Phosphorylation and Calcium Binding Properties of an Arabidopsis GF14 Brain Protein Homolog

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Arabidopsis GF14ω was originally described because of its apparent association with a DNA-protein complex; it is a member of the 14-3-3 kinase regulatory protein family that is conserved throughout eukaryotes. Here, we demonstrated that recombinant GF14ω is expressed in *Escherichia coli* as a dimer. Blot binding and electrophoretic mobility shift analyses indicated that GF14ω binds calcium. Equilibrium dialysis further demonstrated that GF14ω binds an equimolar amount of calcium with an apparent binding constant of 5.5×10^4 M^{-1} under physiological conditions. The C-terminal domain, which contains a potential EF hand motif, is responsible for the calcium binding. The C-terminal domain also cross-reacted with the anti-GF14ω monoclonal antibody. In addition, GF14ω is phosphorylated by Arabidopsis protein kinase activity at a serine residue(s) in vitro. Therefore, GF14ω protein has biochemical properties consistent with potential signaling roles in plants. The presence of a potential EF hand-like motif in the highly conserved C terminus of 14-3-3 proteins together with the calcium-dependent multiple functions attributed to the 14-3-3 proteins indicate that the C terminus EF hand is a common functional element of this family of proteins.

INTRODUCTION

Arabidopsis GF14 is a protein that shows more than 60% identity with mammalian brain 14-3-3 proteins at the amino acid sequence level and is apparently associated with the G-box DNA binding protein complex (Lu et al., 1992). The G-box is a general *cis*-acting DNA regulatory element that is present in promoters of many eukaryotic genes (Lu et al., 1992; Williams et al., 1992) and is involved in quantitative and/or qualitative aspects of promoter activity resulting from diverse stimuli, such as the hypoxic induction of the Arabidopsis alcohol dehydrogenase (*Adh*) gene (McKendree and Ferl, 1992), light induction of the Arabidopsis ribulose bisphosphate carboxylase small subunit (*RbcS*) gene (Donald and Cashmore, 1990), UV light induction of the parsley chalcone synthase gene (Block et al., 1990), abscisic acid induction of the wheat early methionine (*Em*) gene (Marcotte et al., 1989), and other genes (Pla et al., 1992). Thus, it is of interest to determine if the GF14 protein possesses any of the protein kinase and calcium-related biochemical features reported for the mammalian homologs to understand the possible role of the G-box in signal transduction from extracellular stimuli to promoter elements.

The first reported function of 14-3-3 proteins was activation of tyrosine hydroxylase and tryptophan hydroxylase activity by bovine brain 14-3-3 protein in the presence of protein kinase II from rat (Yamauchi et al., 1981; Ichimura et al., 1988; Isobe et al., 1991). Subsequently, kinase C inhibitor proteins from

sheep brain were reported to be 14-3-3 homologs (Aitken et al., 1990; Toker et al., 1990, 1992). Recently, a range of seemingly unrelated experiments resulted in the identification of additional functions for 14-3-3 proteins in yeast, insects, animals, and plants (Aitken et al., 1992), many involving calcium and protein kinases. A human phospholipase A2 activity was attributed to a 14-3-3 homolog with calcium binding activity (Zupan et al., 1992). A member of the 14-3-3 family, designated Exo1, is found to stimulate calcium-dependent exocytosis in permeabilized adrenal chromaffin cells (Morgan and Burgoyne, 1992a), and this effect is synergistically related to protein kinase C (PKC; Morgan and Burgoyne, 1992b), which can itself be activated by the 14-3-3 protein (Isobe et al., 1992). 14-3-3 homologs contribute to the maintenance of calcium-dependent noradrenaline secretion in bovine chromaffin cells (Wu et al., 1992). The bovine brain host factor that activates exoenzyme S (ExoS) from *Pseudomonas aeruginosa* has been shown to be a member of the 14-3-3 family (Fu et al., 1993). In plants, a pea 14-3-3 protein homolog inhibits PKC from sheep brain (Hirsch et al., 1992). Arabidopsis (Lu et al., 1992) and maize (de Vetten et al., 1992) 14-3-3 proteins participate in DNA–protein complexes. These results indicate that 14-3-3 proteins are highly conserved and widespread proteins that exhibit functions that are perhaps interrelated by the involvement of calcium and protein kinases.

It is not known whether plant 14-3-3 homologs biochemically interact with calcium, and in no case has the calcium

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binding constant been determined or the calcium binding site located for the 14-3-3 family of proteins.

Mammalian 14-3-3 isoforms are also known to be phosphorylated *in vitro* by PKC (Toker et al., 1992), and plant 14-3-3 homologs can inhibit mammalian PKC (Hirsch et al., 1992). However, phosphorylation of plant 14-3-3 homologs and interactions with plant protein kinases have yet to be investigated.

Herein, we report that a recombinant Arabidopsis GF14 binds calcium in vitro under physiological conditions, contains a putative EF hand–like motif, and is phosphorylated at a serine(s) by an Arabidopsis protein kinase activity. These biochemical characteristics are consistent with important potential connections between GF14 proteins, signal transductions, and *trans*-acting DNA binding complexes (Lu et al., 1992). The putative EF hand motif is conserved in all of the reported 14-3-3 proteins from both plants and animals. Therefore, this study offers potential insight into the possible molecular mechanisms for the diverse biochemical functions attributed to this highly conserved protein family.

RESULTS

Purification and Dimerization of GF14ω Expressed in *Escherichia coli*

When expressed in pET vector systems, the GF14ω protein is largely soluble in the *E. coli* lysate (Figure 1A, lane 1). After immobilized metal–affinity chromatography (IMAC) purification made possible by the short histidine tract provided by the vector to the N terminus, the GF14ω proteins were ~99% pure, as judged by staining after SDS-PAGE (Figure 1A, lanes 3 and 6). The possible subunit composition of GF14ω was investigated by high-resolution gel filtration chromatography and SDS-PAGE. Superdex-75 column chromatography of GF14ω expressed in *E. coli* resolved a protein peak at ~67 kD (Figure 1B), whereas the monomer is ~32 kD when analyzed by SDS-PAGE (Figure 1C). The treatment of dilute, native recombinant GF14ω with 10 mM glutaraldehyde in solution produced a protein band at ~65 kD when analyzed by SDS-PAGE (Figure 1D, lane 3). With the molecular mass ~65 kD in the native form or cross-linked by glutaraldehyde, and a subunit molecular mass near 32 kD, Arabidopsis GF14ω clearly exists as a *homodimer*.

The C-terminal domain did not elute from the Superdex column as a dimer (data not shown). Thus, the dimerization domain is likely located in the N-terminal 200 amino acids. Because Arabidopsis GF14ω from *E. coli* is soluble, forms a dimer similar to the 14-3-3 proteins from bovine and sheep brains (Isobe et al., 1991; Toker et al., 1992), cross-reacts with anti-GF14 monoclonal antibody (Lu et al., 1992), and activates PKC and tryptophan hydroxylase from rat brain as does the bovine 14-3-3 protein (G. Lu, T. Isobe, and R. J. Ferl, unpublished observation), it is likely that the recombinant GF14ω is properly
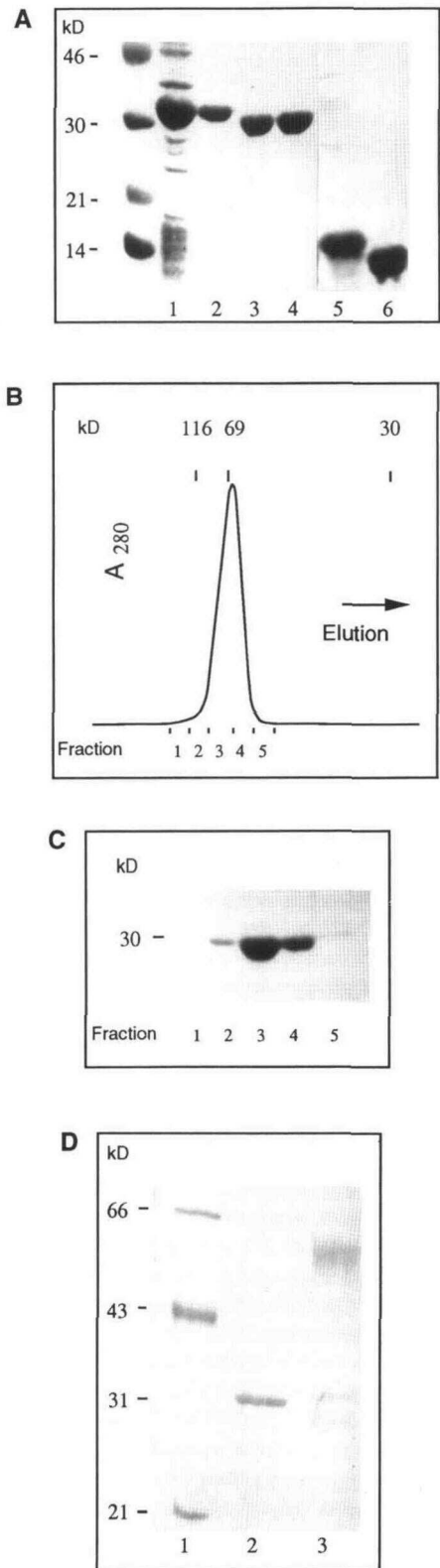

Figure 1. Purification and Characterization of GF14ω.

folded within *E. coli* and may be used to study the biochemical properties of the native GF14 protein.

GF14ω Binds Calcium under Physiological Conditions

The calcium binding activity of GF14ω was tested by three different methods: an electrophoretic mobility shift assay, a protein blot assay, and equilibrium dialysis. As shown in Figure 2A, incubation of GF14ω and $CaCl_2$ caused a shift of a portion of the GF14ω band in the subsequent SDS–polyacrylamide gel (lane 2). The retarded band was returned to its original mobility by the addition of 20 mM EDTA either before or after the incubation of GF14ω with $CaCl_2$ (Figure 2A, lanes 3 and 4). As shown in the protein blot–$^{45}Ca^{2+}$ binding assay of Figure 2B (lane 2), GF14ω protein binds $^{45}Ca^{2+}$ in the presence of KCl and $MgCl_2$.

Equilibrium dialysis analysis (Figure 3A) indicated that the intact GF14ω protein binds 1 mol of calcium per mol of monomer with an apparent binding constant of 5.5×10^4 M^{-1}. The binding activity remains nearly constant over a pH range of 6.5 to 8.5, and ~80% binding activity was detected in the presence of 150 mM KCl (Figure 3B). To test the binding specificity, an equal concentration of Mg^{2+} was added as a divalent cation competitor. The addition of Mg^{2+} caused only a 20% decrease of the binding activity (Figure 3B). Physiological conditions in a resting cell have been considered to be pH 7.4 with ~0.1 mM $MgCl_2$ and 100 to 150 mM KCl (Kilhoffer et al., 1983). Thus, these results indicated that GF14ω has the capability

Figure 2. GF14ω Binds Calcium.

(A) Electrophoretic mobility shift assay. GF14ω demonstrates anomalous migration in an SDS–polyacrylamide gel in the presence of free calcium: lane 1, GF14ω only; lane 2, GF14ω incubated with 10 mM $CaCl_2$; lane 3, GF14ω incubated with 10 mM $CaCl_2$ and 20 mM EDTA; lane 4, GF14ω incubated with 10 mM $CaCl_2$ for 20 min followed by the addition of EDTA to 20 mM. Lane 5 contains protein molecular mass markers, which are indicated at right.

(B) Protein blot–$^{45}Ca^{2+}$ binding assay. GF14ω and BSA were subjected to SDS-PAGE and blotted to a nitrocellulose membrane and then incubated with $^{45}Ca^{2+}$; lane 1, 20 μg of BSA; lane 2, 20 μg of GF14ω.

to bind calcium in a specific manner under potential physiological conditions.

The C-Terminal Domain Contains the Calcium Binding Site and the Epitope Recognized by an Anti-GF14 Monoclonal Antibody

To localize the calcium binding site, we tested the calcium binding activity of the C-terminal domain. As shown in Figure 4A, the C-terminal domain also binds 1 mol of calcium per mol of peptide with a binding constant (3.7×10^4 M^{-1}) similar to the intact GF14ω protein. Clearly, the 59 amino acids of the C-terminal domain include the calcium binding site of GF14ω. Because the C-terminal fragment is a monomer, this result also indicates no cooperativity in the binding of calcium by the intact GF14ω protein.

The immunoblotting results in Figure 4B indicated that anti-GF14 monoclonal antibody reacted to both intact GF14ω and the C-terminal domain fragment. A protein gel blot assay also indicated that the large fragment produced by trypsin digestion of GF14ω cross-reacts to the anti-GF14 antibody (Figure 4C). Amino acid sequence and mass spectroscopy of the large trypsin fragment indicated that the trypsin cleavage site is Lys-247 (data not shown). Because the C-terminal domain and the large tryptic fragment are both immunoreactive with the monoclonal antibody, the epitope recognized by this anti-GF14 monoclonal antibody is located within the C-terminal domain between Gln-200 and Lys-247.

Figure 1. (continued).

(A) Purification of recombinant GF14ω. GF14ω is highly induced by isopropyl-β-D-thiogalactopyranoside and present in the supernatant of the *E. coli* lysate (lane 1, 5 μL of supernatant). After IMAC purification (lane 2, 3 μg), cleavage by thrombin to remove the N-terminal histidine tract (lane 3, 5 μg), and Superdex-75 chromatography (lane 4, 5 μg), the GF14ω was essentially pure. The C-terminal domain peptide is also present in the supernatant of the lysate and was purified by IMAC (lane 5, 5 μg), followed by cleavage by thrombin and IMAC (lane 6, 5 μg).

(B) Native molecular mass by column chromatography. GF14ω was eluted from a Superdex-75 column with a native molecular mass of ~65 kD. The elution peaks for β-galactosidase (116 kD), BSA (69 kD), and the ricin A chain (30 kD) are shown above the A_{280} trace for GF14ω elution.

(C) Monomer molecular mass by SDS-PAGE. SDS-PAGE analysis of the peak fractions demonstrates a monomeric molecular mass of ~32 kD.

(D) Native molecular mass by cross-linking. SDS-PAGE of GF14ω protein cross-linked by glutaraldehyde also indicates that the native form of GF14ω is a dimer. Purified GF14ω at a concentration of 25 ng/mL was incubated in 50 mM sodium acetate, 100 mM KCl, pH 6.0, for 1 hr at 25°C in the presence (lane 3) or absence (lane 2) of 10 mM glutaraldehyde. Samples were subjected to 10% SDS-PAGE, and protein bands were visualized by silver staining. Protein molecular mass markers are in lane 1 and indicated at left.
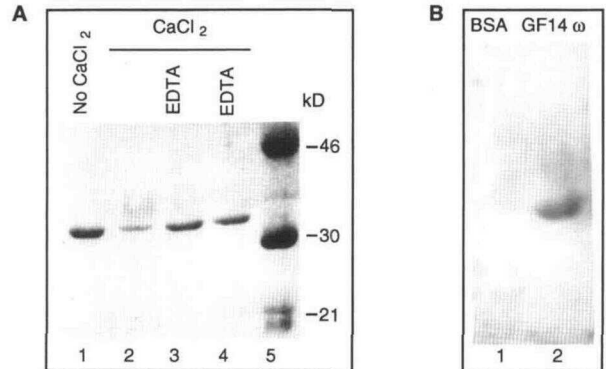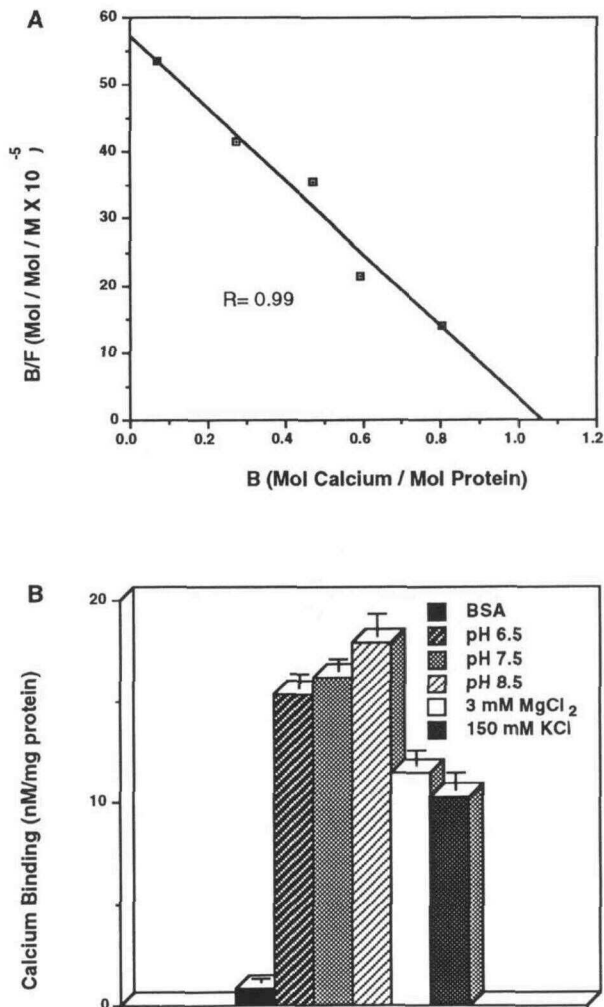
A

B

Figure 3. Calcium Binding Affinity of Purified GF14ω.

(A) Scatchard analysis of the equilibrium dialysis calcium binding data for the full GF14ω protein. B, bound calcium; F, free calcium.
(B) Effects of pH, ion strength, and magnesium on the calcium binding activity of the full GF14ω protein. BSA (0.6 mM) as a negative control was tested in 10 mM Tris-HCl, pH 7.5, containing 100 mM KCl and 3 mM total CaCl₂. The effects of pH, MgCl₂, and KCl were determined at 0.6 mM GF14ω. When used, MgCl₂ and KCl were added to 3 mM and 150 mM, respectively, in the 10 mM Tris-HCl buffer containing 3 mM CaCl₂. The data represent the standard error of two to four determinations.

Tryptic Cleavage Analysis Reveals Altered GF14ω Conformation upon Calcium Binding

To assay the possible effect of calcium binding on the topology of the native protein, GF14ω was incubated with trypsin and analyzed by SDS-PAGE. In the presence of calcium,

GF14ω was completely and specifically cleaved by trypsin at Lys-247 (Figure 5, lane 3). Only ∼50% of the protein was cleaved (Figure 5, lane 2) in very low concentrations of calcium that support trypsin activity but limit calcium binding to GF14ω. This suggests that calcium binding induces a conformational change in GF14ω, exposing Lys-247 to proteolytic attack, a situation similar to that for calmodulin (Babu et al., 1985).
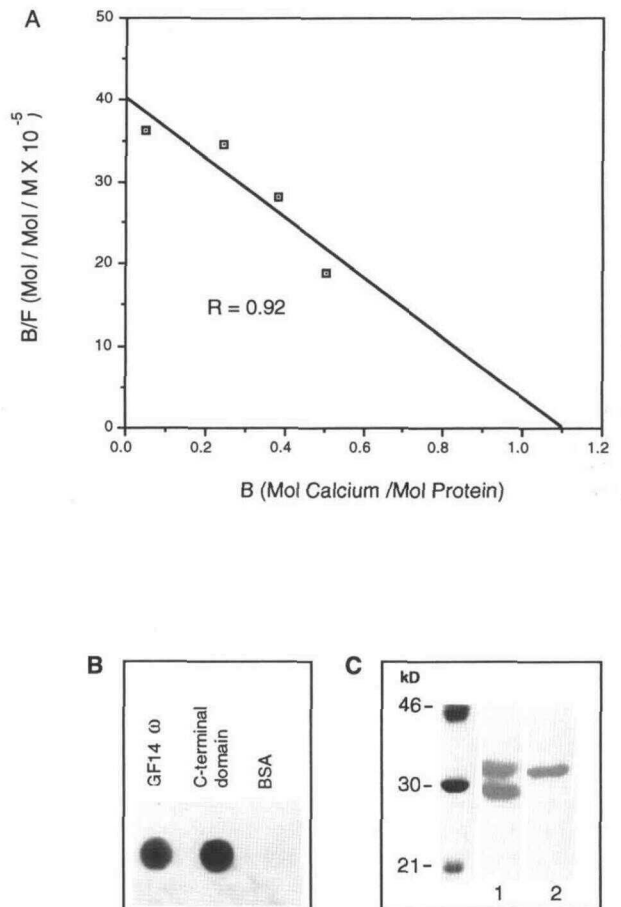
A

B

C

Figure 4. The C-Terminal Domain of GF14ω Contains Both the Calcium Binding Activity and a Monoclonal Antibody Epitope.

(A) Scatchard analysis of calcium binding to the C-terminal domain, which is an ∼10-kD polypeptide from Gln-200 to the C terminus. B, bound calcium; F, free calcium.
(B) Protein dot blot assay of full GF14ω and the C-terminal domain peptide with monoclonal antibody GF14-19. Dot blotting was necessary because the C-terminal domain peptide failed to transfer and bind to nitrocellulose in a standard protein blot.
(C) Protein gel blot assay of intact (lane 2) and partially trypsinized (lane 1) GF14ω protein. The partially trypsinized fragment just below 30 kD resulted from cleavage at Lys-247 and is the N-terminal 247 amino acids. The C-terminal domain begins at Gln-200. Thus, the epitope recognized by the monoclonal antibody is between Gln-200 and Lys-247.
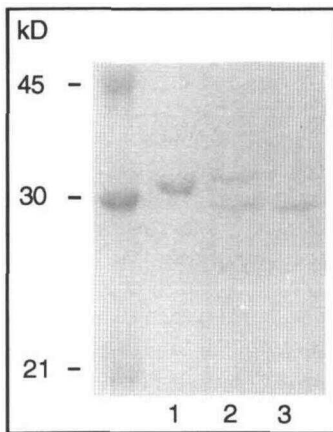
Figure 5. Altered Tryptic Cleavage of GF14ω when Bound to Calcium.

Before SDS-PAGE analysis, GF14ω (lane 1) was incubated with trypsin at an enzyme-to-substrate ratio of 1:100 in the absence of added $CaCl_2$ (lane 2) or in the presence of 5 mM $CaCl_2$ (lane 3).

GF14ω Is Phosphorylated at Serine Residue(s) by Endogenous Protein Kinase Activity

The crude organelle–membrane fraction of Arabidopsis suspension cells was chosen as the protein kinase source because GF14/14-3-3 proteins are soluble proteins (Lu et al., 1992) and free calcium is important for the biochemical effects of GF14/14-3-3 proteins. Thus, use of the washed, crude membrane fraction minimizes effects of any endogenous GF14 and free calcium that would otherwise be part of the crude kinase preparation. In addition, calcium-dependent protein kinases in plants are known to be associated with organelles and membrane systems (Battey, 1990; Harper et al., 1991).

GF14ω is phosphorylated by the extract (Figure 6A, lane 1), even in the absence of added Mg^{2+} (Figure 6A, lane 2). In keeping with the results of Figure 2B, GF14ω is shifted in the presence of calcium (Figure 6A, lane 2). These data suggested that GF14ω can be phosphorylated by a protein kinase from the Arabidopsis organelle–membrane pellet fraction. To identify the phosphorylated amino acid, the soluble phosphorylated GF14ω was separated from the pellet by centrifugation at 14,000g for 5 min. SDS-PAGE analysis of the supernatant indicated that GF14ω is the only phosphorylated protein present in the supernatant (Figure 6A, lane 5). As shown in Figure 6B, high-voltage paper electrophoresis indicated that the phosphorylated amino acid is serine, the same phosphoamino acid that occurs in sheep brain 14-3-3 protein that is phosphorylated by PKC (Toker et al., 1992).

All of the phosphorylated GF14ω was shifted to a slower mobility band in the presence of calcium (Figure 6A, lanes 2 and 6), whereas only a portion of the nonphosphorylated form is shifted by calcium (Figure 2A, lane 2). This suggested that the phosphorylated form of GF14ω has a higher affinity for calcium than the nonphosphorylated form.

DISCUSSION

The GF14/14-3-3 protein family is widespread and highly conserved in eukaryotic cells and has multiple important biological functions that are related to calcium and/or protein kinases (Aitken et al., 1992; Lu et al., 1992). This report definitively demonstrates that a 14-3-3 brain protein homolog in plants, Arabidopsis GF14ω, binds calcium and can be phosphorylated by plant protein kinase activity. These novel observations, especially those involving the stoichiometry and affinity of calcium binding, present insights pertaining to all 14-3-3 homologs and provide important information toward an understanding of the molecular mechanism and interrelationships of the diverse functions attributed to this highly conserved protein family.

Equilibrium dialysis indicates that GF14ω binds calcium in a simple ratio of one calcium atom per monomer of GF14ω and that the calcium binding activity is localized in the C-terminal domain, which is very highly conserved among all known 14-3-3 homologs (Lu et al., 1992). The C-terminal fragment binds calcium with the same stoichiometry and nearly the same affinity as the intact protein, indicating that the calcium binding structure is likely an independent, defined domain located between Gln-200 and the C terminus. Visual inspection of this region revealed the presence of several key acidic residues that are conserved, properly spaced, and centered around a central glycine residue in EF hand calcium binding motifs (Figure 7A). In the classic EF hand motif, these conserved residues are found within the loop of a helix-loop-helix structural domain. Secondary structure predictions for GF14ω as well as previous predictions for 14-3-3 proteins (Toker et al., 1992) suggest that this region of GF14ω contains two α-helixes that flank a loop of 12 residues. The conservation of amino acid sequence in this domain among the various isoforms of 14-3-3 proteins suggests that the structural features of this domain are also conserved.

Figure 7B presents a structural model of the putative calcium binding domain of GF14ω. To produce this model, the amino acid sequence of GF14ω from Gln-220 to Leu-227 was substituted for the corresponding residues of the EF hand in domain III of calmodulin, using the Brookhaven data base structure (Babu et al., 1985) and the molecular graphics program SYBYL. The helix-loop-helix structure of the EF hand was accommodated by the GF14ω sequence without steric hindrance from side chains, and minimization without respect to electrostatic interactions produced little deviation from the calmodulin EF hand structure. The ligands coordinating the calcium atom in GF14ω are, however, rearranged relative to those in calmodulin. In all known examples of EF hand motifs (Kilhoffer et al., 1983), the residue at position X is Asp, and the residue at position −Z is Glu (Figure 7A). This is important because the Asp residue occupying position X presents a single coordinating carboxyl oxygen as a ligand, while the Glu at position −Z presents a bidentate coordination. For GF14ω the situation is reversed, with Glu-208 at position X being the bidentate and Asp-291 at position −Z presenting the single coordination. The
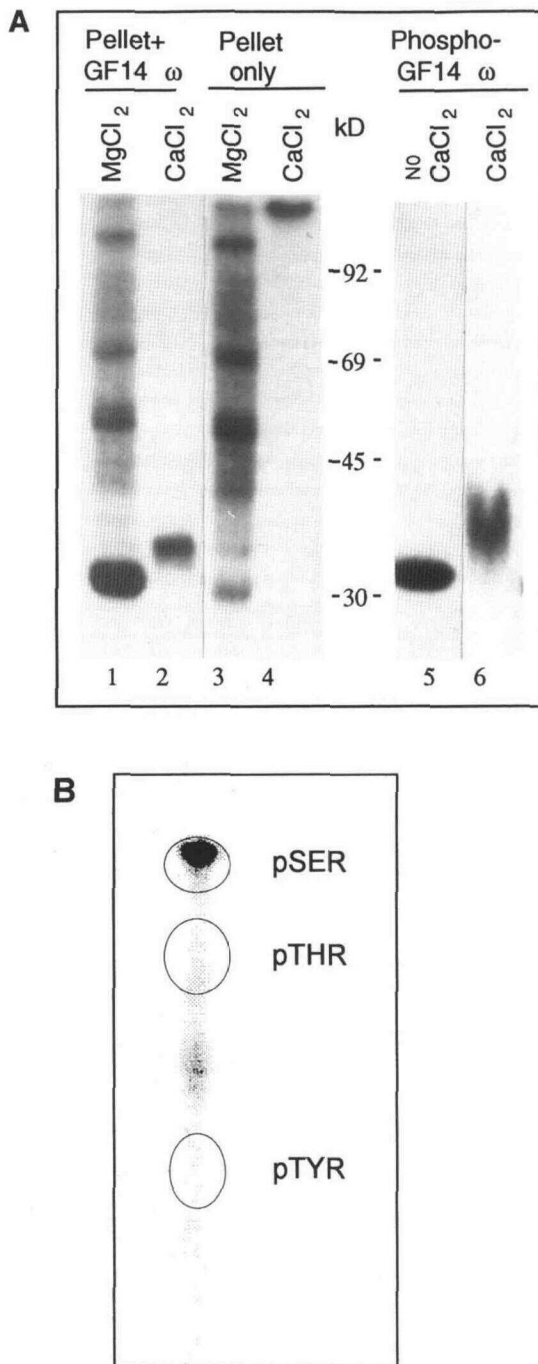
coordination provided by Asp-210 at position Y is conserved between GF14ω and calmodulin. In the model for GF14ω, the remaining calcium coordinations are provided by Glu-214 at position −X. For other EF hands, the remaining coordinations are provided by variable residues at either positions Z, −X, or −Y. Thus, the domain of GF14ω from Gln-200 to Leu-227 bears the structural features of a helix-loop-helix and can meet the calcium coordination requirements for an EF hand (Kilhoffer et al., 1983; Branden and Tooze, 1991).

The calcium binding EF hand is on the surface of the native GF14ω protein molecule. This conclusion is based on the fact that the monoclonal antibody recognizes an epitope within the region Gln-200 to Lys-247 but also binds to the native GF14ω in a fashion that allows antibody supershifting in the electrophoretic mobility shift assays with the G-box DNA element (Lu et al., 1992). Furthermore, the C-terminal domain must be in an orientation that allows antibody binding to occur in addition to interaction with G-box binding factors and the G-box DNA. These conclusions are further supported by the proteolytic cleavage of native GF14ω (Figure 5), which indicates that of the many potential tryptic sites, only the cleavage site at Lys-247 is readily available to trypsin. Thus, the EF hand and the remaining C-terminal amino acids appear to be an appendage of an otherwise compact protein.

The effects of ionic strength and pH on the calcium binding activity of GF14ω are also consistent with the properties of typical EF hand calcium binding proteins (Kilhoffer et al., 1983). In contrast to the low binding affinity exhibited by GF14ω, however, EF hand–type calcium binding proteins normally have high affinity for calcium (Kilhoffer et al., 1983). Two notable exceptions are the calcium-dependent proteases calpain II and the small subunit of calpain I (Emori et al., 1986a, 1986b; Suzuki, 1987). Calpain I and calpain II require micromolar and millimolar levels of calcium for 50% activity, respectively (Kawashima et al., 1984; Emori et al., 1986a; Suzuki, 1987). Although both calpain I and calpain II have calmodulin-like calcium binding domains, the amino acid sequence of the domain has only ~50% overall similarity with calmodulin. The lower affinity for calcium has been attributed, at least in part, to the sequence differences in the calcium binding regions (Kawashima et al., 1984; Emori et al., 1986a; Suzuki, 1987). The relatively low affinity of GF14ω for calcium may also be explained by differences in the calcium binding ligands between the GF14ω and the typical EF hand.

Low-affinity calcium binding sites are thought to have significant biological importance. The affinity of GF14ω (5.5×10^4 M^{-1}) is actually higher than that of calpain II (Kawashima

Figure 6. Phosphorylation of GF14ω by Arabidopsis Protein Kinase Activity in Vitro and Phosphorylated Amino Acid Analysis.

(A) Autoradiography of phosphorylated proteins. Phosphorylation reactions were conducted using the Arabidopsis kinase preparation with (lanes 1 and 2) or without (lanes 3 and 4) GF14ω, with either 5 mM $MgCl_2$ (lanes 1 and 3) or 10 mM $CaCl_2$ (lanes 2 and 4). Lane 5 contains the supernatant after centrifugation of the phosphorylation reaction shown in lane 1, and lane 6 contains the same supernatant incubated with 15 mM $CaCl_2$ before loading on the gel.

(B) Identification of the amino acid phosphorylated in GF14ω. The phosphorylated GF14ω protein shown in lane 5 of **(A)** was cleaved with 6 N HCl, and the resulting phosphoamino acids were separated by one-dimensional paper electrophoresis and revealed by autoradiography. Circles denote the position of coelectrophoresed phosphoserine (pSER), phosphothreonine (pTHR), and phosphotyrosine (pTYR), which were visualized by ninhydrin staining.
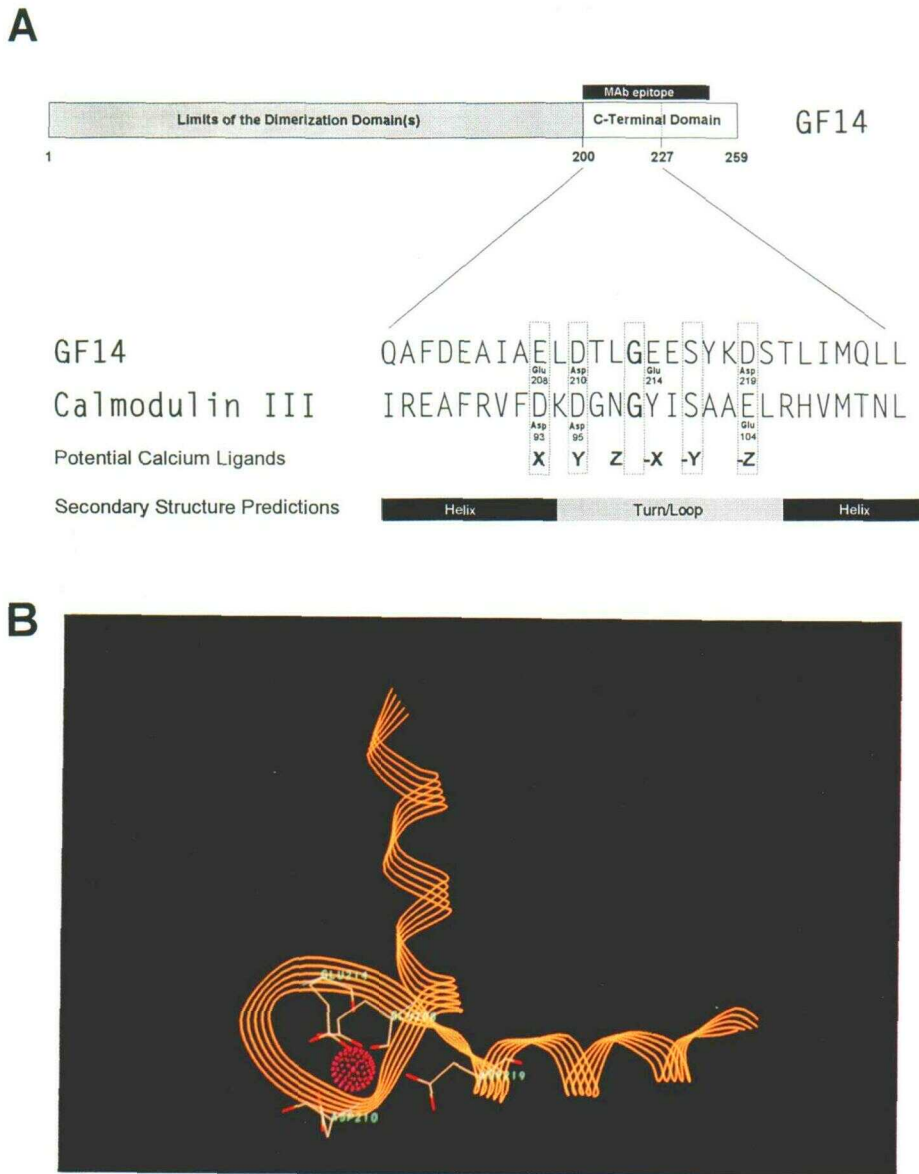
Figure 7. Structural Features of GF14ω and the Potential EF Hand Motif.

(A) Schematic diagram of the GF14ω protein with an expanded representation of the putative EF hand motif. The C-terminal domain is indicated by the open area of the rectangle, whereas the N-terminal 200 amino acids are indicated by the shaded area. The dimerization domain of GF14ω is thought to be located within the N-terminal region because the C-terminal domain fails to dimerize, whereas the intact protein and the large trypsin fragment do dimerize. The limits of the monoclonal antibody (MAb) epitope are indicated within the C-terminal domain and were deduced from the antibody binding to the C-terminal domain and the large tryptic fragment. The expanded region of GF14ω is from the start of the C-terminal domain at Gln-200 to Leu-247. Dotted boxes indicate conserved residues between GF14ω and domain III of calmodulin. Potential calcium ligands (Kilhoffer et al., 1983) are indicated below the amino acid sequences. Secondary structure predictions were based on the analysis of mammalian 14-3-3 proteins (Toker et al., 1992) and analysis of GF14ω using the programs within the GCG package (Devereux et al., 1984) and are indicated by helical (dark rectangles) and turn/loop (shaded rectangle) areas of the region.
(B) A computer-generated model for the putative EF hand from Gln-200 to Leu-227 of GF14ω. Brookhaven atomic coordinates for calmodulin domain III served as a starting model for the EF hand. The GF14ω sequence from residue Gln-200 to Leu-227 was substituted for the corresponding residues of calmodulin domain III using an Evans and Southerland PS390 graphics workstation and the SYBYL (Tripos, St. Louis, MO) program utilities. Optimization with regard to side chain geometry and ligand–calcium spacing was performed, with little main chain movement from the original calmodulin backbone. All ligand–calcium interactions were within standard distances of 2.0 to 4.0 Å.

et al., 1984) and that of the low-affinity site in calreticulins (Michalak et al., 1992), both of which are presumed to have physiological functions in eukaryotic organisms (Suzuki, 1987; Michalak et al., 1992; Huang et al., 1993). Furthermore, GF14ω has similar specific affinity for calcium as the low-affinity sites of troponin C (10^5 M^{-1}), which are responsible for regulatory processes in muscle contraction (Kilhoffer et al., 1983; Ellis et al., 1984). The apparent conformational change triggered by calcium binding, as determined in this study by the effects of calcium on tryptic cleavage, is consistent with a calcium-responsive biological activity for GF14ω.

Phosphorylation also affects the calcium binding properties of several proteins, including lipocortins I and II (Haigler et al., 1987). The phosphorylation of GF14ω by an Arabidopsis membrane-associated protein kinase activity clearly demonstrates that GF14/14-3-3 proteins can be substrates of protein kinases, and the behavior of phosphorylated GF14ω in calcium-dependent SDS-PAGE mobility shift assays may indicate that phosphorylation influences calcium binding. Many of the biochemical functions attributed to 14-3-3 proteins involve phosphorylation. Therefore, GF14 proteins may act as an important nexus in vivo by interrelating transduction pathways involving both calcium and kinase-dependent phosphorylation signals.

Apparently, one terminus of this signal mediation is regulation of promoter activity of certain genes. Regulatory participation in DNA binding complexes is consistent with the known kinase-related properties of the 14-3-3 proteins, because phosphorylation can affect DNA binding and transcriptional activation properties of transcription factors (Sassone-Corsi et al., 1988; Yamamoto et al., 1988; Klimczak et al., 1992). As a part of a trans-acting complex that binds to the general cis-acting G-box element (Marcotte et al., 1989; Block et al., 1990; Donald et al., 1990; Lu et al., 1992; Pla et al., 1992), GF14 proteins may provide important regulatory information on the growth, development, and environmental responsiveness of plants.

METHODS

Expression and Purification of GF14ω Protein

The original Arabidopsis GF14 cDNA (Lu et al., 1992) has been renamed GF14ω to reflect the fact that additional GF14 cDNAs have been isolated. The recombinant GF14ω protein was expressed and purified from Escherichia coli as follows. The coding sequence of GF14ω was amplified by polymerase chain reaction using synthetic oligodeoxynucleotides and subcloned into the NdeI-BamHI site of pET15b (Novagen, Madison, WI). To express the C-terminal domain (amino acids 200 to 259 of GF14ω) containing the potential EF hand motif, the first 200 amino acids of GF14ω were deleted from the cDNA by digesting pET15b-GF14 with NdeI and StuI, which is located at nucleotide number 670 of the GF14ω cDNA clone (Lu et al., 1992). After filling in the ends, the pET15b/GF14ω cDNA was religated.

The pET15b vector provided the expressed GF14ω and the truncated C-terminal domain with a histidine tract at their N termini; this allowed purification by Ni^{2+}-charged immobilized metal–affinity chromatography (IMAC) according to the manufacturer's protocol (Novagen). The histidine tract was removed by human thrombin cleavage, dialysis, and IMAC. Finally, the thrombin was removed by gel filtration on a Superdex-75 column (Pharmacia) in 10 mM Tris-HCl, pH 7.5. The peak fractions were concentrated with Centricon 10 concentrator (Amicon, Beverly, MA) for the full-length molecule and Centricon 3 concentrator for the truncated C-terminal domain. Before loading on the Superdex-75 column, the samples were dialyzed overnight against 10 mM Tris-HCl, pH 7.5, containing 50 mM EGTA at 4°C with three changes to remove the calcium in the samples required for thrombin cleavage, and then against 10 mM Tris-HCl, pH 7.5, for 6 hr with three changes. All the dialysis buffers were prepared with Chelex-100–treated (Sigma) double-distilled water. The protein concentration was determined by the Bradford microassay (Bio-Rad) with BSA as a standard.

Calcium Binding Activity

The protein blot–calcium binding assay was performed as described by Maruyama et al. (1984) using ^{45}CaCl$_2$ (Amersham). The electrophoretic mobility shift of GF14ω in the presence of calcium was assayed by incubating the GF14ω with 10 mM CaCl$_2$ in TEF buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 7.5, containing 25 mM NaF) with or without 20 mM EDTA for 20 min at room temperature, as indicated in the legend of Figure 2B. Samples were then separated by 10% SDS-PAGE, and the protein was visualized by Coomassie Brilliant Blue R 250 staining.

Equilibrium dialysis was performed according to the manufacturer's recommendations (Spectra/Por 5-cell Equilibrium Dialyzer; Spectrum, Houston, TX). The binding buffer was 10 mM Tris-HCl, pH 7.5, 100 mM KCl, with ~1 μCi ^{45}Ca^{2+} per mL containing different concentrations of cold CaCl$_2$ (Sigma). Protein aliquots (0.6 to 1 mM) were added to one chamber, and the dialysis was performed for at least 16 hr at 20°C with rotation. Protein-bound and free calcium concentrations were determined by radioactive counts present in the chambers as measured by liquid scintillation counting. The number of binding sites and the apparent binding constant were calculated by Scatchard plot analysis (Tinoco et al., 1985).

Trypsin Cleavage, Protein Gel Blotting, and SDS-PAGE of Cross-Linked GF14ω

GF14ω was cleaved by porcine trypsin (Sigma) at an enzyme-to-substrate ratio of 1:100 (w/w) in 25 mM Tris-HCl, pH 7.8, with or without 5 mM added calcium for 24 hr at 20°C, and separated by electrophoresis on a 10% SDS-polyacrylamide gel. The control digestion contains minute amounts of calcium, ~1 μM from the trypsin preparation, and trace amounts from the water.

For dot blot analysis, full-length GF14ω protein and the C-terminal domain peptide were blotted onto a nitrocellulose membrane using Minifold I according to the manufacturer's instructions (Schleicher & Schuell). To further determine the epitope recognized by the anti-GF14 antibody, the GF14ω was partially cleaved by trypsin at an enzyme-to-substrate ratio of 1:250 (w/w) and separated on a 10% SDS-polyacrylamide gel. After transferring the protein to nitrocellulose membrane and blocking with 10% nonfat-dried milk, the gel blot and dot blots were probed with anti-GF14 monoclonal antibody and the cross-reactions were detected by ECL protein chemiluminescence (Amersham). To identify the trypsin cleavage site of GF14ω, the large trypsin fragment was

isolated by electroeluting the fragment from the gel and then subjecting it to N-terminal sequencing and mass spectroscopy.

The SDS-PAGE of cross-linked GF14ω protein by glutaraldehyde was performed as described by Sehnke and Johnson (1993). The protein bands were visualized by silver staining using reagents from Bio-Rad.

Phosphorylation of GF14ω and Identification of Phosphorylated Amino Acid

Extracts for phosphorylation studies were prepared from Arabidopsis cell suspension cultures (Lu et al., 1992) by homogenization in TEF buffer (1 mL per 1 g cells) at 4°C with a mortar and a pestle. The homogenate was filtered through four layers of cheesecloth and one layer of Miracloth (Calbiochem). After centrifugation of the filtrate at 5000g for 10 min at 4°C, the pellet was washed three times with TEF buffer and resuspended in TEF buffer (0.5 mL per 1 g of cells). The washed and resuspended pellet was used as a kinase source to phosphorylate the purified recombinant GF14ω protein. The phosphorylation was performed in a mixture containing 2 µL of resuspended Arabidopsis pellet and 3 µg of GF14ω with or without Mg^{2+} and Ca^{2+}, as indicated in the legend of Figure 6. The reaction was started by adding 1 µCi of γ^{32}-P-ATP (Du Pont–New England Nuclear) and incubated for 20 min at room temperature, and then stopped by boiling with an equal volume of 2 × SDS buffer (0.25 M Tris-HCl, pH 6.8, 4% [w/v] SDS, 5% β-mercaptoethanol, 20% glycerol). Samples were separated on a 10% SDS–polyacrylamide gel, and after staining with Coomassie blue, the gel was dried and the phosphorylated peptides were detected by autoradiography.

After phosphorylating 50 µg of GF14ω protein as described above, the mixture was centrifuged at 14,000g for 5 min to separate the soluble GF14ω protein from the Arabidopsis kinase pellet. The clear supernatant was lyophilized and then cleaved with 6 N HCl under an argon atmosphere for 3 hr at 100°C. Amino acids were separated by one-dimensional high-voltage paper electrophoresis (Cooper et al., 1983). Phosphoamino acid markers (Sigma) were coelectrophoresed and identified by ninhydrin staining. The ^{32}P-labeled amino acid was detected by autoradiography and imaging on a scanning densitometer (Molecular Dynamics, Sunnyvale, CA). The digitized radiographic image, together with the outline of the coelectrophoresed markers, was printed on a laser printer.

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