Specificity of ε and Non-ε Isoforms of Arabidopsis 14-3-3 Proteins Towards the H⁺-ATPase and Other Targets

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Abstract

14-3-3 proteins are a family of ubiquitous dimeric proteins that modulate many cellular functions in all eukaryotes by interacting with target proteins. 14-3-3s exist as a number of isoforms that in Arabidopsis identifies two major groups named ε and non-ε. Although isoform specificity has been demonstrated in many systems, the molecular basis for the selection of specific sequence contexts has not been fully clarified. In this study we have investigated isoform specificity by measuring the ability of different Arabidopsis 14-3-3 isoforms to activate the H⁺-ATPase. We observed that GF14 isoforms of the non-ε group were more effective than ε group isoforms in the interaction with the H⁺-ATPase and in the stimulation of its activity. Kinetic and thermodynamic parameters of the binding of GF14e and GF14n isoforms, representative of ε and non-ε groups respectively, with the H⁺-ATPase, have been determined by Surface Plasmon Resonance analysis demonstrating that the higher affinity of GF14n is mainly due to slower dissociation. The role of the C-terminal region and of a Gly residue located in the loop 8 and conserved in all non-ε isoforms has also been studied by deletion and site-specific mutagenesis. The C-terminal domains, despite their high divergence, play an auto-inhibitory role in both isoforms and they, in addition to a specific residue located in the loop 8, contribute to isoform specificity. To investigate the generality of these findings, we have used the SPOT-synthesis technology to array a number of phosphopeptides matching known or predicted 14-3-3 binding sites present in a number of clients. The results of this approach confirmed isoform specificity in the recognition of several target peptides, suggesting that the isoform specificity may have an impact on the modulation of a variety of additional protein activities, as suggested by probing of a phosphopeptide array with members of the two 14-3-3 groups.

Introduction

14-3-3 proteins are a family of evolutionary conserved dimeric proteins that accomplish a wide range of regulatory roles in eukaryotes, including cell cycle control, mitogenesis, and apoptosis [1]. In plants, these proteins regulate primary metabolism, ion transport, cellular trafficking, gene transcription and hormone signalling [2,3].

14-3-3 proteins exist as multiple isoforms and the common theme in their mechanism of action is the ability to associate with target proteins, through binding to consensus motifs [4,5]. Generally, 14-3-3 binding to the target occurs at phospho- Ser or phospho-Thr-containing motifs, RXS(pS/pT)XP and RXY/FXS(pS/pT)XP, defined as mode I and mode II motifs, respectively [5]. More recently, a mode III C-terminal binding motif, where the residue preceding the carboxy-terminal end is a phosphorylated Ser or Thr, has also been defined [6]. A large number of 14-3-3 target proteins have thus far been identified, both in animals and in plants [7]. For some of them the 14-3-3 binding site has also been determined [8].

The crystal structures solved for human [9–11] and tobacco [12] 14-3-3 isoforms demonstrated that 14-3-3 proteins share a very similar tridimensional architecture, with a highly conserved amphipathic groove involved in the ligand binding.

In Arabidopsis thaliana thirteen 14-3-3 isoforms are expressed [13] and are often referred to as GF14 proteins, since they were initially identified as a part of a G-box binding complex [14]. Comparison of the Arabidopsis 14-3-3 isoforms reveals a high degree of amino acid identity, being the differences confined at the N and C termini [15]. Based on a phylogenetic analysis, Arabidopsis 14-3-3s can be divided into two major groups named ε and non-ε. The 14-3-3 ε group has five members – μ (mu), ε (epsilon), π (pi), τ (tota), and Ω (omicron) – while the non-ε group has eight members – κ (kappa), λ (lambda), Ψ (psi), ν (nu), υ (upsilon), Ω (omega), ϕ (phi), and χ (chi). The relatively large number of 14-3-3 isoforms, as well as the abundance of 14-3-3 target proteins, has raised the issue of functional specificity. It is not clear whether 14-3-3s can accomplish specific functions by binding their targets in an isoform-specific manner. Structural analysis does not provide support for the hypothesis of isoform specificity, since the solvent exposed surface of the target-binding pocket is highly conserved among isoforms [11,12,16,17]. In several systems 14-3-3 isoforms were shown to be experimentally interchangeable [18], thus
suggesting functional redundancy. In this respect, the isoform specificity demonstrated in in vivo studies may be the result of differences in the expression patterns rather than residing in the different biochemical properties of 14-3-3 proteins. In accordance, differential expression of 14-3-3 isoforms was observed in different tissues and organs [19], as well as during plant development or in response to different environmental stimuli [20].

On the other hand, several pieces of evidence suggest that 14-3-3 isoforms specifically interact with different target proteins. To mention some examples, in plants, the nitrate reductase [21,22], the plasma membrane H⁺-ATPase [23,24], the sucrose-phosphate synthase [25], phototropin 1 [26] and, more recently, the ABA-responsive-element Binding Factor (ABF) [27]. Interestingly, the differential subcellular distribution of 14-3-3s seems to be dependent upon specific interactions with cellular clients [28].

The reported functional specificity somewhat contrasts with the observation that the target binding pocket is highly conserved in the different isoforms. It has been proposed that the C-terminal region of 14-3-3s, characterized by a high level of divergence among isoforms, can play a role in client binding [29–32]. In addition, 14-3-3s binding to several targets requires the presence of divalent cations and polyamines [33,34] that seem to modulate phosphopeptide binding by affecting the conformation of an EF hand-like motif within loop 8 [35] of the 14-3-3 structure. Accordingly, mutation of a specific amino acid residue in loop 8 affects the divalent cation binding and alters client binding selectivity [21,36].

In the present work, we studied the molecular mechanisms underlying the Arabidopsis 14-3-3 isoforms recognition specificity in the modulation of the activity of the plasma membrane H⁺-ATPase, one of the best characterized plant 14-3-3 client proteins [37]. In addition, we used the SPOT-synthesis technology to assemble a peptide array representing different known putative 14-3-3 binding sites. Results demonstrate that H⁺-ATPase preferentially interacts with non-c 14-3-3 isoforms and show the role of the C-terminal region in the isoform specificity. Furthermore, peptide array studies identified additional targets that are bound by 14-3-3s in an isoform-specific manner.

Materials and Methods

Chemicals

[γ-32P]ATP (specific activity 110 TBq mmol⁻¹) was obtained from Amersham Biosciences (Uppsala, Sweden).

The bL15Vp peptide biotinyl-LKGLDIDTIQQNYTpV (Tp, phosphothreonine) was synthesized by Neosystem (Strasbourg, France).

Streptavidin-agarose magnetic beads, glutathione-sepharose resin, the catalytic subunit of protein kinase A and thrombin were obtained from Sigma (St. Louis, MO, USA).

Restriction enzymes were obtained from Roche Diagnostics (Mannheim, Germany). Pfu turbo DNA polymerase was obtained from Stratagene (La Jolla, CA, USA).

Chemicals for SDS-PAGE were obtained from Bio-Rad (Hercules, CA, USA).

Plant material

Maize caryopses (Zea mays L. Hybrid PR35P12) from Pioneer Italia Hi Bred (Parma, Italy) were germinated and seedlings were grown in the dark for five days, as previously described [38].

Purification of plasma membrane from maize roots

Two-phase partitioned plasma membranes were obtained from 20 g of maize roots as previously described [38].

Purification of endoplasmic reticulum from yeast expressing AHA1

The Arabidopsis H⁺-ATPase isoform 1 (AHA1) was expressed in Saccharomyces cerevisiae, as previously described [39]. Following cell homogenization, membranes were purified by differential centrifugation and endoplasmic reticulum (ER), containing most of the AHA1, was isolated by sucrose gradient centrifugation [38].

Isolation of GF14 isoform cDNA

Arabidopsis GF14e (At1g22300), 6oAt1g78300, g(At4g09000), k(At5g65430), l(At5g10450), µ (At2g42590) and o (At1g34760) cDNAs were obtained by RT-PCR. Total RNA isolated from Arabidopsis seedlings using the RNasy Plant Mini RNA kit (Qiagen) was subjected to retro-transcription by using the SuperScript First-strand Synthesis system (Invitrogen) and PCR with primers corresponding to the coding region sequences, with added terminal EcoRI and BamHI restriction sites for subsequent cloning. All amplified cDNAs were controlled by DNA sequencing (Eurofins MWG Operon, Ebersberg, Germany).

Site-directed mutagenesis of GF14 proteins

pGEX-2TK carrying the GF14e and the GF14o cDNA were used as templates for mutagenesis of GF14e and GF14o proteins. Site-directed mutagenesis of GF14e and GF14o proteins was performed by using the ‘QuickChange site-directed mutagenesis method’ (Stratagene) following the manufacturer’s protocol.

The GF14eΔC and GF14oΔC mutants, deleted of the last 20 and 23 amino acids, were obtained by changing the Asp235 codon of GF14e and the Asp237 codon of GF14o, into the Stop codons, thereby preventing translation of the last 20 and 23 amino acids respectively.

The GF14eN211G and the GF14oG213N mutants were obtained by changing the Asn211 codon of GF14e into the Gly codon and the Gly213 codon of GF14o into the Asn codon, respectively.

The mutagenic primers used were (the mutation generated is underlined): 5’ CTCCACCTTTGTGGACACTTACATGTTATAT-GGAGAAGGAGATG 3’ and its reverse complement for GF14eΔC and 5’ CAATCTCAACTCTTGACATCTTA-GATGCGGATGTGCTG 3’ and its reverse complement for GF14oΔC; 5’ GCTGAACCTTGACAGGCAGACGATCATACAGACG 3’ and its reverse complement for GF14eN211G and 5’ GGAGAGTTGGACACACTTAAATGGAA-GAGGTACAAAGACAG 3’ and its reverse complement for GF14oG213N.

After 18 cycles of PCR (30 s at 95°C, one minute at 55°C, seven minutes at 68°C), 10U of DpnI were added to the mixtures to digest the GF14e and the GF14o cDNA templates and reactions were carried out at 37°C for 2 h. A 20 μl aliquot of each mixture was used to transform E. coli DH5a competent cells. Incorporation of mutations was controlled by DNA sequencing.

Expression of recombinant proteins in E. coli

Wild-type and mutated GF14 proteins were expressed in E. coli BL21 (DE3) strain as Glutathione S-Transferase (GST)-fusion proteins using the pGEX-2TK vector, while the C-terminal domain of the Maize H⁺-ATPase isoform 2 (MH2) (corresponding to the last 103 amino acids) was expressed as a GST-fusion protein using pGEX-2T according to the last 103 amino acids) was expressed as a GST-fusion protein using pGEX-2T following the procedure described by Fullone et al. [40].
A

B

C

14-3-3 Isoform Specificity in Arabidopsis
32P-labeling of GST fusion proteins

Wild type and mutated GF14 proteins were labelled with [γ32P]-ATP on the phosphorylation site present at the junction between GST and the cloned protein, using the catalytic subunit of PKA as already described [40]. Specific activities of 32P-labeled proteins were similar (about 90 GBq mmol⁻¹).

Overlay assay

The overlay assay was carried out according to Fullone et al. [40] with minor modifications; 3 μg GST-fused C-terminal domain of H+-ATPase or 20 μg of maize plasma membranes, containing the H+-ATPase, were separated on SDS-PAGE and blotted on nitrocellulose membrane by semidry electroblotting (Bio-Rad). The membrane was blocked with 5% fatty acid-free milk in 25 mM HEPES-OH, 75 mM KCl, 5 mM MgCl₂, 1 mM dithiothreitol, 0.1 mM EDTA, 0.05% Tween-20, pH 7.5 (buffer HT) and then cut into identical strips which were incubated in the absence or presence of 10 μM FC. Densitometric analysis was performed on four independent overlay experiments and data are the means ± S.E. of three independent experiments.

Binding to bL15Vp peptide

The bL15Vp peptide (0.05 nmol) was immobilized onto 20 μl of streptavidin–agarose magnetic beads and incubated with 0.1 nmol 32P-labeled-GF14 isoforms in the presence or in the absence of 10 μM FC. Radioactivity was measured in a liquid scintillation β-counter (Tri Carb 2100TR Packard).

Surface Plasmon Resonance

The kinetic parameters, association rate constants (kₐ) and dissociation rate constants (kₖ₋ₒ) were determined using the BIAcoreX system for Surface Plasmon Resonance (SPR) detection.

Biotinylated peptide bl15Vp (600 resonance units) was captured on an Biacore SA sensor chip precoated with streptavidin. The analyte (GF14c or GF14c) was applied in the concentration ranges of 10 μM–7.8 nM. The interaction was followed in real time. Experiments were performed in HT buffer in the absence or presence of 10 μM FC at 25°C. Solutions of GF14 protein in the running buffer were injected into the flow cell and passed over the peptide surfaces at a continuous flow rate of 30 μl/min for two minutes. Dissociation was monitored for 5 min. The sensor surface was regenerated for the next sample using a 2 μl pulse of 50 mM glycine-HCl pH 2 to remove GF14 protein bound to the immobilized peptide.

Phospho-hydrolytic activity

The AHA1 phospho-hydrolytic activity of yeast ER membranes was assayed according to Camoni et al. [12] with minor modifications: 10 μg of sucrose gradient-purified yeast ER were pre-incubated with different concentrations of wild-type or mutated GST-GF14 isoforms (ranging from 0 to 4 μM) in 500 μl of 50 mM Tris-MES, 5 mM MgCl₂, 50 mM KNO₃, 5 mM NaN₃, 0.2 mM ammonium molybdate, pH 7.2 (buffer A) in the presence of 10 μM FC. After a 20 minutes incubation, 2 mM ATP was added.

| GF14 Isoform | kₐ (M⁻¹ s⁻¹) | kₖ₋ₒ (s⁻¹) | Kᵦ (nM) |
|-------------|--------------|-------------|---------|
| GF14c       | 5.63 × 10⁶   | 1.27 × 10⁻² | 231     |
| GF14a       | 7.63 × 10⁶   | 3.82 × 10⁻³ | 53      |
| GF14c + FC  | 2.17 × 10⁴   | 3.07 × 10⁻³ | 124     |
| GF14a + FC  | 1.84 × 10⁴   | 5.43 × 10⁻⁵ | 4.1     |

The kinetic parameters, association rate constants (kₐ) and dissociation rate constants (kₖ₋ₒ) were determined using the BIAcoreX system for SPR detection. Biotinylated peptide bl15Vp (600 resonance units) was captured on an Biacore SA sensor chip precoated with streptavidin. GF14c or GF14a were applied in the concentration ranges of 10 μM–7.8 nM in the absence or presence of 10 μM FC at 25°C, with a flow rate of 30 μl/min. Dissociation was monitored for five minutes. The data were evaluated using BIAevaluation 3.1 (Biacore AB). To obtain Kₒ and Rmax values, a 1:1 Langmuir binding model was fitted to the sensograms.

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obtained in the 200 and 250 nm range using 5 m
280 nm and the emission wavelengths ranging from 300 to 500 nm
fluorescence spectra were obtained at the excitation wavelength of
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following subtraction of the buffer background. The measured
spectrum was averaged over four scans and subjected to smoothing
structure prediction
Circular dichroism analysis and protein secondary
Tryptophan fluorescence spectroscopy
Figure 2. Tryptophan fluorescence emission spectra and
circular dichroism spectra of GF14ω and GF14ε. A. Tryptophan
fluorescence spectra were obtained at the excitation wavelength of
280 nm and the emission wavelengths ranging from 300 to 500 nm
using 1.5 μM protein. B. Far-UV CD spectra of GF14ω and GF14ε were
obtained in the 200 and 250 nm range using 5 μM protein. Each
spectrum was averaged over four scans and subjected to smoothing
following subtraction of the buffer background. The measured
ellipticity data were converted to mean molar ellipticity ([θ], degcm²
dmol⁻¹). Continuous line, GF14ε; dotted line, GF14ω.
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Tryptophan fluorescence spectroscopy
Tryptophan fluorescence spectra were recorded with a Perkin
Elmer luminescence spectrometer LS50B at 25 °C using 1.5 μM
GF14 in 1 ml of 25 mM Tris-HCl, 150 mM NaCl, pH 7.5. The excitation
wavelength was 280 nm and the emission wavelength ranged from 300 to 500 nm.

Circular dichroism analysis and protein secondary
structure prediction
Circular Dichroism (CD) measurements were performed using a
Jasco 600 spectropolarimeter, equipped with a thermal controller
calibrated with camphor-sulfonic acid. Far-UV CD experiments
were carried out to explore the conformation of the GF14 proteins
(5 μM). CD spectra were obtained between 200 and 250 nm using

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Estimation of the secondary structure contents was done with the
GOR structure prediction method version IV [43].

Peptide array
The peptides, 13 aminoacids long, were synthesized according
to standard solid phase synthesis protocols [44], using an
automatic SPOT synthesizer (Intavis, Koeln, Germany). In this
approach, peptides are synthesized in array format, bound to
cellulose membranes, which are activated with amino PEG
(Intavis). The chemistry uses Fmoc protected amino-acids, with
protected side chains, which are deprotected at the end of the
synthesis.

Before use, the membranes were wet in ethanol and washed
repeatedly in PBS. Membranes were blocked overnight at 4 °C in
buffer HT containing 5% BSA (blocking buffer).

The binding assay was performed incubating the membranes
with 0.2 μM GST-fused GF14 isoforms, or GST alone as a
negative control, in blocking buffer overnight at 4 °C. After
washing membranes three times for 10 minutes with HT buffer,

Densitometric analysis of positive spots was performed using the
Imagej image processing program [41] and data expressed as
Integrated Densitometric Value (the product of the area and mean
gray value).

Analytical methods
Protein concentration was determined by the method of
Bradford [45], using bovine serum albumin as the standard.

Statistics
Data were analyzed using Microsoft Excel and assessed for
significance by the Student’s t-test.

Results
Non-ε GF14 isoforms preferentially interact with the H⁺-
ATPase
Some isoforms belonging to the ε and non-ε groups were used
to investigate their ability for binding with and activating the H⁺-
ATPase. GF14ε, GF14k, GF14δ, and GF14o isoforms, belonging
to the non-ε group, and GF14ε, GF14k, and GF14o isoforms,
belonging to the ε group were expressed in E. coli as GST-fusion
proteins, purified by affinity chromatography and used in overlay
experiments with the H⁺-ATPase. The plasmalemma H⁺-ATPase
was immobilized onto nitrocellulose membrane and incubated
with identical amounts of 32P-labelled GF14 isoforms. The
proteins were immobilized onto nitrocellulose membrane and
incubated

Densitometric analysis of bands detected in the overlay
experiments was performed using a chemo-luminescence
substrate and the LAS-3000 instrument (Luminescent Image
Analyzer, Fujifilm).

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Imagej image processing program [41] and data expressed as
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proteins, purified by affinity chromatography and used in overlay
experiments with the H⁺-ATPase. The plasmalemma H⁺-ATPase
was immobilized onto nitrocellulose membrane and incubated
with identical amounts of 32P-labelled GF14 isoforms. The
experiment was performed also in the presence of fusicoccin
(FC), a fungal toxin known to activate the H⁺-ATPase by strongly
stabilizing its interaction with 14-3-3 proteins [46–48].

Densitometric analysis of bands detected in the overlay
experiments (see Figure 1A) shows that GF14ε, GF14k and
GF14o interacted with the H⁺-ATPase (left panel) at a very low
extent, both in the presence and in the absence of FC. On the

a path-length of 0.1 cm with a time constant of 1.0 s, a 2 nm
bandwidth and a scan rate of 2 nm min⁻¹ and at 10 mdeg
sensitivity. Each spectrum was averaged over four scans and
subjected to smoothing following subtraction of the buffer
background. The measured ellipticity data were converted to
mean molar ellipticity ([θ], degcm² dmol⁻¹).

Estimation of the secondary structure contents was done with the
gOR structure prediction method version IV [43].
contrary, non-ε isoforms were all able to interact with the H⁺-ATPase in both conditions.

14-3-3 proteins can also interact with the H⁺-ATPase in a phosphorylation-independent manner, but exclusively in the presence of FC [40]. GF14 isoforms were compared in their interaction with the C-terminal domain (103 amino acids) of the H⁺-ATPase, expressed in E. coli as a GST-fusion protein and therefore not phosphorylated. A similar result was obtained, as shown in Figure 1A (right panel), non-ε isoforms bound to C-terminal domain, at difference with all ε group isoforms.

Overlay data were corroborated by results obtained with a phosphorylated biotinyl-peptide (bL15Vp) reproducing the last 15 residues of the MHA2 H⁺-ATPase. This peptide contains the YpTV 14-3-3 binding sequence and it has previously been reported to bind 14-3-3 proteins in pull down assay [32]. bL15Vp was immobilized onto streptavidin-agarose beads and then incubated with 32P-labelled GF14 isoforms in the presence and absence of FC. The results, reported in Figure 1B, showed that GF14 non-ε isoforms interacted with the peptide much more efficiently than ε isoforms and the difference between the two groups was significant both in the absence and in the presence of FC.

GF14 isoforms were also assayed for their ability to activate the H⁺-ATPase. A recombinant AHA1 H⁺-ATPase isoform was expressed in yeast where most of the functional enzyme is located at the endoplasmic reticulum [49] and can be significantly and reproducibly stimulated by exogenous 14-3-3, if FC is added [50]. AHA1 H⁺-ATPase was incubated with different amounts of GF14 isoforms, in the presence of 10 μM FC. The effect of different concentrations of GF14 isoforms on the phosphohydrolytic activity of recombinant AHA1 H⁺-ATPase is shown in Figure 1C. All the non-ε isoforms tested were more active than the ε isoforms. Among the ε isoforms, GF14 μ resulted more effective in stimulating the H⁺-ATPase, while GF14e and GF14o displayed a very low activity at higher concentrations as well, which is in accordance with interaction data reported above.

Results obtained strongly suggest that the H⁺-ATPase binding ability of GF14 isoforms depends on the phylogenetic group to which they belong [13].

Two GF14 isoforms, GF14ε and GF14o, representative of the ε and non-ε groups, respectively, were selected to better characterize the interaction with the H⁺-ATPase.

Firstly, a kinetic and thermodynamic study of the interaction was performed by SPR analysis using the bL15Vp phosphopeptide. The kinetic and dissociation constants, determined for the interaction in the absence and in the presence of FC, are reported in Table 1. As shown, in the absence of FC the equilibrium dissociation constant of GF14o was about 5 fold lower than for GF14ε. This different affinity is mainly due to a slower dissociation rate for GF14o, being that the koff was about 10 fold lower for the GF14o interaction, while the kon resulted of the same order of magnitude for both isoforms. In the presence of FC, the difference in the equilibrium dissociation constants was even more evident and the GF14o affinity to bL15Vp peptide was increased more than 10 fold, in accordance with what had already been determined for maize GF14-6 isoform by SPR [46] and for the tobacco 14-3-3c isoform by isothermal titration calorimetry [12]. The FC effect was on the dissociation phase, being that the koff was strongly reduced by the toxin and the kon almost unaffected.
A control on the possible role of protein folding in the different behaviour of the two isoforms was performed by comparing the tryptophan intrinsic fluorescence spectra of GF14\(v\) and GF14\(e\). As shown in Figure 2A, the emission spectra of GF14\(v\) and GF14\(e\) were superimposed, in accordance with the number and position of all tryptophan residues conserved in the two GF14 isoforms, thus indicating that they shared a very similar folding. Their secondary structure was also analysed by circular dichroism spectroscopy. The Far-UV CD spectra (Figure 2B) showed that the \(\alpha\)-helices were the major component in the secondary structure for both isoforms and were comparable to previously reported spectra \[21,33\]. The \(\alpha\)-helical content of GF14\(e\) was slightly lower than GF14\(v\) and this was also the case for the in silico prediction of secondary structure. In fact, the predicted GF14\(v\) \(\alpha\)-helical content was 64.86\%, whereas that of GF14\(e\) was 54.33\%.

Role of the C-terminal domain in the isoforms specificity towards the \(H^+\)-ATPase

Previous reports have suggested a role for the divergent C-terminal domain of 14-3-3 isoforms in different ligand binding and discrimination \[30,32,33\]. To verify whether the different behaviour of GF14 isoforms in their binding ability and
stimulation of H^+-ATPase may be the consequence of their divergent C terminus, C-terminal deletion mutants were produced and used in interaction studies. GF14e deleted of the last 23 amino acid residue (GF14eΔC) and GF14e deleted of the last 20 amino acid residues (GF14eΔC) (Figure 3) were expressed in E. coli as GST-fusion proteins to be compared to wild type isoforms in binding assay with the immobilized bL15Vp peptide, in the presence or in absence of 10 mM Mg2+.

The association of 14-3-3 proteins with the H^+-ATPase may be the consequence of their divergent C terminus, C-terminal deletion mutants were produced and used in interaction studies. GF14e deleted of the last 23 amino acid residue (GF14eΔC) and GF14e deleted of the last 20 amino acid residues (GF14eΔC) (Figure 3) were expressed in E. coli as GST-fusion proteins to be compared to wild type isoforms in binding assay with the immobilized bL15Vp peptide, in the presence or in absence of 10 mM Mg2+.



For all 14-3-3 target proteins, the interaction with Arabidopsis GF14 proteins was demonstrated. *, peptides reproducing 14-3-3 binding site identified in Arabidopsis 14-3-3 clients. **, peptides gathered from an alignment of identified binding sequence of 14-3-3 clients derived from different plant species and the corresponding Arabidopsis targets. All the other peptides reproduced putative 14-3-3 binding site, present in the indicated Arabidopsis 14-3-3 target, and chosen based on their similarity with the 14-3-3 mode I or mode II binding motif.

NR, nitrate reductase; TPSS, trehalose phosphate synthase 5; F2KP, fructose-6-phosphate,2-kinase/fructose-2,6-bisphosphatase; SPS, sucrose-phosphate synthase; GS1, glutamine synthetase 1; GS2, glutamine synthetase 2; GADPH, glyceraldehydes-3-phosphate dehydrogenase; BZR1, brassinazole-resistant 1 protein; TPK1, two pore K+ channel 1; AHA1, Arabidopsis homologue of the H^+-ATPase channel 1; CPK1, calcium-dependent protein kinase 1; phot1, phototropin 1; phot2, phototropin 2; ABF3, ABA-responsive-element Binding Factor 3; SERK1, somatic embryogenesis receptor-like kinase 1; KAPP, kinase associated protein phosphatase.

So, Spinacia oleracea; Bo, Brassica oleracea; Vf, Vicia faba.

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NR, nitrate reductase; TPSS, trehalose phosphate synthase 5; F2KP, fructose-6-phosphate,2-kinase/fructose-2,6-bisphosphatase; SPS, sucrose-phosphate synthase; GS1, glutamine synthetase 1; GS2, glutamine synthetase 2; GADPH, glyceraldehydes-3-phosphate dehydrogenase; BZR1, brassinazole-resistant 1 protein; TPK1, two pore K+ channel 1; AHA1, Arabidopsis homologue of the H^+-ATPase channel 1; CPK1, calcium-dependent protein kinase 1; phot1, phototropin 1; phot2, phototropin 2; ABF3, ABA-responsive-element Binding Factor 3; SERK1, somatic embryogenesis receptor-like kinase 1; KAPP, kinase associated protein phosphatase.

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For all 14-3-3 target proteins, the interaction with Arabidopsis GF14 proteins was demonstrated. *, peptides reproducing 14-3-3 binding site identified in Arabidopsis 14-3-3 clients. **, peptides gathered from an alignment of identified binding sequence of 14-3-3 clients derived from different plant species and the corresponding Arabidopsis targets. All the other peptides reproduced putative 14-3-3 binding site, present in the indicated Arabidopsis 14-3-3 target, and chosen based on their similarity with the 14-3-3 mode I or mode II binding motif.

NR, nitrate reductase; TPSS, trehalose phosphate synthase 5; F2KP, fructose-6-phosphate,2-kinase/fructose-2,6-bisphosphatase; SPS, sucrose-phosphate synthase; GS1, glutamine synthetase 1; GS2, glutamine synthetase 2; GADPH, glyceraldehydes-3-phosphate dehydrogenase; BZR1, brassinazole-resistant 1 protein; TPK1, two pore K+ channel 1; AHA1, Arabidopsis homologue of the H^+-ATPase channel 1; CPK1, calcium-dependent protein kinase 1; phot1, phototropin 1; phot2, phototropin 2; ABF3, ABA-responsive-element Binding Factor 3; SERK1, somatic embryogenesis receptor-like kinase 1; KAPP, kinase associated protein phosphatase.

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stimulatory effect of Mg$^{2+}$ on wild type GF14ω was significantly higher than that observed on GF14ω213N. These results strongly suggest that the Gly residue in the loop 8 of non-ε GF14 isoforms has a relevant role in their binding properties; this concurs with data reported on the isoform GF14 ω where substitution of Asn213 residue with Gly produced a mutant displaying a more flexible C-terminal region and a different target specificity compared to wild type [36].

**Isoform specificity of GF14 proteins towards other Arabidopsis targets**

The different behaviour of ε and non-ε isoforms towards the H+-ATPase could be more generally shared by other 14-3-3 Arabidopsis targets. To investigate this possibility, a phosphopeptide array, obtained by using the SPOT-synthesis technology, was tested with different GF14 isoforms. The peptide array consisted of eight repeats of 21 tridecapeptides (reported in Table 2) containing known or putative 14-3-3 binding sites of Arabidopsis target proteins chosen from the literature data. Some peptides were designed based on 14-3-3 binding site identified in orthologous 14-3-3 clients in different plant species, others were designed on putative binding sites identified in the Arabidopsis target protein either on the basis of homology with 14-3-3 mode I or mode II binding motifs or because they were known to be in vivo phosphorylated. As peptides are immobilized by their C-termini, the mode III binding sites where the C-terminus is directly involved in the interaction were not considered.

All peptides were phosphorylated on a single Ser or Thr residue, positioned in the center of the sequence. Binding assay was performed by incubating each array with a GST-GF14 isoform or GST alone, as a control. Peptide-bound proteins were revealed by an anti-GST antibody conjugated to peroxidase.

As shown in Figure 6, GF14 isoforms differently bound to the peptides. As expected, GF14 isoforms bound to peptides reproducing the 14-3-3 binding site of nitrile reductase (NR: 1) [51], trehalose-phosphate synthase 5 (TPS5: 2, 3) [51], brassinazole-resistant 1 protein (BZR1: 12) [52], two pore K$^+$ channel (TPK1: 13) [53], phototropin 2 (Phot2: 17) [54] and ABA-responsive-element Binding Factor 3 (ABF3: 18) [55], in agreement with the literature data identifying these sequences as 14-3-3 binding sites, thus confirming the validity of this approach for investigating the in vitro interaction of 14-3-3 proteins with their targets.

Some of the hypothetical 14-3-3 binding sites, although related to mode I or mode II binding motifs, did not interact in the assay: no binding was observed with the putative binding sequence of Arabidopsis sucrose-phosphate synthase (SPS: 7) and cytosolic glutamine synthetase (GS1: 8) – gathered from alignment with the binding site in the Spinacia oleracea [56] and Brassica oleracea [57] orthologs respectively –, the putative 14-3-3 binding site of Arabidopsis Glu-tRNA synthetase (11) [58], calcium-dependent protein kinase-1 (CPK-1: 14) [59] and embryogenesis receptor-like kinase 1 (SERK1: 20) [60]. Nevertheless this result was not totally unexpected, since these peptides contain residues in specific positions negatively affecting 14-3-3 binding, as Yaffe et al. [5] and, more recently, Panini et al. [61] demonstrated using degenerate peptide libraries. The Glu-tRNA synthetase peptide (11) has two negatively charged residues at −1 and −2 positions, with respect to the phosphorylated residue, as well as an Asn residue at +1 position, all features known to reduce the affinity with 14-3-3 proteins. Similarly, the CPK-1 peptide (14) contains a Glu residue at −1 and a Lys residue at +1 position, while the SERK1 peptide (20) contains a Pro residue at −1 position, which markedly hamper 14-3-3 binding.

Interaction was instead detected with the putative binding sequences of fructose-6-phosphate,2-kinase/fructose-2,6-bisphosphatase (F2KP: 4 and 5) [62], glyceraldehydrolase-3-phosphate dehydrogenase (GAPDH: 10) [58], ATP synthase β-subunit (15) [63], phototropin 1 (Phot1: 16) [64], AAA-ATPase Cdc48 (19), PP2C kinase-associated protein phosphatase (KAPP: 21) [60] and, even to a very low extent, the plastidic glutamine synthetase (GS2: 9) [58].

Interestingly both the F2KP putative binding sites interacted with GF14 proteins. These two sequences have been demonstrated to be in vivo phosphorylated, but the direct evidence of their involvement in 14-3-3 binding has thus far been lacking and only the sequence surrounding the Ser303, corresponding to peptide 5, has been proposed as 14-3-3 binding site for its similarity with the 14-3-3 mode I binding motif [62].

In addition the ATP synthase β-subunit sequence has been supposed to be a 14-3-3 binding site based on its homology with the mode I motif [63] and here is shown to be directly involved in 14-3-3 binding.

Notably, in some cases GF14 isoforms differently interacted with the same peptide. Densitometric analysis of the positive spots allowed for better evaluating the differences in the GF14 interactions (Figure 7). Interestingly, with some peptides GF14 isoforms belonging to the same phylogenetic ε and non-ε group displayed similar interaction capability, suggesting the existence of a “group specificity”, similarly to that observed with the AHA1 H+-ATPase. In particular, with the ATP synthase β-subunit (15), phot2 (17) and ABF3 (18) peptides the interaction of all GF14 ε isoforms was higher respect to that of all non-ε isoforms, whereas F2KP (4) and GS2 (9) peptides interacted exclusively with isoforms belonging to the non-ε group.

It is worth noting that GF14ω, among the non-ε isoforms, was more active in most of the interactions tested. Moreover, with BZR1 (14), TPK1 (15) and KAPP (21) peptides GF14ω displayed a different behaviour compared to the other non-ε members, in fact
its interaction was the highest while the other non-ε isoforms are less active than ε isoforms.

Discussion

The existence of multiple isoforms of 14-3-3 proteins has raised the question on their functional role. Besides the different spatial expression pattern in various tissues and organs, during the plant development or in response to external stimuli, some isoform specificity in target recognition has been reported [65].

In the present work, the plasma membrane $H^+$-ATPase was chosen to investigate the functional role of Arabidopsis GF14 isoforms, being one of the best characterized 14-3-3 client in plants. We demonstrate that GF14 isoforms bind to the $H^+$-ATPase and stimulate its activity in an isoform-specific manner; in particular, both in binding and stimulation of the enzyme, all tested GF14 non-ε isoforms resulted more active than GF14 ε group. Previous data showed some different results on isoform specificity towards the $H^+$-ATPase [23,66]. A rationale for this may be that those experiments had been performed with peptides reproducing the AHA2 (Arabidopsis $H^+$-ATPase isoform 2) C-terminus, while in the present work a peptide reproducing the maize MHA2 C-terminus and the AHA1 enzyme were used in interaction and enzymatic activation studies, respectively. Differences in target sites, even slight, as those present in the $H^+$-ATPase C-termini, could affect binding selectivity.

The observation that isoforms that are more active in $H^+$-ATPase binding and activation belong to the same phylogenetic group allows us to propose a “group specificity” towards the $H^+$-ATPase. Results from the peptide array interaction studies are in agreement with the different behaviour of GF14 isoforms depending on the target peptide. Interestingly, also in these

Figure 7. Data analysis of the peptide array binding assay. Densitometric analysis of spots derived from the experiment in Figure 6. Data are expressed as Integrated Densitometric Value (the product of the area and mean gray value).
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experiments isoforms belonging to the same group shared similar binding affinities to some peptides reproducing binding sites of several targets and supporting the hypothesis of a “group specificity” in several interactions. This appears particularly evident for one of the two 14-3-3 binding sites (peptide 4) in F2KP that interacts exclusively with non-ε isoforms. It is interesting to note that its sequence is not a 14-3-3 mode I motif. The presence in F2KP of two 14-3-3 binding sites exhibiting a different GF14-specificity raises questions regarding their functional role. The regulation of F2KP activity appears in fact of great interest for the pivotal role played by fructose-2,6-bisphosphate in the control of plant sugar metabolism.

Additional peptides, such as the ATP synthase β-subunit (peptide 15) and Phot2 (peptide 17) show a slight preferential interaction with isoforms belonging to the ε group. The interaction of ATP synthase β-subunit peptide with ε group isoforms is in accordance with previous studies demonstrating that GF14ε and μ isoforms, but not GF14α, can be localized within the plastidic stroma, where they also participate in the regulation of starch synthase activity [67,68].

A similar preferential interaction with isoforms belonging to the ε group is evident also for the ABF3 peptide [18]. Nonetheless, studies of ABFs interaction with 14-3-3 proteins of Thellungiella saluginosa, a close relative of Arabidopsis, performed by yeast two-hybrid system show a specific interaction with non-ε isoforms. Since in these studies ABF2 and ABF4, but not ABF3, were used [27] it is possible that different target isoforms could display different specificity towards 14-3-3 isoforms.

Besides the “group specificity”, it is also of interest that isoforms belonging to the same phylogenetic group displayed a different behaviour in the interaction with some peptides. In particular, GF14α was often the most active among the non-ε isoforms and, in several cases, such as TPK1 (13) and BZR1 (14) peptides, the difference compared to the other isoforms was considerable. Accordingly, Lutz et al. [53] showed, by SPR analysis, that TPK1 interaction had a Kd that was more than 10 fold lower for GF14α than GF14ε, another non-ε isoform. Interestingly, although GF14k is strongly related (93% identical in the primary structure) to GF14α, it does not exhibit the same high binding affinity.

The specific biochemical binding properties of GF14α may explain the peculiar behaviour of this isoform in vivo: GF14α, but not GF14k, seems to be required for stomatal opening mediated by Phot2 [54].

At variance with results on peptides used in the array, GF14α does not exhibit a higher activity compared to the other GF14 non-ε isoforms in the interaction with the H+-ATPase and in its stimulation (Figure 1).

A more detailed study on the kinetic and thermodynamic properties of the interaction between the ε and non-ε isoforms and the H+-ATPase was performed by SPR analysis. The results concerning the interaction between the peptide reproducing the 14-3-3 binding site of the H+-ATPase and GF14ε or GF14α, as representative of ε and non-ε isoforms, respectively, demonstrate that GF14α has a higher affinity and that this was due to a slower dissociation rate of the complex, as can be deduced by comparing the kinetic parameters determined for both isoforms (Table 1). The different affinity of GF14 isoforms was particularly evident when FC was added, the Kd values determined for GF14α (Kd = 4.1 nM) was about 25 fold lower than that of GF14ε and similar to that reported in literature for a maize 14-3-3 isoform (Kd = 7 nM) [46]. As known, FC stabilizes 14-3-3/H+-ATPase complex, slowing down the dissociation phase, as demonstrated by the decrease in koff values of two order of magnitude for GF14α and one order of magnitude for GF14ε. Notably, FC seems to have a negative effect on the association rate, being that the k÷ values for both isoforms were reduced, even slightly, after toxin addition. This finding indicates that FC slows down the 14-3-3/H+-ATPase complex formation, but strongly reduces its dissociation.

During the past number of years, the molecular mechanism underlying the isoform binding specificity has been investigated but a full clarification has not yet been obtained. Structural data reported for mammalian and plant 14-3-3 proteins indicate that residues directly involved in the ligand binding are highly conserved among isoforms [11,12,17]. Obsil and co-workers [69] observed that in the crystal structure of the 14-3-3ζ Serotonin N-acetyltransferase complex, of the 37 residues of 14-3-3 contacting the target, 34 are identical in the mammalian 14-3-3 isoforms and the other four conservatively substituted. Despite that, in two serotonin N-acetyltransferase were preferentially found associated with 14-3-3ε and 14-3-3ζ isoforms, suggesting that structural characteristics outside the binding groove may be responsible for isoform binding specificity.

Results shown in the present work indicate that both the C-terminal domain and a specific Gly residue, within the loop 8 of 14-3-3s, play a role in the GF14 isoform specificity.

The primary structure of 14-3-3 isoforms show the high divergence of C-terminal domains which has already been proposed to be involved in ligand binding and discrimination. Our results with GF14ε and GF14γ deletion mutants demonstrate that the C-terminal region plays an autoinhibitory role, which accords with literature data obtained with other 14-3-3 isoforms [30–32]. It is worth noting that the effect of the C-terminal removal is higher on GF14ε compared to GF14α (Figure 4), thus suggesting that the C-terminal region can have a role in the isoform specificity because of a different extent of autoinhibition. Otherwise, it can be directly involved in ligand interaction as demonstrated by a peptide reproducing the C-terminal region of a maize 14-3-3 affecting H+-ATPase activity [32] and by the observation that the GF14α C-terminal tail is required for nitrate reductase inhibition [33].

The position of the C-terminal region, and consequently the groove accessibility to the target, can be altered by binding of divergent cations at the loop 8 of 14-3-3. A Gly residue, within this loop, is necessary for the correct folding of the EF hand-like domain involved in cation binding; interestingly this residue is specifically conserved in non-ε isoforms. Substitution of this Gly in GF14ε isoform with an Asn residue, present at the same position in GF14ε, reduces GF14ε binding to the H+-ATPase (Figure 5). This effect is due to a lower Mg2+ sensitivity, since it can be detected only in its presence. Accordingly, replacement of Asn for Gly within the loop 8 of GF14ε increases its Mg2+-dependent interaction with the H+-ATPase. This result well confirms data previously obtained by Schnke et al. [36] with the GF14 μ isoform and its target nitrate reductase, suggesting that the Gly in the loop 8 might affect the C-terminal flexibility of 14-3-3 proteins.

Conclusions

This paper demonstrate that Arabidopsis 14-3-3 isoforms, classified in ε and non-ε groups, display a “group specificity” in the target recognition. This is observed in the FC-mediated activation of the H+-ATPase, one of the best-characterized 14-3-3 clients in plants, and confirmed by probing several targets by SPOT peptide array method. The results reported show that the isoform binding specificity is dependent on structural characteristics outside the binding groove, in particular the C-terminal domain and a specific Gly residue, within loop 8 of 14-3-3s. The
C-terminal region can play a role in the isoform-specificity because of a different extent of autoinhibition. Moreover, the different C-terminal flexibility and the extent of $\alpha$-helix content appears to play an important role in their different activity.

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Author Contributions

Conceived and designed the experiments: RP SV PA. Performed the experiments: RP SV LC SM SP PT. Analyzed the data: RP SV LC SM SP PT. Contributed reagents/materials/analysis tools: SV GC SM PT PA. Wrote the paper: SV GC PA.

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