Dimerization of *Arabidopsis* 14-3-3 proteins: structural requirements within the N-terminal domain and effect of calcium

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Abstract The structural requirements for dimerization of RCI14A and RCI14B, two 14-3-3 isoforms from *Arabidopsis thaliana*, have been analyzed by testing truncated forms of RCI14A for dimerization with full-length RCI14A and RCI14B. The results show that only the fourth helix of the truncated partner is essential for dimerization, which represents a difference from what is known for animal isoforms. On the other hand, the effect of calcium has been tested in RCI14A homodimerization. Millimolar concentrations of calcium exert a negative, dose-dependent effect that involves the C-terminal domain of RCI14A and might modulate interactions with other cellular components or among *Arabidopsis* 14-3-3 isoforms.

Key words: 14-3-3 protein; Protein dimerization; Calcium; *Arabidopsis*

1. Introduction

The 14-3-3 proteins were discovered in the late 1960s as a family of highly abundant mammalian brain proteins [1]. Since then, they have been described in many eukaryotic organisms [2,3]. They are highly conserved and have been proposed to have a variety of functions, being now accepted as a novel type of chaperones that modulate interactions between components of signal-transduction pathways [2,3]. The broad spectrum of activities ascribed to 14-3-3 proteins could be related to the coexistence of several isoforms that can act as homo- or heterodimers [2]. In this way, different 14-3-3 proteins have been shown to form dimers in vivo and in vitro, and mammalian isoforms ζ and τ have been crystallized as dimers [4,5]. In both proteins, the monomers consist of nine antiparallel helices organized in two structural domains, and dimerization takes place at the N-terminal domain, which contains the first four helices [4,5]. Deletion experiments with mammalian isoform ζ have shown that dimerizations cannot take place between a full-length monomer and a truncated one lacking the first three helices [6], suggesting that these are essential to support dimerization. The C-terminal domain of 14-3-3 proteins, which contains the last five helices, appears to be involved in interactions with other cellular components [6,7].

In plants, genes encoding 14-3-3 proteins have been isolated from several species [3]. In all cases, the deduced polypeptide sequences revealed a high degree of conservation with animal isoforms, which made it possible to predict a structure of nine antiparallel helices [3]. There are, however, certain features that appear to be characteristic of plant isoforms, such as an EF-hand-like motif located in the C-terminal domain, where calcium binding has been reported [8,9]. The functions of plant 14-3-3 proteins have not been clearly established yet. The expression of some of the genes encoding plant isoforms is tissue-specific and developmentally regulated [10], while others are modulated by environmental stimuli [10–12]. In *Arabidopsis*, 10 genes encoding 14-3-3 isoforms have been described [10,13]. We previously reported the isolation of cDNAs corresponding to two of them, *RCI1A* and *RCI1B*, formerly called *RCI1* and *RCI2*, respectively [10]. The expression of these two genes is induced by low temperature in a development-independent way, the kinetics of *RCI1A* and *RCI1B* mRNAs accumulation by low temperature being correlated with the increase in freezing tolerance that occurs during the cold acclimation process in *Arabidopsis* [10,14].

The high level of sequence conservation between all 14-3-3 proteins suggests that the structural requirements for dimerization are probably similar in plant and animal variants. Interactions among plant 14-3-3 isoforms have been the object of study by using non-denaturing PAGE and the yeast two-hybrid system [15]. This made it possible to detect homo- and heterodimers among eight *Arabidopsis* isoforms. In the same report, deletion analyses showed that, as in the case of animal isoforms (see above), dimerization takes place at the N-terminal domain, since a truncated GF14χ isoform, consisting of the first four helices, can form dimers with full-length GF14χ and ψ from *Arabidopsis* and with pGAD14 from maize [15]. Thus, it seems that interactions between *Arabidopsis* 14-3-3 isoforms occur through the four helices that compose their N-terminal domains. It is not established, however, if all four helices, or only some of them, are essential for dimerization. Moreover, dimerization with *Arabidopsis* GF14ε required a longer truncated GF14χ containing the N-terminal domain plus the fifth helix [15]. Whether this requirement, which has not been described for animal isoforms so far, is just an exceptional circumstance for interaction between GF14χ and GF14ε or a more common situation for plant 14-3-3 dimerizations remains to be studied.

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Abbreviations: BSA, bovine serum albumin; DTT, 1,4-dithiothreitol; GST, glutathione transferase; IPTG, isopropyl-β-D-thiogalactoside; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; TCA, trichloroacetic acid

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In this report, we present a detailed analysis of the structural requirements for dimerization between *Arabidopsis* isoforms RCI14A and RCI14B (referred to as GF14ψ and GF14λ in [13]), which are the gene products of *RCI1A* and *RCI1B*, respectively [10]. While isoform RCI14A (hereafter 1A) has been analyzed in dimerization studies with other isoforms [15], the dimerization requirements of isoform RCI14B (hereafter 1B) have not been studied so far. By using truncated forms of 1A, we show that only the fourth helix is essential for dimerization. Interestingly, helices 1, 2, 3 and 5 seem not to be required for these interactions, which represents a denoting difference from what is known for animal 14-3-3 isoforms. Furthermore, since it has been reported that binding of calcium at the C-terminal domain of GF14ω induces a conformational change in this isoform [8,16], we have also analyzed the possibility that calcium could modulate the interaction among plant 14-3-3 isoforms. Here, we show that calcium interaction with the C-terminal domain of 1A has a dose-dependent, negative effect on dimerization.

2. Materials and methods

2.1. Generation of radiolabelled proteins

DNA manipulations were performed according to standard protocols [17]. The complete coding sequences of *RCI1A* and *RCI1B* [10], as well as truncated forms of *RCI1A* (1NZ, 1C, 1ZC and 1ΔZ) were used to obtain the corresponding polypeptides (Fig. 1A). All subclones except 1C were obtained by PCR with *Taq* polymerase, using suitable oligonucleotides as primers. In all cases, constructs were sequenced to ensure that no mutations had been introduced during the PCR reactions. To generate constructs 1NZ, 1ΔZ and 1ZC, codons 127–255 (1NZ), 3–72 (1ZC) or 84–123 (1ΔZ) were deleted. Construct 1C was obtained after deletion of codons 3–121 by digestion of the full-length construct with suitable restriction enzymes and religation. Radiolabelled proteins 1A, 1B, and truncated forms of 1A, were synthesized in vitro by coupled transcription-translation of the corresponding cDNAs cloned into Bluescript KS(−), using Promega TNT-coupled wheat germ extract system with [^{35}S]Met and T3 RNA polymerase, according to the manufacturer's protocol (Fig. 1B).

2.2. Purification of GST fusion proteins

The expression vector pGEX-2T (Pharmacia) was used to obtain glutathione transferase (GST) fusion proteins in *Escherichia coli*. Fusion proteins GST-1A and GST-1B, as well as control GST protein, were isolated by affinity chromatography in glutathione-Sepharose from IPTG-induced *E. coli* cultures, according to the protocol provided by the supplier.

2.3. Dimerization assays

Purified proteins GST-1A, GST-1B or GST were immobilized in glutathione-Sepharose for in vitro dimerization assays. Fifty μl of resin with 15 μg of the immobilized protein were mixed with 200 μl of HND buffer (20 mM HEPES pH 7.2; 50 mM NaCl; 5 mM DTT; 1 mg/ml BSA) containing $0.5–1\times10^6$ cpm of a radiolabelled protein. Mixtures were incubated for 1 h at 4°C with gentle agitation, and non-bound proteins were washed out with 0.05% Nonidet P-50 in PBS buffer (4 mM NaH_2PO_4, 16 mM Na_2HPO_4, pH 7.3; 150 mM NaCl). Radioactive proteins bound to the Sepharose-immobilized fusion proteins were eluted by addition of 2×SDS-PAGE loading buffer and boiling. Samples were loaded onto 12.5% SDS-PAGE gels and proteins were detected by fluorography.

For quantitative binding experiments, the concentration of in vitro translated polypeptides was determined previously. A fraction of each translation mixture was placed on a piece of 3MM paper and polypeptides were immobilized by incubation in ice-cold 10% TCA. The remaining [$_{35}$S]Met was removed by boiling in 10% TCA containing 1 mg/ml methionine. The 3MM papers were washed with ethanol, dried, and radioactivity measured in a scintillation counter. Subsequently, equal molar amounts of each radiolabelled polypeptide were subjected to dimerization assays with GST-1A, GST-1B or GST. After washing the resin, bound polypeptides were eluted as described above. To determine the relative amount of polypeptides retained in the resin, radioactivity was measured in the eluates. Relative binding efficiency was calculated considering the number of methionines in each polypeptide, and was expressed in relative units, where one unit was defined as the amount of radiolabelled 1A bound to GST-1A. In dimerization assays in the presence of $CaCl_2$ or $MgCl_2$, the relative amount of radioactive protein bound to the resin was directly quantified by laser scanning densitometry (Molecular Dynamics, UK) of fluorographs exposed within the linear range of the film.

3. Results

3.1. The fourth antiparallel helix of protein 1A is sufficient to support dimerization with a full-length partner

In order to determine whether dimerization of 1A and 1B proteins could be detected in our experimental conditions, we performed dimerization assays between Sepharose-immobilized fusion proteins GST-1A and GST-1B, and in vitro synthesized radiolabelled 1A and 1B products (see Section 2 and Fig. 1). As a negative control for binding specificity, we carried out the same assays using Sepharose-immobilized GST protein. The results showed that radiolabelled 1A or 1B were retained by GST-1A (Fig. 2A, lanes b and e) and GST-1B (lanes c and f) but not by GST (lanes a and d). In all cases, 0.5–1% of the radiolabelled protein loaded was detected in dimerization assays with GST-1A and GST-1B, as estimated by densitometry (not shown). The small amount of radiolabelled proteins that associates with the resin-immobilized ones is probably due to the fact that 14-3-3 proteins dimerize in solution [8]. Direct interaction among 14-3-3 dimers has not been detected [15], which indicates that the interactions described here represent dimerizations between a radiolabelled monomer and a resin-immobilized partner. In control assays

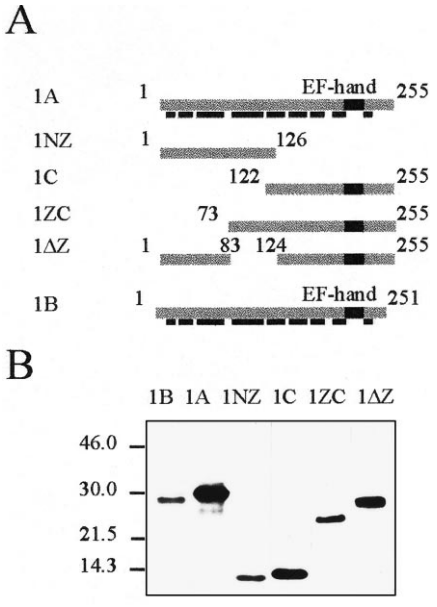

Fig. 1. Polypeptides used in this work. A: Schematic representation of the different polypeptides. Black boxes indicate the location of the EF-hand-like motif. Blocks under the sequences indicate the antiparallel helices described in [4,5]. Numbers refer to the first and last amino acids of each truncated form, respectively. Positions flanking the deletion in 1ΔZ are also indicated. B: Fluorography after SDS-PAGE of in vitro translated polypeptides shown in A. Molecular weight markers are indicated on the left.

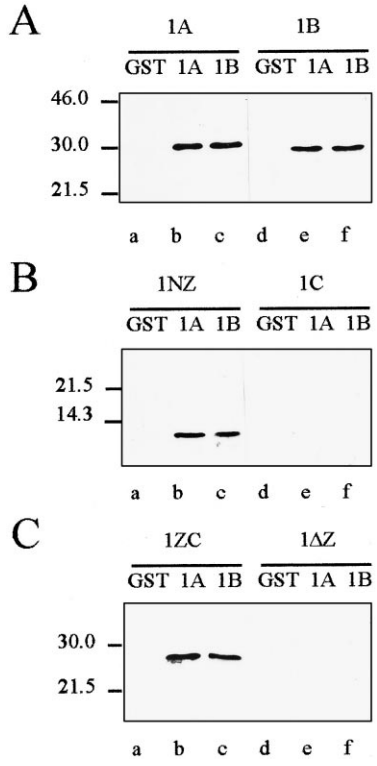

Fig. 2. In vitro dimerization assays with 1A, 1B and truncated forms of 1A. A: Radiolabelled proteins 1A (lanes a–c) and 1B (lanes d–f) were incubated with equal amounts of Sepharose-immobilized GST (lanes a and d), GST-1A (lanes b and e) and GST-1B (lanes c and f). Proteins retained in the resin after washing (see Section 2) were separated by SDS-PAGE and revealed after fluorography of the gels. B: Radiolabelled polypeptides 1NZ (lanes a–c) and 1C (lanes d–f) were tested for dimerization with Sepharose-immobilized proteins GST (lanes a and d), GST-1A (lanes b and e) and GST-1B (lanes c and f) as described in A. C: The same as in B, but using radiolabelled polypeptides 1ZC (lanes a–c) and 1ΔZ (lanes d–f). Molecular weight markers are indicated on the left.

with Sepharose-immobilized GST, less than 0.001% of the protein loaded was detected, which represents the background of unspecific binding (not shown). In all experiments, 1A and 1B bound nearly equally to either GST-1A or GST-1B, suggesting that homo- and heterodimers are similarly formed in our experimental conditions.

Since dimerization of plant 14-3-3 proteins is mainly determined by their N-terminal domains [15], truncated polypeptides corresponding to the full N-terminal (1NZ) and C-terminal (1C) domains were synthesized (see Section 2 and Fig. 1). These polypeptides were used to determine whether dimerizations among 1A and 1B required the four helices from the N-terminal domain or, as in the case of GF14χ and GF14ε [15], an additional helix from the C-terminal domain was necessary. Dimerization assays between Sepharose-immobilized fusion proteins GST-1A and GST-1B and radiolabelled polypeptides 1NZ and 1C were carried out. Similar assays with Sepharose-immobilized GST protein were performed as controls for binding specificity. Polypeptide 1NZ was retained by GST-1A (Fig. 2B, lane b) and GST-1B (lane c) but not by GST (lane a), showing the same behavior as the full-length 1A and 1B proteins (see above). By contrast, polypeptide 1C was not detected after incubation with any of the three Sepharose-immobilized proteins (lanes d–f). These results indicated that the sequences of 1A located within the first four helices of the protein were sufficient to support homodimerizations as well as heterodimerizations with 1B.

Deletion experiments with mammalian 14-3-3 isoform ζ have shown that dimerization cannot take place when one of the monomers lacks the first three helices [6]. In order to test whether dimerizations between 1A and 1B have the same requirement, we generated a truncated form of 1A where the first three helices from the N-terminal domain were deleted. The polypeptide obtained, called 1ZC (see Section 2 and Fig. 1), included the full C-terminal domain of the protein plus the fourth helix of the N-terminal one. The results of dimerization tests showed that polypeptide 1ZC was retained by GST-1A (Fig. 2C, lane b) and GST-1B (lane c) but not by GST (lane a), and therefore behaved like the full-length protein. Thus, the first three helices of isoform 1A were not necessary to support homo- and heterodimerizations with full-length 1A and 1B, respectively. To confirm the importance of the fourth helix from the N-terminal domain in dimerization, we generated polypeptide 1ΔZ, which comprises the 1A full-length protein except for 40 amino acids including the fourth helix (see Section 2 and Fig. 1). Dimerization assays showed that 1ΔZ was not retained by any of the Sepharose-immobilized proteins (Fig. 2C, lanes d–f), indicating that the fourth helix of the truncated partner was necessary for dimerization.

In order to compare the binding efficiency of the different polypeptides used in this work, quantitative dimerization assays between radiolabelled products and Sepharose-immobilized GST proteins were performed. The results of interactions between GST-1A and the radiolabelled polypeptides (Fig. 3A)

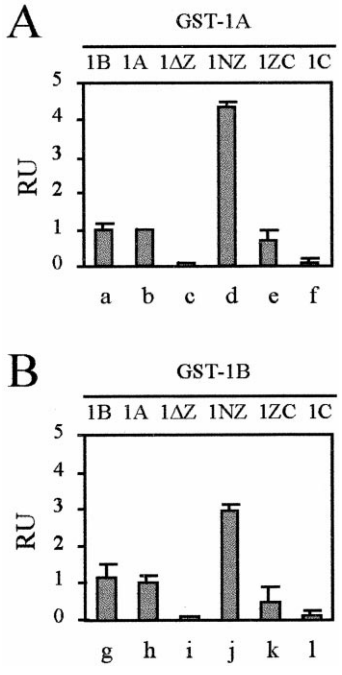

Fig. 3. Quantification of polypeptide binding in dimerization assays. Equal molar amounts of radiolabelled polypeptides 1B (lanes a and g), 1A (lanes b and h), 1ΔZ (lanes c and i), 1NZ (lanes d and j), 1ZC (lanes e and k) and 1C (lanes f and l) were tested for dimerization with Sepharose-immobilized proteins GST-1A (A) or GST-1B (B) as described in Fig. 2. Relative binding efficiency was quantified as described in Section 2. Bars represent S.E.M. of two independent experiments. RU: relative units. One unit was defined as the amount of radiolabelled 1A bound to GST-1A.

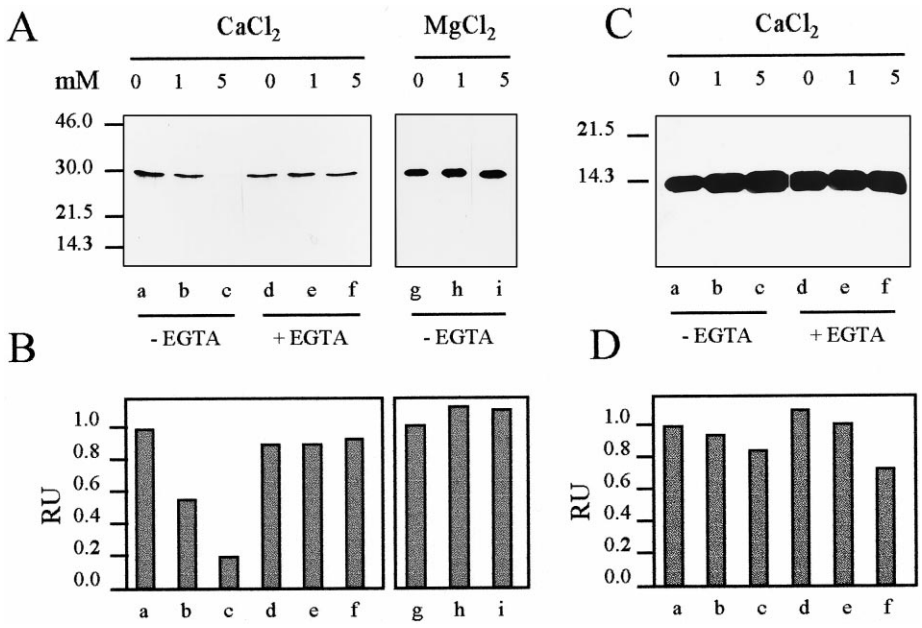

Fig. 4. Effect of calcium on 1A homodimerization. Radiolabelled 1A and 1NZ polypeptides were tested for dimerization with Sepharose-immobilized GST-1A, as described in Fig. 2, in the presence of different concentrations of $CaCl_2$ (lanes a–f) or $MgCl_2$ (lanes g–i), and in the absence (lanes a–c and g–i) or presence of 20 mM EGTA (lanes d–f). A and C: Fluorography of the gels obtained in representative experiments with radiolabelled 1A (A) and 1NZ (C) polypeptides. Molecular weight markers are indicated on the left. B and D: Relative quantification of the dimerization assays shown in A and B, respectively. RU: relative units. One unit was defined as the amount of radioactivity detected in homodimerizations, as shown in lane a of both panels.

showed that similar amounts of 1B (lane a), 1A (lane b) and 1ZC (lane e) were retained by the resin, while the retained levels of radiolabelled 1NZ were substantially higher (lane d). In contrast, the amounts of radiolabelled 1ΔZ and 1C retained by the resin were barely detectable (lanes c and f). The same results were obtained when GST-1B was used to test interactions with the radiolabelled polypeptides (Fig. 3B). Altogether, these results indicate that polypeptides containing both the fourth helix and the C-terminal domain of 1A have comparable efficiencies for dimerization. Polypeptide 1NZ, which contains the first four helices of 1A but lacks the C-terminal domain, has a higher efficiency, indicating that the presence of the C-terminal domain in the full-length protein reduces the efficiency of the interactions.

3.2. Dimerization of protein 1A is affected by calcium

Binding of Ca^{2+} has been reported for *Arabidopsis* 14-3-3 variant GB14ω [8]. The region of GB14ω involved in Ca^{2+} binding is located within the C-terminal domain, where a EF-hand-like motif was identified [8]. An analysis of the sequence of 1A and 1B reveals that they contain this motif at positions 207–218 and 211–222, respectively (Fig. 1A). In GB14ω, binding of Ca^{2+} appears to induce a conformational change [8,16]. We hypothesized that such a change could modify the dimerization capability of the protein. To test this possibility, the effect of different Ca^{2+} concentrations in homodimerization assays between radiolabelled 1A and Sepharose-immobilized GST-1A was studied. The experiments were performed as described above, with binding buffers containing $CaCl_2$ concentrations in the range used in previous GB14ω-Ca^{2+} interaction studies [8]. As a control, the same reactions were carried out in the presence of 20 mM EGTA. Fig. 4A,B shows the results of a representative assay, in which 0, 1 and 5 mM $CaCl_2$ were tested. The presence of Ca^{2+} in the buffer decreased the amount of radioactive protein retained proportionally to $CaCl_2$ concentration (lanes a–c). This effect was not observed when 20 mM EGTA was included (lanes d–f). Interaction assays performed in the presence of $MgCl_2$ (lanes g–i) showed that magnesium did not affect the dimerization, indicating that the effect is specific of Ca^{2+}.

If the effect of Ca^{2+} in dimerization was due to an interaction with the C-terminal domain of 1A, which contains the EF-hand-like motif, Ca^{2+} should not have a specific effect in dimerization between polypeptides lacking this region. To test this hypothesis, we performed dimerization assays between radiolabelled polypeptide 1NZ and Sepharose-bound GST-1A in the presence of 0, 1 or 5 mM $CaCl_2$ (Fig. 4C,D). In these experiments, non-substantial differences in binding efficiency were observed when Ca^{2+} was included in the buffer (lanes a–c). Similar results were obtained in the presence of 20 mM EGTA (lanes d–f), indicating that, in the case of 1NZ, the small differences observed were not due to a Ca^{2+}-specific effect in dimerization. Therefore, our results show that Ca^{2+} affects the formation of 1A homodimers, an effect that appears to be specifically related with the C-terminal region of the protein.

4. Discussion

The 14-3-3 proteins are always found as dimers [2]. Mammalian isoform ζ has been crystallized, as a dimer, in complex with two peptide ligands [7]. In addition, mutant monomeric forms of isoform ζ could not enable Raf to be activated [18]. These results suggest that dimerization of 14-3-3 proteins should be essential for at least some of their functions. A central role for dimerization has been proposed for the N-terminal domain of 14-3-3 proteins [4,5]. Crystal structure of mammalian isoforms ζ and τ has shown that the N-terminal

domains of the two monomers are located in an antiparallel fashion and have two contact regions, each one corresponding to helices 1–2 of one monomer that interact with helices 3–4 of the other [4,5]. Deletion analysis of isoform ζ has shown that the elimination of the first three helices impairs dimerization with a full-length partner [6], indicating that the two contact regions between the N-terminal domains are necessary to stabilize the dimer.

The sequences of plant 14-3-3 isoforms show a high degree of homology with animal isoforms [3,19], which suggests that dimerization could have similar structural requirements. In this way, heterodimerization experiments among several *Arabidopsis* isoforms revealed that the N-terminal domain is sufficient in most cases for interactions. However, in one of the isoforms considered, GF14ε, the fifth helix was also required to ensure dimerizations [15]. This result implies that some sequences located at the C-terminal domain could have a direct role in certain dimerizations, and suggests that the spatial arrangement between the two monomers might not be exactly the same in all cases.

In this work, we have investigated the structural requirements for dimerization of plant 14-3-3 proteins using *Arabidopsis* isoforms 1A and 1B. Isoform 1A has been reported to dimerize with isoform GF14χ [15]. According to these authors, the interaction is mediated by the four helices that constitute the N-terminal domain of GF14χ. The dimerization requirements of isoform 1B have not been studied so far. We have analyzed the capacity of 1A and 1B to homo- and heterodimerize, as well as the regions that are essential for these interactions. The results revealed that 1A and 1B can dimerize with similar efficiency regardless of which protein was immobilized in the Sepharose resin. Similar conclusions were reported for eight 14-3-3 variants from *Arabidopsis*, including 1A, using other experimental approaches [15]. Thus, it seems that most 14-3-3 proteins from *Arabidopsis* can form homo- and heterodimers. It is still unclear whether all these dimerizations have a functional meaning since, in the context of a plant cell, interactions with other cellular components could modulate the dimerization specificity of the different subunits [2].

A truncated isoform 1A containing the first four antiparallel helices that compose its N-terminal domain was able to dimerize with full-length 1A and 1B. Therefore, the sequences that are essential for dimerization among 1A and 1B are restricted to the N-terminal domain, as in the case of animal isoforms and most plant isoforms tested [6,15]. This result suggests that the requirement of sequences located at the C-terminal domain is specific for the interaction between isoforms GF14χ and GF14ε [15]. On the other hand, we show that a polypeptide containing the C-terminal domain of isoform 1A is not competent for dimerization. This result harmonizes with the general idea that the C-terminal domain of 14-3-3 proteins is not directly involved in dimerizations, but in interactions with other cellular components [2,3].

In contrast to the results reported for mammalian isoform ζ [6] (see above), a truncated 1A obtained after deletion of helices 1–3 (polypeptide 1ZC) was able to form dimers with both 1A and 1B, the efficiency of dimerization being comparable to that obtained with full-length proteins. Thus, the fourth α-helix of the truncated monomer was sufficient to drive in vitro dimerizations with full-length partners. The three-dimensional structure proposed for 14-3-3 dimers [4,5] predicts that dimerization between 1ZC and 1A or 1B would rely solely on interaction of helices 1–2 of the full-length protein with helix 4 of 1ZC. On the other hand, deletion of the fourth α-helix from isoform 1A (polypeptide 1ΔZ) impaired homo- and heterodimerizations with full-length partners. This result seems to indicate that the fourth helix of the truncated protein is essential for dimerization. Alternatively, the change in the relative spacing of the two protein domains caused by the deletion in 1ΔZ could induce a conformational change that would impair the formation of dimers. Taken together, our results suggest that one contact region between the N-terminal domains from isoforms 1A and 1B is sufficient to keep the two partners together as long as the relative spacing of the N-terminal and C-terminal domains of the proteins remains intact. This is compatible with the finding that, in plant 14-3-3 proteins, interactions with other proteins take place not only at the C-terminal domain, but also through sequences located at the N-terminus [20].

The results from quantitative dimerization assays seem to indicate that removal of the C-terminal domain favors dimerization. This implies that the C-terminal domain could have an indirect role in in vivo dimerization of 14-3-3 proteins. A conformational change induced by interaction of the C-terminal domain with calcium has been described for the 14-3-3 *Arabidopsis* isoform GF14ω [8,16]. Here, we show that the formation of 1A homodimers is negatively affected by calcium, and that this effect is not observed when one of the monomers lacks the C-terminal domain. These results suggest that the interaction of the C-terminal domain from *Arabidopsis* isoform 1A with calcium causes a reduction in the dimerization capability of the protein. Alternatively, calcium binding could have a positive effect on dimerization. Namely, calcium could cause the immobilized 14-3-3 and free solution 14-3-3 dimers to be so strongly bound that there would no longer be any exchange between the two groups of dimers. The fact that dimerization is not affected by magnesium indicates that the effect of calcium is specific, and not due to unspecific interactions with negatively charged residues. Calcium is a well-known second messenger in a variety of intracellular signal transduction pathways [21]. Transient increases in cytosolic calcium concentration have been described to occur after different stimuli, including chilling [22], and calcium signalling has also been involved in long-term response to low temperatures leading to cold acclimation [23,24]. The effect of calcium in 1A dimerization could be related to a possible role of this protein in the process of cold acclimation by modulating its interaction with different cellular components, including other 14-3-3 isoforms. Experiments to elucidate the function of 1A and 1B are under way in our laboratory, and will help to clarify their potential role in cold acclimation as well as in other stress conditions.

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