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Specific Interactions with TBP and TFIIB in Vitro Suggest That 14-3-3 Proteins May Participate in the Regulation of Transcription When Part of a DNA Binding Complex

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The 14-3-3 family of multifunctional proteins is highly conserved among animals, plants, and yeast. Several studies have shown that these proteins are associated with a G-box DNA binding complex and are present in the nucleus in several plant and animal species. In this study, 14-3-3 proteins are shown to bind the TATA box binding protein (TBP), transcription factor IIB (TFIIB), and the human TBP–associated factor $hTAF_{II}32$ in vitro but not $hTAF_{II}55$. The interactions with TBP and TFIIB were highly specific, requiring amino acid residues in the box 1 domain of the 14-3-3 protein. These interactions do not require formation of the 14-3-3 dimer and are not dependent on known 14-3-3 recognition motifs containing phosphoserine. The 14-3-3–TFIIB interaction appears to occur within the same domain of TFIIB that binds the human herpes simplex virus transcriptional activator VP16, because VP16 and 14-3-3 were able to compete for interaction with TFIIB in vitro. In a plant transient expression system, 14-3-3 was able to activate GAL4-dependent β-glucuronidase reporter gene expression at low levels when translationally fused with the GAL4 DNA binding domain. The in vitro binding with general transcription factors TBP and TFIIB together with its nuclear location provide evidence supporting a role for 14-3-3 proteins as transcriptional activators or coactivators when part of a DNA binding complex.

INTRODUCTION

The proteins comprising the 14-3-3 family were initially characterized by Moore and Perez (1967) as acidic, soluble proteins that are highly abundant within bovine brain tissues. Now, ~50 14-3-3 genes have been identified from animals, plants, and yeast by researchers in very different fields of study (Ferl, 1996; Wang and Shakes, 1996). In many cases, multiple 14-3-3 genes encode different isoforms, which are ubiquitously expressed in many different cell types. As many as 10 14-3-3 isoforms have been identified from Arabidopsis (Wu et al., 1997b). Sequence comparison of 14-3-3 cDNAs indicates that 14-3-3 proteins are highly conserved and widely distributed phylogenetically (Wang and Shakes, 1996). X-ray crystallographic studies indicate that two isoforms possess nearly identical structures, suggesting that all isoforms have very similar structural features (Liu et al., 1995; Xiao et al., 1995).

Although members of the 14-3-3 protein family possess highly conserved sequences and structures, their biological functions appear to be highly diversified in different systems. The 14-3-3 proteins have been recognized for some time as regulators of a series of kinases important in multiple signaling pathways. These include Raf (Freed et al., 1994; Irie et al., 1994; Li et al., 1995), Ras (Gelperin et al., 1995; Rommel et al., 1996), Bcr (Reuther et al., 1994; Braselmann and McCormick, 1995), and protein kinase C (Toker et al., 1992; Dellambra et al., 1995). In animal brain cells, 14-3-3 proteins can activate phosphorylated tyrosine tryptophan hydroxylase, which is an important enzyme involved in neural transmission (Yamauchi et al., 1981; Ichimura et al., 1987). In permeabilized adrenal chromaffin cells, 14-3-3 proteins are able to mediate calcium-dependent exocytosis (Morgan and Burgoyne, 1992). In yeast, 14-3-3 proteins are required for cell viability (Van Heusden et al., 1994) and play a role in a mechanism that acts as checkpoint for mitotic DNA damage repair (Ford et al., 1994). 14-3-3 proteins also appear to have a chaperone-like function because they can facilitate protein translocation through the mitochondrial membrane by their ATPase activity (Alam et al., 1994).

In plants, 14-3-3 proteins include the receptor for the phytotoxin fusicoccin (Marra et al., 1994; de Boer, 1997) and a

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protein that specifically inhibits nitrate reductase activity from spinach cells (Bachmann et al., 1996). In several species, including dicots and monocots, 14-3-3 proteins are found in a sequence-specific DNA binding complex in vitro (de Vetten et al., 1992; Lu et al., 1992; Schultz et al., 1998) and are localized in the nucleus in vivo (Bihn et al., 1997). Although the 14-3-3 proteins appear to be involved in a wide range of biological functions, a common theme regarding function is their propensity for interactions with other proteins (Ferl, 1996).

Biochemical characterization of the 14-3-3 proteins has shown that they are able to form homodimers or heterodimers through their N-terminal dimerization domains (Jones et al., 1995; Luo et al., 1995; Wu et al., 1997a). The 14-3-3 protein dimer is cup shaped, containing a spacious internal area and negative residues located in the C-terminal portion of each monomer. These charged residues align on the surface to form a negative groove (Liu et al., 1995). This topological feature, which is thought to be a site for protein–protein interactions (Liu et al., 1995), should be universal among 14-3-3 proteins because the negative residues are conserved in numerous isoforms.

Many proteins contain binding sites for 14-3-3 proteins that include phosphoserine as part of the specific recognition motif RSXpSXP, where X represents any amino acid and pS denotes phosphoserine (Muslin et al., 1996). For these types of interactions, the binding of 14-3-3 proteins inhibits dephosphorylation of the phosphoserine in the partner protein (Bachmann et al., 1996; Banik et al., 1997). Due to its widespread occurrence, phosphoserine binding has been thought to serve as the primary mechanism through which 14-3-3 proteins participate in a variety of diverse cellular functions (Muslin et al., 1996). Domain mapping for 14-3-3 proteins has identified a C-terminal region, called box 1, that is able to independently mediate phosphoserine binding (Ichimura et al., 1997). The isolated box 1 domain cannot form a dimer structure but still retains full capacity for binding other proteins (Ichimura et al., 1995, 1997). Therefore, the importance of the dimeric structure and the role of the negative groove of 14-3-3 proteins in protein–protein interactions remain unclear.

Although phosphoserine binding can explain many interactions involving 14-3-3 proteins, there is a growing list of exceptions. For example, the C1 domain of myosin II heavy-chain-specific protein kinase C, which was shown to interact with a 14-3-3 protein, does not contain the phosphoserine motif (Matto-Yelin et al., 1997). In the case of the glucocorticoid receptor, the potential RSXpSXP motif is present only in its activation domain, not in the ligand binding domain to which 14-3-3η preferentially binds (Wakui et al., 1997). Another example is the SGHSL motif in a platelet adhesion receptor, glycoprotein Ib-IX, which interacts with 14-3-3ζ and does not appear to be phosphorylated (Gu and Du, 1998). In addition, 14-3-3ζ has also been shown to bind to a nonphosphorylated peptide containing the consensus WLDL, which has been isolated by phage display (Petosa et al., 1998). These exceptions suggest that phosphoserine binding is but one of several possible modes through which partner proteins interact with 14-3-3 proteins.

The 14-3-3 proteins are also found as part of a transcriptional DNA binding complex. In several plant species, including Arabidopsis, maize, and rice, 14-3-3 proteins have been reported to associate with G-box DNA binding complexes in vitro (de Vetten et al., 1992; Lu et al., 1992; Schultz et al., 1998). In agreement with these results, 14-3-3 proteins were found in the nuclei of both maize and Arabidopsis cells by using confocal microscopy (Bihn et al., 1997). In another study, human (h) 14-3-3η was shown to associate with the glucocorticoid receptor in a yeast two-hybrid screen. The association with 14-3-3η was through direct interaction with the ligand binding domain (ligand bound state) of the glucocorticoid receptor. Overexpression of 14-3-3η in COS-7 cells stimulates the glucocorticoid receptor–dependent transcription in a ligand-dependent manner (Wakui et al., 1997). Another example of 14-3-3 involvement in transcription is seen in mouse embryogenesis. Here, 14-3-3ε is found in a complex with the homeodomain transcription factor TLX-2 and is thought to modulate its activity (Tang et al., 1998). More recently, one Arabidopsis 14-3-3 protein was shown to strongly activate transcription in yeast cells when it was fused to the LexA DNA binding domain (Wang et al., 1999).

These findings raise the possibility that 14-3-3 proteins may directly participate in the regulation of transcription; however, the precise role for 14-3-3 proteins in transcriptional regulatory complexes is not understood. In theory, 14-3-3 proteins may alter the function of activator proteins, such as the glucocorticoid receptor, TLX-2, and the G-box binding factor, or they may be more directly involved in the recruitment of general transcriptional factors to the preinitiation complex (PIC).

Eukaryotic mRNA transcription requires the recruitment of the PIC onto the promoter. This recruitment is usually accomplished through protein–protein interactions between an activator and various members of the PIC (Colgàn et al., 1993, 1995; Roberts et al., 1993; Hadzic et al., 1995; Nakshatri et al., 1995). The activator can be a sequence-specific DNA binding protein itself or a component of a multiprotein–DNA complex. In many cases, the TATA box binding protein (TBP) and the general transcription factor IIB (TFIIB) serve as important targets for those activator proteins with acidic (Roberts et al., 1993; Kim et al., 1994), proline-rich (Kim and Roeder, 1994), or glutamine-rich activation domains (Colgàn et al., 1995; Nakshatri et al., 1995). To explore the possible function(s) of the 14-3-3 proteins in transcription, we evaluated in vitro the potential for these proteins to directly interact with TBP and TFIIB and assessed in vivo their abilities to activate transcription. The 14-3-3 proteins are shown to have specific affinities for Arabidopsis and human TBP, hTFIIB, and $hTAF_{II}32$ (for TBP-associated factor) in vitro. In addition, the transiently expressed GAL4 DNA binding domain–14-3-3 chimeric protein can activate GAL4-dependent β-glucuronidase (*GUS*) gene expression in plants.

RESULTS

Plant 14-3-3 Proteins Interact with hTFIIB in Vitro

To test whether plant 14-3-3 proteins directly participate in transcriptional activation by the promoter complex with which they are associated, we used glutathione *S*-transferase (GST) pull-down assays to examine possible interactions between 14-3-3 proteins and components of the PIC. The high degree of amino acid similarity between plant and animal 14-3-3 proteins allowed us initially to use hTFIIB and later to switch to Arabidopsis TFIIB when this cDNA clone was isolated (Baldwin and Gurley, 1996).

In the first in vitro binding experiment, we tested the interaction between hTFIIB and maize 14-3-3 protein (ZmGF14-12) (de Vetten et al., 1992; de Vetten and Ferl, 1994). hTFIIB was expressed in *Escherichia coli* as a GST fusion and immobilized on glutathione beads. Maize 14-3-3 (His tagged) was expressed in *E. coli* and purified by using nickel beads. Possible interaction between hTFIIB and maize 14-3-3 was examined by incubating the free maize 14-3-3 protein with bead-immobilized GST–hTFIIB or GST control proteins. After extensive washing of nonbound molecules, the bound 14-3-3 was detected by protein gel blotting using the anti–14-3-3 polyclonal antibody. As shown in Figure 1A, maize 14-3-3 was retained on GST–hTFIIB beads but not on the GST control beads. Further experiments showed that this interaction was stable under high salt concentrations of up to 0.5 M, although binding was gradually reduced with increasing salt concentrations (data not shown).

In similar experiments, we also observed interactions between hTFIIB and five Arabidopsis 14-3-3 isoforms (Figure 1B). As seen with the maize 14-3-3 protein, the Arabidopsis isoforms specifically bound to the GST–hTFIIB beads (Figure 1B) but not to the GST beads (Figure 1B, lanes c). However, the binding affinities for hTFIIB appeared to vary among isoforms, as indicated by their differential retention on the GST–hTFIIB beads (Figure 1B), with the highest binding efficiency seen for the Arabidopsis isoform AtGF14ϕ and the lowest for isoform AtGF14φ. Taken together, these results suggest that the capacity of 14-3-3 proteins to interact with TFIIB is a conserved phenomenon.

The Conserved C-Terminal Core of hTFIIB Binds the Arabidopsis 14-3-3 Proteins

hTFIIB is known to interact with activator proteins in vitro, and the two repeat motifs located in the conserved C terminus are important for binding to herpes simplex viral protein VP16 (Roberts et al., 1993). We generated a series of C- or N-terminal deletion mutants of hTFIIB and evaluated their potential for interactions with AtGF14ϕ. All hTFIIB deletion mutants were expressed in *E. coli* as GST fusions and immobilized on the glutathione beads. The free AtGF14ϕ protein was then incubated with the bead-immobilized hTFIIB constructs containing approximately equal amounts of proteins. For the C-terminal deletion series of hTFIIB, 14-3-3 protein interactions occurred with the construct containing amino acids 1 to 202 (Figure 2A, lane 2), but no interactions were evident with TFIIB constructs containing amino acids 1 to 123 (lane 3) or amino acids 1 to 65 (lane 4). Therefore, the hTFIIB region between amino acids 124 and 202 is required for 14-3-3 binding (cf. lanes 3 and 2). For the N-terminal deletion series of hTFIIB, the 14-3-3 interaction was exhibited by immobilized GST–hTFIIB constructs containing amino acids 65 to 316 (lane 7), amino acids 124 to 316 (lane 8), or amino acids 262 to 316 (lane 9) but not to those containing amino acids 293 to 316 (lane 10).

These results show that a second 14-3-3 binding domain in hTFIIB is localized within the region from amino acids 262 to 292 (Figure 2A, cf. lanes 9 and 10). Consistent with the results obtained by the C-terminal deletion series (cf. lanes 3 and 2), deletion of amino acids 124 to 261 in the N-terminal deletion series resulted in a significant reduction in binding

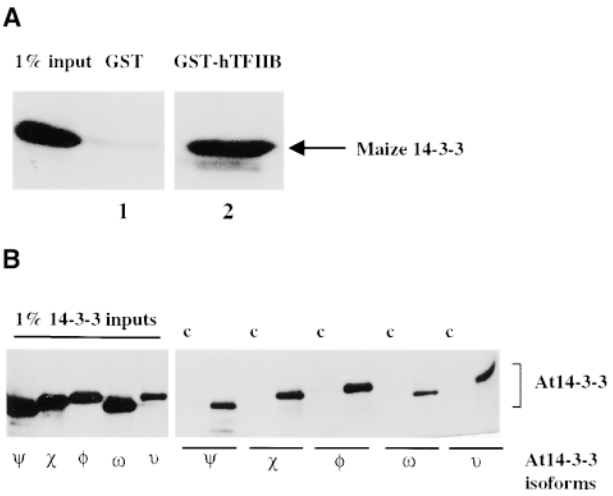

Figure 1. In Vitro Interactions of hTFIIB with 14-3-3 Proteins from Maize and Arabidopsis.

The hTFIIB coding sequence was expressed in *E. coli* as a GST fusion. GST–hTFIIB or GST was immobilized on glutathione–agarose beads and incubated with different isoforms of maize and Arabidopsis 14-3-3 proteins purified from *E. coli*. Binding reactions were conducted at room temperature for 3 hr in a buffer containing 0.10 M KCl. 14-3-3 isoforms were incubated with equal amounts of GST or GST–hTFIIB during the binding reaction. After extensive washing, bound 14-3-3 proteins were eluted and detected by protein gel blotting using the anti–14-3-3 polyclonal antibody. Input lanes in this figure (and subsequent Figures 2 through 7) represent a portion of the free ligand protein, as indicated.

(A) Maize 14-3-3 protein (ZmGF14-12) interacted with GST–hTFIIB (lane 2) but not with the GST control (lane 1).

(B) Five Arabidopsis 14-3-3 isoform proteins interacted with GST–hTFIIB but not with GST alone (control lanes; c).

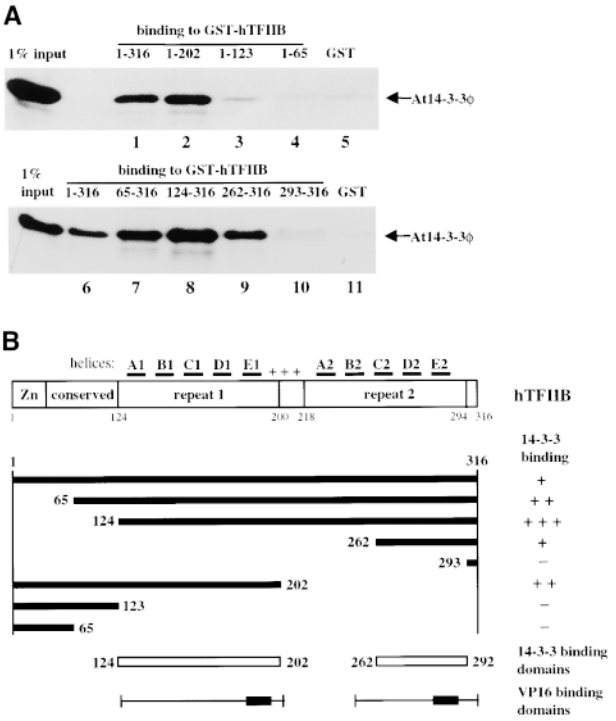

Figure 2. Identification of 14-3-3 Binding Domains within hTFIIB by Deletion Analysis.

(A) A series of GST-fused C- or N-terminal deletion mutants of hTFIIB were tested for their ability to interact with the Arabidopsis 14-3-3 protein AtGF14ϕ. Free 14-3-3 protein was incubated in binding reactions identical to those used for Figure 1 with equal amounts of immobilized GST–hTFIIB deletion mutants. The protein gel blot probed with the anti–14-3-3 polyclonal antibody showed that hTFIIB possesses two regions capable of interacting with the 14-3-3 protein: one is located from amino acids 124 to 202 and the other from amino acids 262 to 292.

(B) Summary diagram of the results in **(A)** showing locations of binding domains for 14-3-3 and VP16. (+) denotes binding, and (–) indicates no binding.

(cf. lanes 9 and 8). Both interaction domains are located in the conserved core of hTFIIB, with amino acids 124 to 202 in repeat 1 and amino acids 262 to 292 in repeat 2. These regions roughly correspond to domains previously reported to bind VP16 (Roberts et al., 1993) (Figure 2B).

The VP16–hTFIIB interaction in vitro was previously shown to be enhanced by removal of two regions located at the N and C termini of hTFIIB, respectively. This phenomenon was attributed to disruption of the intramolecular interaction between the N-terminal region and the C-terminal core of hTFIIB, which blocks access to binding domains within TFIIB (Roberts and Green, 1994). In the present study, we also observed a similar enhancement in binding to 14-3-3 resulting from C-terminal and N-terminal deletions of hTFIIB (Figure 2A, cf. lanes 2 and 1, 7 and 8, and 6). This deletion-dependent increase in binding was most pronounced after removal of the entire N-terminal region of hTFIIB (amino acids 1 to 123; cf. lanes 8 and 7).

The Arabidopsis 14-3-3 Protein and VP16 Show Similarities When Interacting with hTFIIB

To further investigate the similarity between 14-3-3–hTFIIB and VP16–hTFIIB interactions, we tested a mutation of hTFIIB and one of VP16, both of which are known to affect the TFIIB–VP16 interaction and to inhibit transcriptional activation in vitro (Lin et al., 1991; Roberts et al., 1993). The hTFIIB mutation is within the E1 helix of repeat 1, consisting of substitutions of two arginine residues by two glutamic acids, R185E/R193E. Both the wild type and the R185E/R193E mutant of hTFIIB were tagged with the T7 epitope at their C-terminal ends and were added to binding reactions containing immobilized GST–VP16 or GST–AtGF14ϕ as crude *E. coli* extracts. The VP16 mutant VP16Δ456/F442P had the C-terminal activation motif deleted, and the critical residue phenylalanine 442 of the N-terminal activation motif was substituted by a proline. The GST–VP16 and GST–VP16Δ456/F442P proteins served as positive and negative controls for hTFIIB binding, respectively, in Figure 3.

As expected, wild-type hTFIIB and the R185E/R193E mutant showed no interaction with GST–VP16Δ456/F442P (lanes 1 and 2). However, hTFIIB showed clear evidence of interactions with both GST–VP16 (lanes 3 and 4) and GST–AtGF14ϕ (lanes 5 and 6). The affinities of hTFIIB for wild-type VP16 and AtGF14ϕ were approximately the same based on band intensities (Figure 3). In addition, the R185E/R193E mutation of hTFIIB impaired interactions with VP16 and 14-3-3 to a similar degree (Figure 3). These results suggest that both VP16 and 14-3-3 may use the same or similar

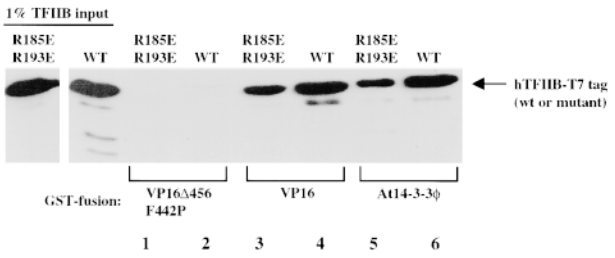

Figure 3. Comparison of hTFIIB and Mutated hTFIIB Interactions with Immobilized 14-3-3 (AtGF14ϕ), VP16, and Mutant VP16.

Free hTFIIB (T7 tagged) or the R1853/R193E mutant were incubated at 4°C for 1 hr with immobilized GST–VP16Δ456F442P (as a negative control), GST–VP16, and GST–14-3-3 in binding buffer containing 0.15 M KCl. The hTFIIB point mutation R185E/R193E inhibited interactions with VP16 and the 14-3-3 protein to a similar degree. Bound hTFIIB was detected on protein gel blots by using the anti-T7 antibody. WT, wild-type hTFIIB protein.

mechanisms in binding to hTFIIB. In addition, the binding of hTFIIB to GST–14-3-3 in this experiment is reciprocal to that of 14-3-3 to GST–hTFIIB, as shown in Figure 2, confirming that the GST moiety was not involved in the interaction between hTFIIB and AtGF14ϕ.

Because amino acid residues R185 and R193 of hTFIIB were implicated in the binding of both VP16 and Arabidopsis 14-3-3, TFIIB interactions with these two proteins may be mutually exclusive. To test this idea, we conducted binding competition experiments, as shown in Figure 4. In the presence of an excess amount of free 14-3-3 (AtGF14ϕ), binding of hTFIIB to immobilized GST–VP16 was greatly inhibited (Figure 4A, cf. lanes 3 and 2). Hypothetically, the observed inhibition could be due to competition between GST–VP16 and 14-3-3 for binding to hTFIIB or competition between hTFIIB and 14-3-3 for binding to GST–VP16. In the former case, most of the hTFIIB molecules would be complexed with 14-3-3 molecules in solution and removed during washing steps. In the latter case, 14-3-3 would bind to GST–VP16 and be detected by anti–14-3-3 antibody. This possibility was excluded because no additional 14-3-3 band was seen in the same blot reprobed with the anti–14-3-3 antibody (data not shown). We conclude that the greatly reduced binding of hTFIIB to VP16 was due to competition between VP16 and Arabidopsis 14-3-3 for binding to hTFIIB.

In a reciprocal experiment, T7-tagged 14-3-3 was immobilized on beads containing covalently linked protein A using anti-T7 immunoaffinity, as diagramed in Figure 4B. Free hTFIIB in a crude bacterial extract was then incubated with immobilized 14-3-3 (AtGF14ϕ) in the presence of equal amounts of either GST (Figure 4B, lane 2) or GST–VP16 (lane 1) in excess. Bound hTFIIB was detected in a protein gel blot using the anti-hTFIIB monoclonal antibody (Promega, Madison, WI). The difference in binding between lanes 1 and 2 (Figure 4B) clearly shows that VP16 strongly inhibited binding of hTFIIB to 14-3-3, which is consistent with competition between VP16 and 14-3-3 for interactions with hTFIIB.

h14-3-3ϵ Shows Affinity for hTFIIB, hTBP, and hTAF$_{II}$32 but Not for hTAF$_{II}$55

Due to the lack of cloned plant TAF$_{II}$s and to avoid possible difficulties in interpretation resulting from the use of components from plants and humans, we evaluated homotypic interactions between h14-3-3 (h14-3-3ϵ) with human general transcription factors and cofactors. The GST–h14-3-3ϵ protein was immobilized on glutathione beads and incubated with bacterially expressed hTBP and hTFIIB or with hTAF$_{II}$32 and hTAF$_{II}$55 synthesized using a coupled transcription/translation system. h14-3-3ϵ interacted with hTBP (Figure 5A, cf. lanes 4 and 3), hTFIIB (cf. lanes 2 and 1), and hTAF$_{II}$32 (Figure 5B, cf. lanes 1 and 2) but not with hTAF$_{II}$55 (lane 3). This multiple interaction pattern of h14-3-3ϵ with the human PIC components parallels that previously observed for VP16 (Roberts et al., 1993; Kim et al., 1994; Chiang and Roeder, 1995; Klemm et al., 1995).

h14-3-3ϵ Contains Two Domains That Bind TFIIB

Using human proteins, we identified regions of 14-3-3ϵ required for binding TFIIB. Deletion mutants for h14-3-3ϵ were

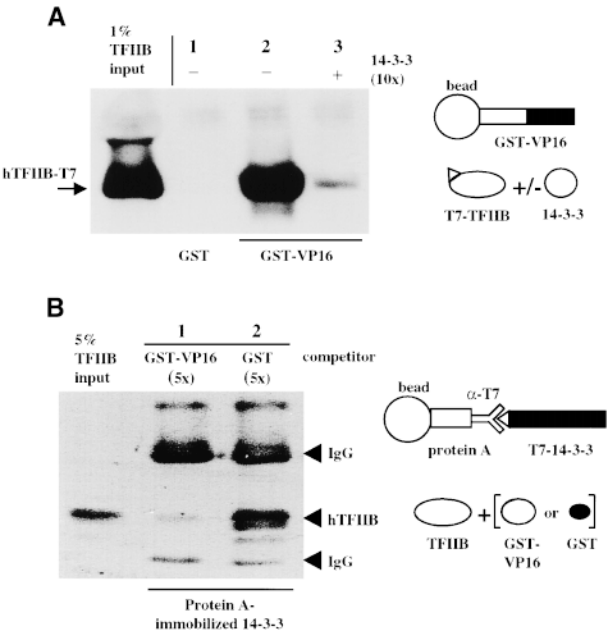

Figure 4. Competition between 14-3-3 (AtGF14ϕ) and VP16 for hTFIIB Binding.

Diagrams in **(A)** and **(B)** show experimental configuration of immobilized and free proteins. In **(A)**, GST–VP16 was immobilized on Sepharose beads and incubated with T7-tagged hTFIIB in the presence or absence of 14-3-3 protein. In **(B)**, T7-tagged 14-3-3 was immobilized on protein A–Sepharose beads through interactions with a monoclonal antibody specific for the T7 epitope tag joined to the N terminus of AtGF14ϕ. hTFIIB was incubated with the beads in the presence of GST–VP16 or GST.

(A) T7-tagged hTFIIB was incubated at room temperature for 3 hr with immobilized GST–VP16 in the absence ([−], lane 2) or presence ([+], lane 3) of a 10-fold (10×) excess (compared with VP16) of 14-3-3 protein in binding buffer containing 0.15 M KCl. Bound hTFIIB was detected on protein gel blots by using an anti-T7 antibody. Lane 1 shows hTFIIB binding to GST alone.

(B) hTFIIB was incubated with T7-tagged 14-3-3 proteins immobilized on protein A–Sepharose beads in the presence of GST–VP16 or GST. GST–VP16 and GST were in fivefold (5×) excess to the immobilized 14-3-3 protein in binding buffer containing 0.15 M KCl at 4°C for 3 hr. Bound hTFIIB was detected using anti-hTFIIB monoclonal antibody (Promega). Large and small chains of T7 antibody released from the Sepharose beads were also detected by the secondary antibody in the protein gel blot, as indicated.

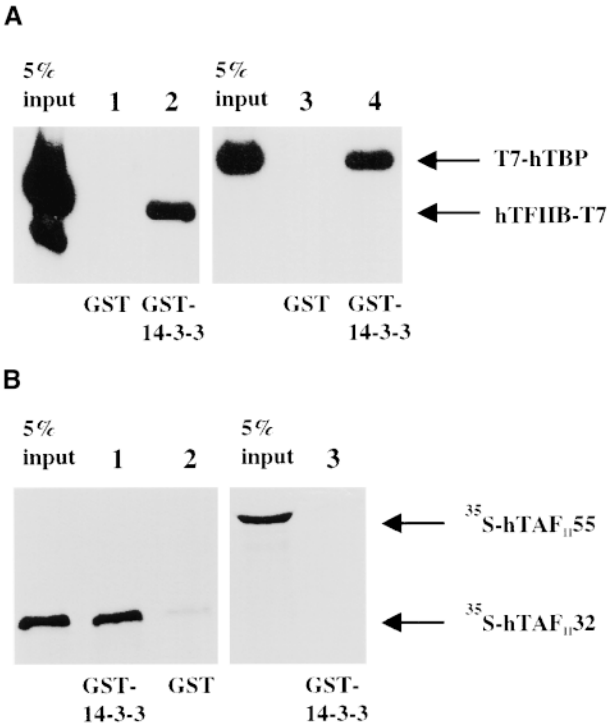

Figure 5. Immobilized h14-3-3ϵ Interacts with hTBP, hTFIIB, and $hTAF_{II}32$ but Not with $hTAF_{II}55$.

GST, GST–14-3-3, T7-hTBP, and T7-hTFIIB proteins were expressed in *E. coli*. $hTAF_{II}32$ and $hTAF_{II}55$ proteins (^{35}S-methionine labeled) were in vitro translated using the rabbit reticulocyte TNT system (Promega). Binding reactions were conducted at 4°C for 3 hr in buffer containing 0.15 M KCl. Input amounts (5%) of free ligand protein are shown.

(A) Binding reactions between immobilized h14-3-3ϵ and hTBP and TFIIB. Bound T7-hTFIIB and T7-hTBP were detected on protein gel blots by using the anti-T7 antibody.

(B) Binding reactions between immobilized h14-3-3ϵ and ^{35}S-labeled $hTAF_{II}32$ (lanes 1 and 2) and $hTAF_{II}55$ (lane 3). ^{35}S-labeled proteins were detected by 2,5-diphenyl-oxazole–enhanced autoradiography.

constructed as GST fusions so that the truncations did not intrude into regions with α-helical structure (each 14-3-3 monomer includes nine α helices). TFIIB showed affinity for both the N- and C-terminal halves of the immobilized 14-3-3 protein (Figure 6, lanes 6 and 7). Further progressive deletion of the C-terminal half of 14-3-3 (construct 7; helices 5 through 9) identified a single domain involved in binding TFIIB corresponding to helix 7, with helices 5, 6, 8, and 9 being dispensable (cf. lanes 4, 5, 11, 12, 13, and 16 with lane 15). Progressive deletion of the N-terminal helices (construct 6; helices 1 through 4) indicated that a second TFIIB binding domain is located within helices 2 and 3 (cf. lane 10 with lanes 8 and 9). Helices 2 and 3 appear to comprise a single functional unit required for TFIIB binding, because constructs containing only one helix were unable to bind (lanes 2 and 3).

Alanine Substitutions in 14-3-3 Helix 7 Identify Amino Acids Critical for Binding TBP and TFIIB

A detailed mutagenesis of 14-3-3 helix 7 was performed to identify amino acid residues required for binding TBP and TFIIB. These studies were conducted using plant proteins 14-3-3 (AtGF14ϕ), TBP2, and TFIIB from Arabidopsis. Unlike mutation by deletion, alanine substitution was assumed to have minimal effect on the overall structure of the 14-3-3 protein. Helix 7 of 14-3-3 was targeted for mutation due to its strong evolutionary conservation, being identical between many 14-3-3 isoforms of different origins, including AtGF14ϕ, h14-3-3ϵ, and h14-3-3η. In the context of full-length 14-3-3, residue pairs of helix 7 (amino acids 177-PIRLGLALNFSVFYYEI-193) were systematically substituted with two alanines in eight constructs to scan the entire helix.

As indicated by lanes 5 through 7 in Figure 7, a clear footprint in 14-3-3 binding activity was obtained for both AtTFIIB and AtTBP2. The F186A/S187A mutation nearly eliminated interactions with AtTFIIB and AtTBP2 (cf. lanes 6 and 1), indicating that these two residues are critically important for the interaction. Flanking mutations (L184A/N185A and

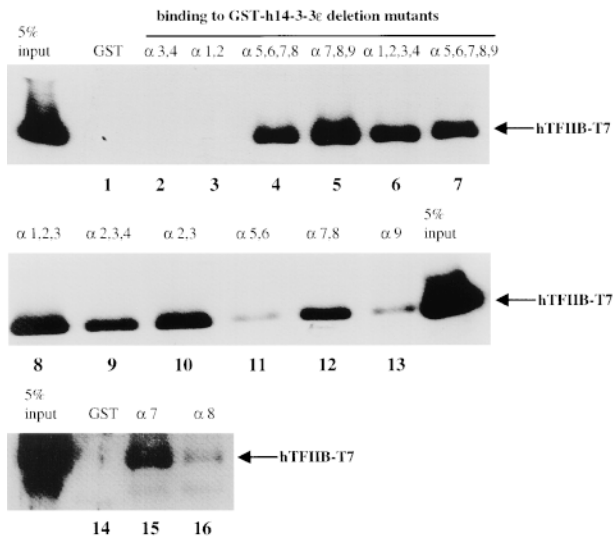

Figure 6. Identification of hTFIIB Binding Domains within h14-3-3ϵ.

A series of 14-3-3 deletion mutants was generated by polymerase chain reaction–based cloning such that individual α helices remained intact. Mutant proteins were immobilized as GST fusions and incubated at 4°C for 3 hr with T7-hTFIIB in binding buffer containing 0.15 M KCl. Bound T7-hTFIIB was detected on protein gel blots by using the anti-T7 antibody. Regions of hTFIIB interacting with h14-3-3ϵ were located on helices 2 and 3 (lane 10) and helix 7 (lane 15).

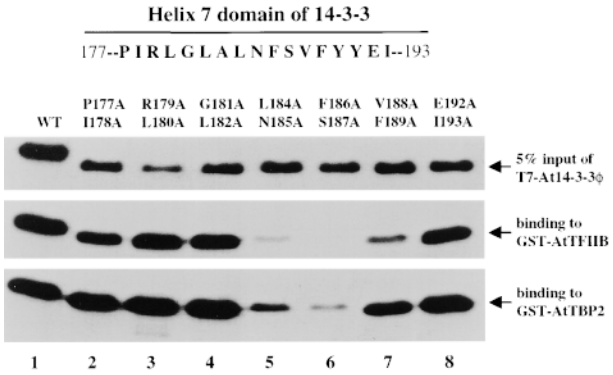

Figure 7. Analysis of Alanine Substitution Mutations within Helix 7 of the 14-3-3 Protein.

In the context of full-length AtGF14φ protein, every two amino acid residues of helix 7 were substituted with two alanine residues using the Altered Sites II-Ex1 in vitro mutagenesis system (Promega). These T7-tagged free mutant proteins were released from GST by thrombin digestion and incubated at room temperature for 2 hr with equal amounts of immobilized GST–AtTFIIB or GST–AtTBP2 in binding buffer containing 0.15 M KCl. Bound proteins were detected in protein gel blots by using the anti-T7 antibody. A clear footprint of binding disruption is seen in lanes 5 to 7, with residues F186 and S187 being the most critical for the interaction (lane 6). WT, wild type.

V188A/F189A) showed partial loss of binding capacity for AtTFIIB and AtTBP2 (cf. lanes 5 and 1 and lanes 7 and 1). Other residues more distal to F186 and S187 appeared to have no involvement in interactions with AtTBP2 or AtTFIIB, because corresponding mutant proteins still showed strong binding (cf. lanes 2, 3, 4, 7, and 8 with lane 1). Mutations for residues Y190 and Y191 were not obtained. As a whole, these results clearly show that the in vitro interactions between AtGF14φ and either AtTBP2 or AtTFIIB were highly specific and involved residues in the region from Leu-184 to Phe-189 of helix 7.

The GAL4 DNA Binding Domain–14-3-3 Chimera Stimulated *GAL4–GUS* Reporter Gene Expression in Onion Cells

The transcriptional potential of 14-3-3 proteins was assessed in plant cells by monitoring the activity of a GAL4 DNA binding domain–14-3-3 fusion protein in transient assays. Fusion of the GAL4 DNA binding domain to the N terminus provided a means of tethering the 14-3-3 protein to the promoter in addition to providing a nuclear localization signal. Transcriptional activity was monitored using a *GUS* reporter gene driven by a minimal cauliflower mosaic virus 35S promoter containing multiple copies of the GAL4 DNA binding site upstream. A second reporter composed of the luciferase gene driven by a maize ubiquitin promoter (Christensen and Quail, 1996) was used as an internal control for transformation efficiency. The reporter and effector constructs used in this study are diagramed in Figure 8A, and a representative experiment is shown in Figure 8B. In several repeated experiments using onion epithelial cells, coexpression of GAL4 DNA binding domain–14-3-3 (AtGF14φ) using a low amount of DNA (2 μg) showed no significant stimulation of GUS expression compared with GAL4 DNA binding domain alone. However, when high amounts of DNA (20 μg) were used, activity was stimulated approximately fivefold over background levels. Although the GAL4–14-3-3 protein is transcriptionally active in onion cells, the level of stimulation was low compared with a strong activator such as the GAL4 activation domain.

DISCUSSION

Using in vitro GST pull-down assays, we observed direct physical interactions between 14-3-3 proteins and TBP or 14-3-3 proteins and TFIIB in plants and humans. In addition, h14-3-3ε interacted with $hTAF_{II}32$ but not with $hTAF_{II}55$. These results suggest that the 14-3-3 proteins, when associated with a DNA binding complex or a DNA binding domain, may directly participate in the transcriptional activation of genes through contacts with the PIC. In agreement with this prediction, several studies have shown that 14-3-3 proteins associate with transcriptional regulatory complexes such as those containing VP1, EmBP1 (Schultz et al., 1998), the TLX-2 homeodomain protein (Tang et al., 1998), or the glucocorticoid receptor (Wakui et al., 1997).

Interactions between 14-3-3 proteins and general transcription factors appear to be a conserved phenomenon rather than being isoform specific. The seven 14-3-3 proteins from Arabidopsis, maize, and humans that were tested all had affinity for TFIIB, which is consistent with the highly conserved protein structure of the family. Although we did not test h14-3-3η in this study, it seems probable that it also has the ability to interact with TBP and TFIIB, because it shows 100% amino acid identity in helix 7. In addition, these interactions do not appear to use phosphoserine or any other previously characterized 14-3-3 recognition motifs such as RSXpSXP (Muslin et al., 1996), XXXSXXSXXXSXXSX (Vincenz and Dixit, 1996), RX(Y/F)XpSXP (Andrews et al., 1998), RXXSXpSXP (Liu et al., 1997), RXSX(S/T)XP (Yaffe et al., 1997), SGHSL (Gu and Du, 1998), or WLDLE (Petosa et al., 1998), because these sites are not present in TBP, TFIIB, or $hTAF_{II}32$.

Two isolated regions of 14-3-3 proteins are able to stably associate with TBP and TFIIB in vitro: helices 2 and 3, and helix 7. In the context of the full-length protein, a single point mutation within helix 7 almost abolished binding with TFIIB and TBP, suggesting that helices 2 and 3 make little additional contribution to interactions between the intact 14-3-3

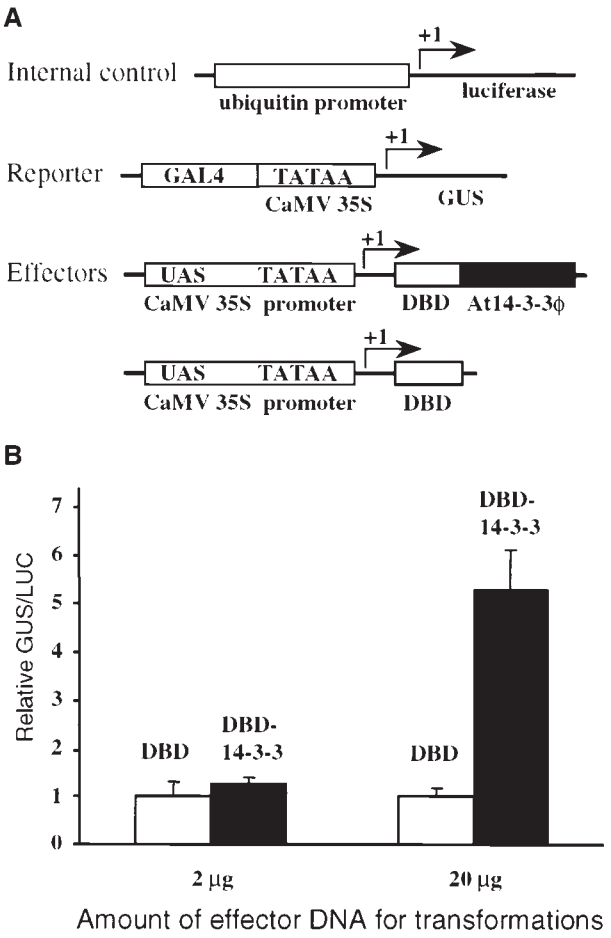

Figure 8. Evaluation of Transcriptional Activity of GAL4 DNA Binding Domain–AtGF14ϕ in Onion Epithelial Cells.

(A) Diagram of constructs used for in vivo transient expression assays. Expression of the *GUS* reporter gene was driven by the cauliflower mosaic virus (CaMV) 35S minimal promoter containing 10 copies of the GAL4 element inserted upstream of TATAA. Expression of the effectors GAL4 DNA binding domain (DBD) and GAL4 DNA binding domain–14-3-3 (AtGF14ϕ) was driven by the wild-type 35S promoter. The luciferase reporter driven by the maize ubiquitin promoter was used as an internal control for normalizing GUS activities to transformation efficiency.

(B) Effector and internal control plasmids were delivered into onion epidermal cells by particle bombardment. GUS and luciferase enzymatic activities were determined after a 40-hr incubation of transformed tissue at 25°C in the dark. Activities of GAL4-dependent transcription are shown as arbitrary units of the GUS/luciferase (LUC) ratio. Bars represent an average of three trials with standard deviations indicated. Compared with the GAL4 DNA binding domain construct alone, GAL4 DNA binding domain–AtGF14ϕ stimulated GUS expression fivefold when high amounts of effector DNAs (20 μg) were used, but no stimulation was observed with low amounts of effector DNAs (2 μg).

protein and TFIIB or TBP. 14-3-3 proteins consist of nine antiparallel helices, with four helices being highly invariant and forming the inner surface of an amphipathic groove (Liu et al., 1995; Xiao et al., 1995). Helices 3 and 5 generally interact with charged or polar groups in the target protein, and helices 7 and 9 interact with hydrophobic residues (Yaffe et al., 1997; Petosa et al., 1998). Although multiple helices of 14-3-3 are involved in binding to phosphoserine and nonphosphoserine target sites (Yaffe et al., 1997; Petosa et al., 1998), in vitro interactions can still occur with isolated pairs or single helices. For example, helix 9 was identified as the binding site for the platelet adhesion factor GPIbα (Gu and Du, 1998). Similarly, box 1, consisting of helices 7 and 8, was shown to bind several proteins, including tyrosine tryptophan hydroxylase (Ichimura et al., 1995), Raf-1, and Bcr (Ichimura et al., 1997). Although we demonstrate that isolated helix 7 and helices 2 and 3 are sufficient for in vitro binding of TBP and TFIIB, these results do not rule out the possibility that other helices also make close contact in the context of the full protein.

Binding studies conducted with the alanine mutations of helix 7 indicated that a stretch of amino acid residues from Leu-184 to Phe-189 were essential for binding TBP and TFIIB (Figure 7). The most severe disruptions of binding occurred with the F186A/S187A mutation, which alters two residues that are not oriented toward the amphipathic groove by analogy to the structure determined for h14-3-3ζ (Phe-174 and Ser-175) (Liu et al., 1995). Because the helix 7 mutations were assessed in the context of the intact protein, the requirement of Phe-186 and Ser-187 for binding may be related to their potential structural role in positioning helix 7 rather than their participation in direct interactions with the target proteins. However, the disruptions in binding seen with mutations in flanking residues such as L84/N185 and V188/F189 most likely interfere with helix 7 contacts with the target sites on TBP and TFIIB because these residues are accessible within the amphipathic groove of 14-3-3 (Liu et al., 1995).

The biological significance of in vitro interactions between 14-3-3 proteins and general transcription factors is supported by the close similarity between 14-3-3 and TFIIB interactions and those of the mammalian viral activator VP16 with TFIIB. As with VP16 (Roberts et al., 1993), deletion analysis of hTFIIB revealed two 14-3-3 binding domains localized to repeats 1 and 2 of the C-terminal conserved core. In addition, both 14-3-3 proteins (i.e., AtGF14ϕ) and VP16 interacted with the E1 helix of TFIIB in a similar manner. The R185E/R193E mutation within this helix disrupted binding for both proteins, and competition experiments demonstrated that VP16–hTFIIB and At14-3-3–hTFIIB interactions were mutually exclusive. The similarity of At14-3-3–hTFIIB and VP16–hTFIIB interactions in vitro further suggests that 14-3-3 proteins may be directly involved in the activation of transcription.

The potential of 14-3-3 proteins to influence transcription was evaluated by using the GAL4 DNA binding domain–14-

3-3 chimeric protein to activate *GAL4–GUS* reporter gene expression in vivo. In onion epidermal cells, the At14-3-3 protein showed weak transcriptional activity, with approximately fivefold stimulation of *GUS* expression compared with activity obtained using the GAL4 DNA binding domain alone. Although relatively low, this level of activity is similar to that observed for h14-3-3η stimulation of glucocorticoid receptor–dependent transcription in human cells (Wakui et al., 1997).

In summary, accumulating evidence suggests that 14-3-3 proteins may serve as coactivators in transcription in addition to their postulated roles in signal transduction. Although it is possible that 14-3-3 proteins may participate in transcriptional regulation by forming associations between protein members of a multicomponent transactivator complex, the affinity of 14-3-3 proteins for general transcription factors shows that 14-3-3 proteins also have the potential to act as a bridge between activators and general transcription factors, a process that may facilitate recruitment of the PIC to the promoter.

METHODS

Protein Expression: Growth and Induction in *Escherichia coli*

The cDNAs encoding 14-3-3 proteins, transcription factor IIB (TFIIB), or the TATA box binding protein (TBP) were inserted into the polylinkers of pGEX-2TK (Pharmacia, Piscataway, NJ), pET-15b, pET-24b, and pET-24d (Novagen, Madison, WI) *E. coli* expression vectors. The vector pGEX-2TK has the coding sequence for glutathione *S*-transferase (GST) followed by a thrombin cleavage site and a kinase site. The His (pET-15b) and T7 (pET-24b and pET-24d) tag coding sequences are located after the start codons, respectively. To maintain the correct reading frame, we introduced extra amino acids into either the C- or N-terminal ends of the protein coding regions by subcloning. The cDNA clone for human (h) 14-3-3ε was isolated from a human cDNA library (Clontech, Palo Alto, CA) using sequence-specific polymerase chain reaction primers.

For protein expression, pGEX-2TK constructs were transformed into *E. coli* BL21 and selected by ampicillin resistance. The transformed cells were grown overnight and then diluted 1:100 in 50 mL of Luria broth containing 10 μg mL^{-1} ampicillin and grown for an additional 3 hr at 37°C before isopropyl-1-thio-β-D-galactoside (IPTG) induction. The expression of GST fusion proteins was induced by 0.01 or 0.1 mM IPTG at 37°C for 2 hr. The pET constructs were transformed into *E. coli* strain BL21(DE3). Transformants grown overnight were diluted 1:100 in 50 mL of Luria broth containing the appropriate antibiotics and grown to an optical density of 0.6 ($OD_{600} = 0.6$) before IPTG induction. The expression of His- or T7-tagged fusion proteins was induced by 0.4 mM IPTG for 3 hr at 37°C. All IPTG-induced *E. coli* cells were collected by centrifugation, washed with cold protein binding buffer (PB), and suspended in 1 mL of cold PB (PB is 20 mM Hepes, pH 7.5, 150 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, 0.5 mM EDTA, and 0.05% Nonidet P-40). The cells were disrupted by sonication for three 1-min bursts and then centrifuged for 30 min at 12,000 rpm. Soluble proteins in the supernatants were either used directly in protein binding assays or purified by their specific affinity tags.

Protein Purification from *E. coli* Lysates

GST fusion proteins from *E. coli* lysates were purified by incubation with glutathione–agarose beads (Pharmacia, Piscataway, NJ) at 4°C for 30 min with continuous rotation. The beads were pelleted by brief microcentrifugation and then washed with cold PB plus 0.5 M KCl for 10 min followed by a final PB wash without added KCl. The beads containing the purified GST fusion proteins were suspended in PB plus 20% glycerol and stored at −20°C for use. When necessary, GST fusion proteins were eluted from beads by 0.2 M free glutathione at room temperature for 1 hr and then dialyzed against 0.5 L of PB for 40 hr with three changes. In some cases, recombinant proteins were cleaved from the GST moiety by thrombin digestion in PB at 4°C overnight. His-tagged proteins were purified by and eluted from Ni^{2+} beads, according to the manufacturer's recommendations (Novagen). T7-tagged proteins were purified by protein A–conjugated beads coated with the T7 antibody, which served as a bridge between protein A and the T7 tag by immunoaffinity. The pelleting and washes for T7-tagged proteins were the same as those for GST fusion proteins. The quantity of each individual protein was estimated using a Coomassie Brilliant Blue R 250–stained SDS–polyacrylamide gel.

In Vitro Protein Translation

$hTAF_{II}32$ and $hTAF_{II}55$ proteins were obtained by in vitro translation and labeled using ^{35}S-methionine with a transcription/translation–coupled (TNT) rabbit reticulocyte system, according to the manufacturer's protocol (Promega). The TAF cDNAs were cloned into the pET-24d vector (Novagen), which utilizes the T7 promoter.

In Vitro GST Pull-Down Assay

To study the interactions of 14-3-3 proteins with the general transcription factor proteins, we fused one of the two interacting proteins with GST and purified it on glutathione–agarose beads, as described above. The second protein from an *E. coli* lysate or from rabbit reticulocytes was then incubated with the beads for 3 hr at either 4°C or room temperature in 600 μL of 1 × PB containing 0.5% BSA with continuous rotation. The beads were then extensively washed with PB (four times in 1 mL each). The beads were pelleted by spinning in a minicentrifuge for 15 sec after each washing. The bound protein molecules were finally resolved by SDS-PAGE and detected by an appropriate antibody with an enhanced chemiluminescence system (Pharmacia) or by 2,5-diphenyl-oxazole–enhanced autoradiography. The percentages of free protein input for the binding reactions are indicated.

Site-Specific Point Mutagenesis

An altered site II mutagenesis system (Promega) was used to generate alanine substitution mutants for 14-3-3 proteins, according to the manufacturer's protocol. The polylinker of the pALTER-Ex1 vector (Promega) was engineered so that the T7 tag coding sequence (MASMTGGQQMG) was placed immediately after the start codon. Arabidopsis 14-3-3φ cDNA (Wu et al., 1997b) was subcloned into the pALTER-Ex1 vector in frame with the T7 tag coding sequence. All

mutants were confirmed by DNA sequencing (Microbiology Department Sequencing Facility, University of Florida, Gainesville) and subcloned into pGEX-2TK to express the T7-tagged proteins.

Transient Expression Assay

Transient assays were conducted using epidermal peels from large yellow onions (from the supermarket). The middle layers of the onions were cut into square pieces of ~2 × 2 cm in size. The epidermal layers were then peeled off and placed on individual Murashige and Skoog plates (Murashige and Skoog, 1962) immediately before use.

The reporter and effector plasmid DNAs were precipitated onto gold particles (1.6 μm; Bio-Rad) as follows. The combined DNAs in a volume of 35 μL was mixed with 37 μL of gold suspension (40 mg mL^{-1} deionized water) in a 1.5-mL microcentrifuge tube. Next, 50 μL of 2.5 M $CaCl_2$ and 20 μL of 100 mM spermidine were deposited in separate droplets on the inner wall of the tube and then immediately mixed with the DNA and gold particles by vortexing for 20 sec. The particles were pelleted for 5 sec, and the supernatant was discarded. The DNA-coated gold particles were then washed in 200 μL of 100% ethanol by sonication for 5 sec using a small sonicator bath, collected by centrifugation for 5 sec, and resuspended in 100% ethanol to a final volume of 80 μL. Each DNA–gold preparation contained 5 μg of the internal control plasmid and 5 μg of the reporter plasmid in addition to various amounts of effector plasmid. The internal control plasmid consisted of a luciferase reporter driven by the maize ubiquitin promoter (Ubi-LUC) (Christensen and Quail, 1996). The reporter plasmid was composed of the β-glucuronidase (*GUS*) gene driven by 10 GAL4 elements upstream of the cauliflower mosaic virus 35S minimal promoter (Odell et al., 1985). The effector plasmids encoding either GAL4 DNA binding domain or the GAL4 DNA binding domain–14-3-3 fusion were expressed by the wild-type 35S promoter.

The DNA–gold particles were delivered into onion epidermal cells by using the Bio-Rad particle bombardment system at 1100 psi. For each bombardment, 5 μL of well-suspended DNA–gold particles was pipetted onto the carrier disk and allowed to air dry before use. The bombarded onion tissues were allowed to recover for 40 hr in the dark at 25°C and then were harvested by grinding for 1 min using a mortar and pestle in extraction buffer containing 200 mM Na_2HPO_4/NaH_2PO_4, pH 7.8, 4 mM EDTA, 2 mM DTT, 5% glycerol, and 1 mg mL^{-1} BSA. The supernatants were collected by centrifugation for 15 min at 12,000 rpm, and aliquots were assayed for GUS and luciferase activities.

GUS and Luciferase Assays

Luciferase assays were conducted as an internal control using a multipurpose scintillation counter to measure single-photon emission from the enzymatic reaction in which 5 μL of maize cell extract was incubated with 25 μL of luciferase substrate (Promega) at room temperature. Light emission was measured immediately after the mixing of extract and substrate. To measure GUS activity, we incubated 50 μL of the original extract with 75 μL of 2 mM 4-methylumbelliferyl β-D-glucuronide (GUS substrate) at 37°C for 2.25 hr. The reaction was stopped by mixing 50 μL of the reaction mixture with 950 μL of 0.2 M Na_2CO_3. The product of the enzymatic reaction, 4-methylumbelliferone, was measured using a spectrofluorophotometer, with excitation and emission wavelengths of 365 and 445 nm, respectively. The 4-methylumbelliferone concentration after 15 min was considered background and subtracted from the final 4-methylumbelliferone concentration after the 2.25-hr reaction. Activities were expressed as a ratio of GUS units to counts per minute of luciferase photon emission.

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