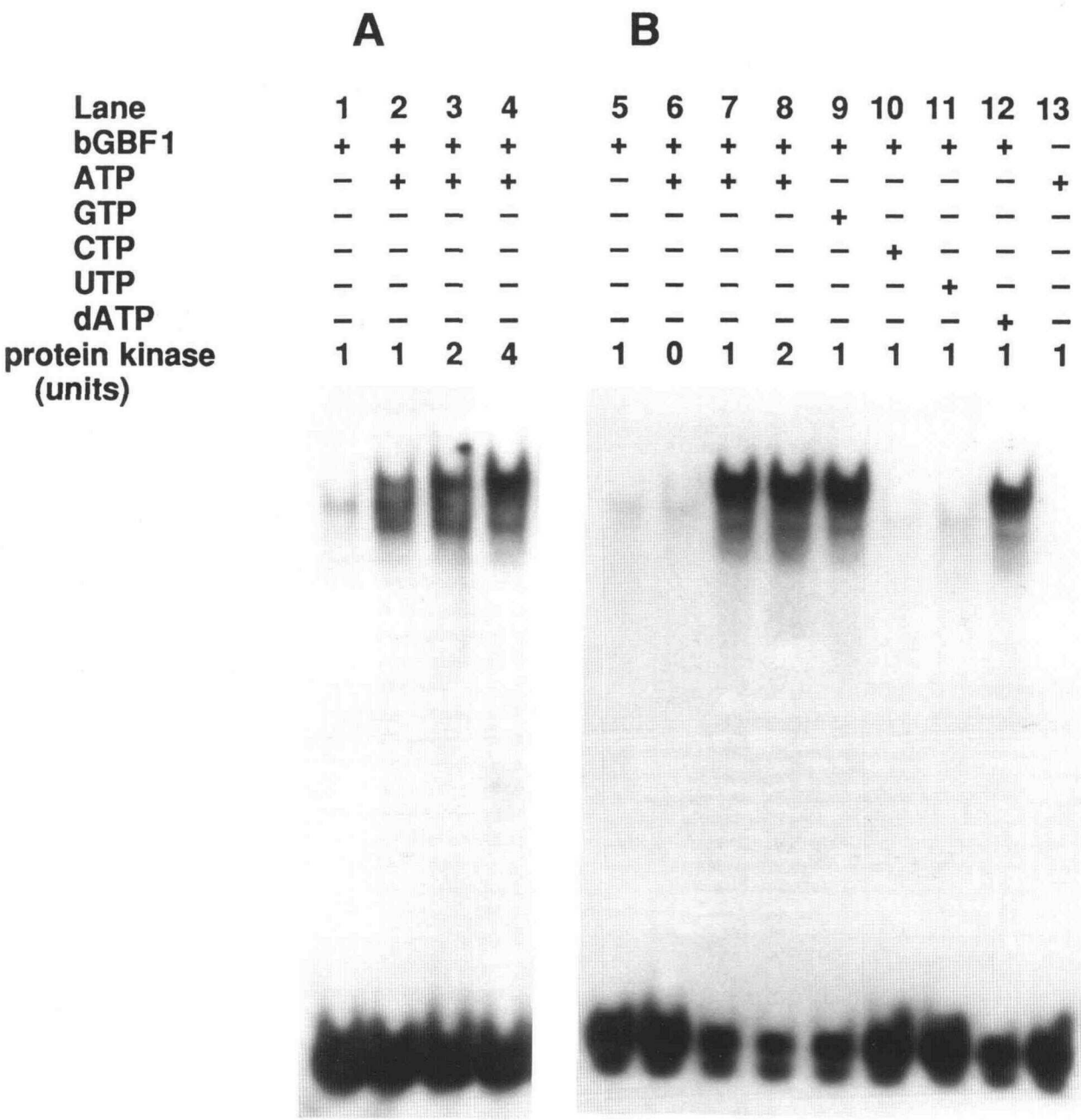

Figure 8. Effect of Phosphorylation on the Binding of Native bGBF1 to G-Box DNA.

The following components were preincubated for the times given below at room temperature in 10 μL of a buffer containing 50 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 10 mM NaF, and 50 μg/mL BSA: lane 1, 80 ng bGBF1 and 1 unit of kinase; lane 2, 80 ng bGBF1, 1 unit of kinase, and 400 μM ATP; lane 3, 80 ng bGBF1, 2 units of kinase, and 400 μM ATP; lane 4, 80 ng bGBF1, 4 units of kinase, and 400 μM ATP; lane 5, 80 ng bGBF1 and 1 unit of kinase; lane 6, 80 ng bGBF1 and 400 μM ATP; lane 7, 80 ng bGBF1, 1 unit of kinase, and 400 μM ATP; lane 8, 80 ng bGBF1, 2 units of kinase, and 400 μM ATP; lane 9, 80 ng bGBF1, 1 unit of kinase, and 400 μM GTP; lane 10, 80 ng bGBF1, 1 unit of kinase, and 400 μM CTP; lane 11, 80 ng bGBF1, 1 unit of kinase, and 400 μM UTP; lane 12, 80 ng bGBF1, 1 unit of kinase, and 400 μM dATP; lane 13, 1 unit of kinase and 400 μM ATP. After the incubation, EDTA was added to the final concentration of 10 mM, and 2 μL of the incubation mixture was added to 25 μL of gel retardation buffer containing the radiolabeled G-box probe and assayed as described in Methods.

(A) Ten-minute incubation.

(B) Thirty-minute incubation.

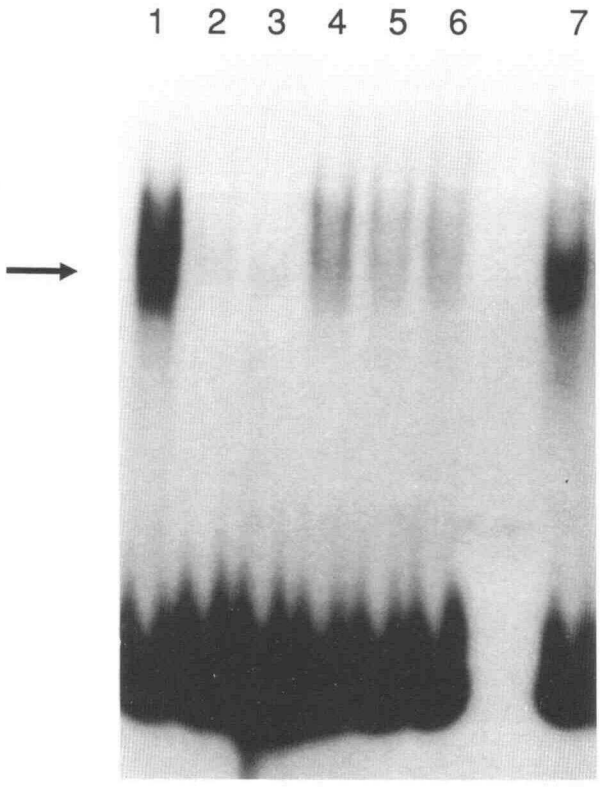

Figure 9. Phosphorylation Status of Endogenous Plant GBF.

Dephosphorylation was with calf alkaline phosphatase. Eight microliters of desalted nuclear extract from broccoli was mixed with 8 μL of 50 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$ and was incubated at room temperature in the presence of the following additions: lane 1, no additions; lane 2, 0.8 units of phosphatase; lane 3, 0.08 units of phosphatase; lane 4, 0.08 units of phosphatase, 10 mM NaF, and 0.5 mM KPO_4; lane 5, 0.08 units of phosphatase, 1 unit of kinase, and 400 μM ATP; lane 6, 0.02 units of phosphatase. Four microliters of the incubation mixture was analyzed by gel retardation with the radiolabeled G-box probe. Lane 7 shows gel retardation with 4 μL of nuclear extract of an independent preparation that showed a different pattern of complex migration and was not sensitive to alkaline phosphatase. The arrow points out the position of the faster migrating dephosphorylated protein–DNA complex.

relatively high K_m, of 100 μg/mL (Yan and Tao, 1982b), but this protein has not been subsequently identified. More recently, Grasser et al. (1989) demonstrated phosphorylation of maize high-mobility group proteins by casein kinase II, but further characterization of these substrates is necessary.

In our laboratory, studies with crude nuclear fractions indicated that the specific DNA binding activity of the nuclear factor AT-1 was inhibited as a result of phosphorylating treatment apparently mediated by casein kinase II (Datta and Cashmore, 1989). Because the gene for this factor has not yet been cloned, it is not possible to investigate directly the phosphorylation of this factor. The availability of a cloned factor and an abundant bacterially expressed protein used in the present work facilitated such studies to a great extent and made possible more precise and unequivocal conclusions.

Many of the substrates of casein kinase II identified in animal systems are proteins involved in gene expression, such as simian virus 40 large T antigen, RNA polymerases I and II, DNA polymerases I and II, DNA topoisomerases I and II, nucleolin, high-mobility group proteins, and others (Pinna, 1990; Tuazon and Traugh, 1991). This range of substrates is also in keeping with the partial nuclear localization of the kinase, a fact that was also observed for the plant enzyme (Erdmann et al., 1982, 1985). The involvement of casein kinase II in phosphorylation of transcription factors was suggested recently by the studies of the serum response factor that binds to the serum response element. These studies demonstrated that serum response factor was phosphorylated in vivo on serine residues and that phosphatase treatment in vitro abolished its DNA binding activity (Prywes et al., 1988). The DNA binding activity of recombinant serum response factor produced in *E. coli* was enhanced by incubation with nuclear extracts and nucleotide and deoxynucleotide triphosphates, and the protein kinase involved was identified as casein kinase II (Manak et al., 1990). A significant effect of this phosphorylation treatment was also reported therein with UTP as a phosphate donor, which is quite puzzling because this is not a property of casein kinase II. In our study, the observed effects of phosphorylation on DNA binding are correlated with the actual phosphate donors of casein kinase II. Although dATP has never been directly demonstrated to be used by casein kinase II as a phosphate donor (probably due to the very high cost of the radiolabeled form), the data of Baydoun et al. (1981) demonstrated that the contribution of the 2′ and 3′ ribosyl OH groups to the binding of the nucleotide to casein kinase II is marginal.

The data presented in this article are in compliance with the first of the criteria proposed by Krebs and Beavo (1979) for the functionality of regulatory phosphorylation: GBF1 phosphorylation in vitro takes place at a high rate, with high affinity for the kinase, and to high stoichiometry. The high level of exclusive bGBF1 phosphorylation in crude extracts demonstrates that it very successfully competes for access to the kinase with endogenous substrates. The low K_m value determined for GBF1 as substrate clearly reflects this high affinity and is comparable to the values determined for other substrates of casein kinase II, for which the in vivo role has been implicated by a much larger body of data. For instance, casein kinase II phosphorylates DNA topoisomerase I with a K_m of 0.3 μM (Mills et al., 1982), DNA topoisomerase II with a K_m of 0.4 μM (Ackerman et al., 1985), nucleolin with a K_m of 7 nM (Caizergues-Ferrer et al., 1987), and the high-mobility group-14 protein with a K_m of 11 μM (Caizergues-Ferrer et al., 1987).

The system described in this article fulfilled also the second criterion of Krebs and Beavo (1979) because phosphorylation of GBF1 stimulated 10- to 20-fold its binding to the

G-box sequence and resulted in lower mobility of the DNA–protein complex. Although endogenous plant GBF, as defined and measured by the retardation of a G-box oligonucleotide, appears to be composed of a family of related proteins (Schindler, et al., 1992a), and the exact contribution of the individual protein GBF1 is not known yet, the isolated nuclear GBF population had its DNA binding activity reduced approximately 10- to 20-fold by dephosphorylation with alkaline phosphatase. The DNA binding activity was not completely abolished, in agreement with the presence of DNA binding activity in the unphosphorylated bacterial protein. The in vivo status of GBF phosphorylation will have to be studied in more detail; in particular, it should be determined whether different mobilities of plant GBF preparations and their different sensitivities to alkaline phosphatase reflect actual changes in the level of phosphorylation and whether this level is regulated somehow.

Many of the aspects of functional significance of phosphorylation in this particular plant system should be viewed in the context of the still unresolved questions about the function of casein kinase II in general (Pinna, 1990; Tuazon and Traugh, 1991). In particular, the question of whether the enzyme performs any functions in regulatory processes typical of a signal transduction cascade or whether it acts constitutively and incorporates functional features into proteins whose amino acid sequence alone is not sufficient for full biological activity needs to be answered. An interesting contribution in this area is the study of Rihs et al. (1991), which suggests that casein kinase II may introduce functional determinants for nuclear localization, in addition to the nuclear targeting sequences, by phosphorylating sites close to these targeting sequences. Our current work on the determination of the phosphorylation sites in GBF1 will show whether they are in the proximity of potential nuclear targeting sequences.

In conclusion, the identification of GBF1 phosphorylation by casein kinase II and its characteristics presented in this article provide a starting point for addressing questions about the biological role of such phosphorylation in a well-defined plant system and for investigating the regulation of transcription.

METHODS

Plant Material

Broccoli (*Brassica oleracea* var *italica*) was purchased in fresh state from local wholesale distributors.

Chemicals

γ-^{32}P-ATP (specific activity 3000 Ci/mmol) was obtained from Amersham Corp.; calf alkaline phosphatase (1700 units per milliliter), poly(dI:dC), heparin-Sepharose, and DEAE-Sephacel from Pharmacia LKB Biotechnology Inc.; iminodiacetic acid-agarose, isopropyl-thiogalactoside (IPTG), histone IIIS, and dephosphorylated casein from Sigma. Other chemicals were analytical grade.

Cloning of *GBF1* in the Expression Vector pDS56

The coding region of *GBF1* (Schindler et al., 1992b) was cloned as a protein fusion into a derivative of pDS56, pDS-MCS (Bujard et al., 1987), that contained a polylinker cloning site inserted into its BamHI site. The construct was transformed into *Escherichia coli* MC1061 containing plasmid pDMI,1. The transformants were grown at 37°C in Luria broth medium in the presence of 100 μg/mL ampicillin and 25 μg/mL kanamycin.

Purification of bGBF1 by Metal Affinity Chromatography

A culture of the *E. coli* strain bearing the expression construct was incubated overnight, diluted 1:5 in Luria broth medium, and grown for 40 min at 37°C. IPTG was added to the final concentration of 1 mM, and the growth was continued for 2 hr. Bacteria were harvested by centrifugation, and the pellet was washed in 0.1 culture volume of TE buffer (10 mM Tris-HCl, pH 8.0). The washed pellet was suspended in 0.02 volume of 6 M guanidine hydrochloride in 50 mM potassium phosphate buffer, pH 8.0 (buffer A), and the suspension was stirred for 20 to 30 min on ice until it clarified. The lysate was centrifuged at 100,000*g* for 30 min, and the supernatant was loaded on an iminodiacetic acid–agarose column equilibrated with 100 mM $NiCl_2$ and extensively washed with distilled water. The column volume was 0.01 of the volume of the bacterial culture. The column was washed with 10 volumes of buffer A and eluted with 6 M guanidine hydrochloride in 50 mM potassium phosphate buffer, pH 6.0. The eluate fractions were analyzed for the presence of a 39-kD polypeptide by SDS-PAGE; those containing this polypeptide were pooled, diluted to 0.1 mg/mL protein, and sequentially dialyzed against 1 M, 0.1 M, and 0 M guanidine hydrochloride in 50 mM Tris-HCl, pH 8.0. If the sample was not diluted, precipitation of the protein occurred, but some of the precipitate could be dissolved in 0.5 M KCl, 50 mM Tris-HCl, pH 8.0, at a concentration of 0.05 mg/mL.

Purification of Native bGBF1 by Chromatography on Heparin-Sepharose

The growth of bacteria, IPTG induction, and cell harvesting were performed as described in the previous paragraph. The washed bacterial pellet was suspended in 0.02 culture volume of TE buffer. The suspension was subjected to four cycles of freezing in liquid nitrogen and thawing. The lysate was centrifuged at 100,000*g* for 30 min, and the supernatant was loaded on a heparin-Sepharose column (0.02 volume of the bacterial culture). The column was washed with 10 volumes of 50 mM Tris-HCl, pH 8.0, and eluted with a linear gradient of 0 to 2 M KCl in the same buffer. The fractions were screened for the presence of a predominant 39-kD polypeptide of bGBF1 by SDS-PAGE or assayed by gel retardation for the binding to a G-box containing DNA probe as described in Schindler and Cashmore (1990). bGBF1 eluted between 0.9 and 1.2 M KCl.

Purification of GBF Kinase

Ten kilograms of broccoli heads were processed in 1-kg portions by homogenization in 3 L of 250 mM solution of sucrose in a buffer containing 50 mM Tris-HCl, pH 7.0, 5 mM NaF, 5 mM EDTA, 50 μg/mL phenylmethylsulfonyl fluoride (buffer B). Crude nuclei were pelleted by centrifugation at 700*g* for 10 min. Combined nuclear pellets were lysed by two washes in hypotonic solution–buffer B (without sucrose), and chromatin was pelleted each time at 4000*g* for 10 min. Chromatin was extracted by resuspension in 400 mM KCl in buffer B (50 mL of buffer per 1 kg of plant material), and the extract was clarified by centrifugation at 10,000*g* for 20 min. The supernatant was brought to 50% saturation in ammonium sulfate, and the pellet was collected by centrifugation and dissolved in buffer B (10 mL of buffer per 1 kg of plant material). This fraction was further purified by batch adsorption on 10 mL of heparin-Sepharose. The batch was washed in buffer B and was poured into a column. The column was eluted with 16 volumes of a linear gradient of 0 to 1.5 M KCl in buffer C (50 mM Tris-HCl, pH 8.0, 5 mM 2-mercaptoethanol). The fractions containing bGBF1 kinase activity were pooled and dialyzed overnight against a 50-fold excess of buffer C. The dialysate was loaded on a 1-mL column of DEAE-cellulose, which was washed with 10 volumes of buffer D (buffer C with 15% glycerol) and eluted with 8 volumes of a linear gradient of 0 to 400 mM KCl in buffer D.

Protein Kinase Assay

Protein kinase activity was assayed in 25 μL of an assay solution containing 50 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 20 mM KCl, 2 μM ATP, 1 μCi γ-^{32}P-ATP, and 0.25 μg of bGBF1. After adding up to 5 μL of enzyme preparation, the assay was incubated for 30 min at room temperature. At earlier stages of protein kinase purification, phosphorylation products were analyzed by SDS-PAGE. In routine assays with the fractions that showed only bGBF1 phosphorylation, the samples were spotted on 2 × 2 cm squares of Whatman 3MM filter paper, which were subsequently washed three times for 10 min in 10% trichloroacetic acid, 1% sodium pyrophosphate, rinsed in ethanol, dried, and counted for Cerenkov radiation in a scintillation counter. One unit of activity is defined as the amount of enzyme incorporating 1 pmol of phosphate into bGBF1 during a 30-min incubation time.

Determination of Protein Concentration

Protein concentration was determined using a commercial reagent from Bio-Rad using the method of Bradford (1976).

Identification of Phosphoamino Acids

Radiolabeled phosphoamino acids were separated and identified by thin-layer electrophoresis at pH 3.5 as described by Cooper et al. (1983).

Protein Electrophoresis

Gel electrophoresis under denaturing conditions was performed in the presence of SDS as described by Laemmli (1970). The stacking and separating gels contained 4 and 10% (w/v) polyacrylamide, respectively (acrylamide/bis-acrylamide, 38:2 w/w). The gels were stained overnight with Coomassie brilliant blue R 250 (0.005% dye in 10% acetic acid, 10% isopropanol) and destained for 1.5 hr in three changes of 10% acetic acid, 5% methanol.

Electrophoretic Mobility Shift Assay

DNA-protein binding was performed in 30 μL of a solution containing 10 mM Tris-HCl, pH 8.0, 40 mM KCl, 1 mM EDTA, 0.07 mg/mL poly(dI:dC), 0.3 mg/mL bovine serum albumin, and 8 fmol (10,000 cpm) of radioactively labeled oligonucleotide probe. The sequence of this oligonucleotide was published in Schindler and Cashmore (1990) (the sequence labeled "*rbcS-3A*" in Figure 1D therein), as were the protocols used for cloning, preparation, and labeling of the probe. After a 30-min incubation at room temperature, the samples were separated in a 4% polyacrylamide gel in 25 mM Tris, 190 mM glycine, pH 8.3. The gels were dried and autoradiographed. Determination of band intensity was performed using a computing densitometer from Molecular Dynamics (Sunnyvale, CA).

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