Binding of 14-3-3 Protein to the Plasma Membrane H\(^{+}\)-ATPase AHA2 Involves the Three C-terminal Residues Tyr\(^{946}\)-Thr-Val and Requires Phosphorylation of Thr\(^{947}\)*

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14-3-3 proteins play a regulatory role in a diverse array of cellular functions such as apoptosis, regulation of the cell cycle, and regulation of gene transcription. The phytotoxin fusicoccin specifically induces association of virtually any 14-3-3 protein to plant plasma membrane H\(^{+}\)-ATPase. The 14-3-3 binding site in the Arabidopsis plasma membrane H\(^{+}\)-ATPase AHA2 was localized to the three C-terminal residues of the enzyme (Tyr\(^{946}\)-Thr-Val). Binding of 14-3-3 protein to this target was induced by phosphorylation of Thr\(^{947}\) (KD = 88 nm) and was in practice irreversible in the presence of fusicoccin (KD = 7 nm). Mass spectrometry analysis demonstrated that AHA2 expressed in yeast was phosphorylated at Thr\(^{947}\).

We conclude that the extreme end of AHA2 contains an unusual high-affinity binding site for 14-3-3 protein.

The 14-3-3 proteins are extremely well conserved proteins that have been found in all eukaryotic cells (see Refs. 1 and 2 for review). Typically, each organism has a number of isoforms (3), and in certain cell types these proteins can be very abundant (4).

The ability to bind to other proteins is a common feature of 14-3-3 proteins, and more than 30 interacting proteins have been identified (5). In addition, an increasing number of target proteins has been found to contain more than one binding site (6), and activate proteins such as tyrosine and tryptophan hydroxylases (7, 8) and myosin II heavy-chain-specific protein kinase C (9) inhibit or activate their targets; they inhibit nitrate reductase modulators of enzyme activity (5, 6). 14-3-3 proteins either have the ability to bind more than one general function 14-3-3 proteins may be to serve as direct modulators of enzyme activity (5, 6). 14-3-3 proteins either inhibit or activate their targets; they inhibit nitrate reductase (7, 8) and myosin II heavy-chain-specific protein kinase C (9) and activate proteins such as tyrosine and tryptophan hydroxylases (10), exoenzyme S (11), the glucocorticoid receptor (12), Raf-1 kinase (13, 14), the DNA-binding transcription factor p53 (15), and the plasma membrane H\(^{+}\)-ATPase (16).

The generally accepted paradigm is that the interaction between the 14-3-3 protein and its target is inducible. According to one generally accepted model, this protein-protein interaction is regulated by protein kinase-mediated phosphorylation on serine residues in the target protein. Thus, following phosphorylation of a number of binding motifs, the affinity for 14-3-3 proteins is increased by orders of magnitude (17). However, other mechanisms are known to regulate the 14-3-3 protein-target protein association. Fusicoccin (FC), a wilt-inducing toxin produced by the fungus Fusicoccum amygdali, induces a tight association between 14-3-3 proteins and the plant plasma membrane H\(^{+}\)-ATPase (18, 19). This proton pump generates H\(^{+}\)-gradients across the plant plasma membrane, which are used for driving solute uptake through H\(^{+}\)-coupled symporters (20). FC binding sites are absent from fungi and animals (21), suggesting that a specific function of one or more plant 14-3-3 proteins is to bind FC. This suggestion turned out to be invalid, following the discovery that the FC binding site is a complex between two proteins: a 14-3-3 protein and the C-terminal regulatory domain of the plant plasma membrane H\(^{+}\)-ATPase (16, 22). Apparently, all isoforms of 14-3-3 protein, no matter whether they are of plant, human, or yeast origin, form complexes with plant plasma membrane H\(^{+}\)-ATPase and participate in generating a FC binding site (16).

In this study, we have identified a binding site for 14-3-3 protein in the C-terminus of the Arabidopsis plasma membrane H\(^{+}\)-ATPase AHA2. The binding site does not resemble any other 14-3-3 binding motif, and binding was dependent upon phosphorylation of a threonine residue. Interestingly, the 14-3-3 binding site is identical to the part of the H\(^{+}\)-ATPase contributing to the FC receptor.

MATERIALS AND METHODS

Chemicals—FC was prepared as described (23). [\(^{3}\)H]FC was obtained according to Ballio et al. (24).

Expression of AHA2 in the Yeast Saccharomyces cerevisiae—The yeast strain RS-72 was transformed and cultured essentially as described previously (25). In RS-72 (MATa ade1–100 his4–519 leu2–3, 112), the natural constitutive promoter of the endogenous yeast plasma membrane H\(^{+}\)-ATPase, PMA1, has been replaced by the galactose-dependent promoter of GAL1. The cells were harvested and plasma membranes isolated as described (25, 26).

GFP-14-3-3-Cyan Fluorescent Protein (CFP)—Fusion Protein Construction and Expression—The construction and expression of the

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1 The abbreviations used are: FC, fusicoccin; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; GST, glutathione S-transferase; MALDI-TOF, matrix-assisted laser desorption ionization/time of flight; CFP, cyan fluorescent protein; Mes, 4-morpholineethanesulfonic acid; BTP, 1,3-bis[tris(hydroxymethyl)methylamino]propane.
MRGSH$_{14}$-tagged GF14-14-3-3 protein was described previously (16). The primers 5'-GAAGATCTCGGTGGGACGAGGGCGGACG-3' and 5'-CCGGATCCGGTTGATGTCGTCGTCGTCG-3' were employed in a PCR reaction to amplify CFP from pSETB (Invitrogen). The resulting product was digested with BamHI and BglII and ligated into the pRSETB vector (Invitrogen). The resulting vector plasmids are described for expression of an N-terminally MRGSH$_{14}$-tagged GF14-14-3-3 protein with a C-terminal fusion to CFP (pMP-970). The fusion protein was expressed in Escherichia coli and extracted using standard procedures.

Site-directed Mutagenesis—Mutations in the 3' end of the coding region of AHA2 were produced by cassette mutagenesis as described (26). Oligonucleotide primers containing mismatches were employed in a PCR reaction and the resulting products digested with restriction enzymes and substituted with the corresponding fragment in plasmid pMP-658 (26). All mutations were verified by DNA sequencing of the cassettes.

SDS-PAGE and Western Blotting—Proteins were dehydrationpurified according to Chang and Slanyan (27) with slight modifications. SDS-PAGE and Western blotting were performed as described (18).

14-3-3 Protein Overlay Assay—The nitrocellulose membranes were blocked by incubation for 1 h in 2% dry milk powder in 20 mM Mes/KOH, pH 6.5, 130 mM NaCl, and 10 mM MgSO$_4$ (binding buffer) for 30 min. The membranes were washed in binding buffer adjusted by 1.5-biasis hydroxyethyltrimethylammonio/propane (BTP) to different pH values as incubated in a 1:200 dilution of a crude protein extract of E. coli expressing GF14-14-3-3-CFP (see above) in binding buffer either in the presence or absence of FC (10$^{-5}$ M). Before analysis, membranes were washed twice in binding buffer. Samples were analyzed on a Storm scanner (Molecular Dynamics) using the blue-fluorescence mode at 600 V and 200 mm resolution. Alternatively, bound 14-3-3 protein was detected immunologically. After incubation with MRGSH$_{14}$-tagged GF14-14-3-3 protein, the blot was incubated with a primary anti-RGS9$_{24}$ antibody, followed by incubation with a secondary anti-IgG antibody conjugated with horseradish peroxidase and developed with enhanced chemiluminescence.

Two-hybrid Screens—S. cerevisiae strain Y190 (MATa gal1 gal10 his3 trpl-901 ade2-101 wu85-32 leu2-3,112 + URA::GAL–lacZ, LYS2–GAL–His3–cyH) was used as a host for the two-hybrid vectors pACT2 and pAS1-CYH (28) and constructs derived from these plasmids. pACT2 and pAS1-CYH have open reading frames encoding the GAL4 activation domain and the GAL4 DNA binding domain, respectively, but pAS1-CYH has been under control of the alcohol dehydrogenase ADH1 promoter. Plasmids derived from pACT2 contained open reading frames encoding the full-length AHA2 were excised from the gel, washed, followed by in-gel reduction and in-gel digestion with trypsin. In-gel digestion with trypsin was performed at 37 °C overnight, and the peptides were subsequently extracted. The resulting peptide mixture was dried in a vacuum centrifuge and redissolved in 0.1 M acetic acid or 5% formic acid prior to mass spectrometry analysis. Phosphopeptides present in a tryptic digest of AHA2 wild type were purified by immobilized metal affinity chromatography (31). Sample desalting/concentration of peptides prior to mass spectrometry analysis was accomplished by nanoscale reversed-phase columns (32).

Mass Spectrometry—Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectra were acquired on a Bruker REFLEX mass spectrometer (Bruker-Daltonics, Bremen, Germany) equipped with a nitrogen laser and delayed ion extraction technology. 3,5-Dihydroxybenzoic acid or 2,5-dihydroxybenzoic acid were used as matrix. Nanoelectrospray tandem mass spectrometry (33) was performed on a Q-TOF hybrid instrument (Micromass, Manchester, United Kingdom). Peptides purified by nanoscale immobilized metal affinity chromatography were concentrated by Nanopore (33) and recombined in solution. Purified FC (10$^{-5}$ M) was added in parallel for each sample. As an additional control, GST-14-3-3 protein (1 μg in 200 μl) was immobilized on to 30 μl of glutathione-Sepharose 4B (Amersham Pharmacia Biotech) beads and the binding assay was carried out in the same conditions as described above. As expected, no radioactivity was associated to the 14-3-3 protein (data not shown). A slightly different strategy (16) was employed to assay binding of [3H]FC to yeast membranes expressing AHA2. Non-specific binding was detected by running a parallel sample for which an excess of unlabeled FC (10$^{-4}$ M) was added. All the samples were made as triplicates.

Sample Preparation for Mass Spectrometry—In-gel tryptic digestion was performed as previously described. The protein bands containing AHA2 were excised from the gel, washed, followed by in-gel reduction and in-gel digestion with trypsin. The tryptic digest was performed as described previously (30). The protein bands containing AHA2 were excised from the gel, washed, followed by in-gel reduction and in-gel digestion with trypsin. The tryptic digest was performed as described previously (30). The resulting product was digested immunologically. After incubation with MRGSH$_{14}$-tagged GF14-14-3-3 protein, the blot was incubated with a primary anti-RGS9$_{24}$ antibody, followed by incubation with a secondary anti-IgG antibody conjugated with horseradish peroxidase and developed with enhanced chemiluminescence.

RESULTS

Binding of 14-3-3 Protein to AHA2 Expressed in Yeast Membranes Is Phosphatase-sensitive—Full-length Arabidopsis plasma membrane H$^+$-ATPase AHA2 as well as a C-terminal fragment of AHA2 were tested for their ability to associate with 14-3-3 protein under various conditions. Full-length AHA2 was expressed in S. cerevisiae plasma membranes, whereas a peptide consisting of the 98 C-terminal amino acid residues of AHA2 was expressed in E. coli as a GST fusion protein. The proteins were run on SDS-PAGE, transferred to a nitrocellulose membrane, and the immobilized polypeptides allowed to interact with soluble 14-3-3 protein. The 14-3-3 protein employed in the overlay assay had been fused to CFP, a spectral variant of green fluorescent protein (34), to allow for direct visualization of binding.

In the overlay assay, 14-3-3 protein bound to only one polypeptide (molecular mass 97 kDa) in the yeast plasma membranes (Fig. 1C, lane 1). This polypeptide corresponds to AHA2 since it was recognized by an anti-plant H$^+$-ATPase monoclonal antibody (Fig. 1B), and no binding was observed in a control yeast strain not expressing AHA2 (data not shown). Binding was pH-dependent and was optimal at slightly acidic pH (pH 6.3), whereas substantially less binding was observed at more neutral pH values (pH 7.3). This result correlates with

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The known pH dependence of \( H^+ - \text{ATPase} \) activity showing its maximum at pH 6.5 (20). In the overlay assay, no binding to the C-terminal fragment of AHA2 was observed at any pH value (Fig. 1, C, lane 3, and D).

To test the possibility that binding of 14-3-3 protein to full-length AHA2 was dependent on phosphorylation of residues in AHA2, solubilized yeast plasma membranes expressing AHA2 were subjected to treatment with alkaline phosphatase to remove any phosphate group covalently bound to polypeptides. This treatment did indeed completely abolish 14-3-3 binding to AHA2 (Fig. 1C, lane 2). This effect was not due to degradation of AHA2 by a protease contaminant in the phosphatase preparation, because FC-dependent binding of 14-3-3 protein (Fig. 1C, lanes 6 and 8) requires the three C-terminal amino acids of AHA2 (see below). The fact that binding of 14-3-3 protein to AHA2 was phosphatase-sensitive implied that the heterologously-expressed AHA2 polypeptide became phosphorylated in yeast, and that phosphorylation might be essential for binding of 14-3-3 to AHA2.

Thr\(^{947}\) in the C terminus of AHA2 Gets Phosphorylated in S. cerevisiae—In order to investigate the possibility that AHA2 was a target for a yeast protein kinase, proteins of yeast plasma membranes expressing AHA2 were separated by SDS-PAGE, and the band corresponding to AHA2 was excised and subjected to trypsinolysis. Phosphopeptides were purified from the AHA2 tryptic peptide mixture by nano-scale immobilized metal affinity chromatography and analyzed by MALDI-TOF mass spectrometry. A \( M_r \) 1410.6 phosphopeptide candidate was identified and subsequently sequenced by nanoelectrospray tandem mass spectrometry. The resulting mass spectrum displayed a number of sequence specific peptide fragment ion signals identifying the peptide GLDIETPSHYTV (inset). X-terminal and C-terminal peptide sequence ions are indicated with \( b \) and \( y \), respectively, and their calculated masses are shown adjacent to the amino acid sequence. Additionally, a number of ion signals corresponding to peptide fragments which have lost the phosphate group \( H_2PO_4^- \) by \( \beta \)-elimination (indicated with asterisks) were detected. This data identified the phosphorylated residue as the penultimate Thr in the AHA2 C-terminal peptide GLDIETPSHYTV.

**Fig. 1.** The association between 14-3-3 protein and AHA2 depends upon phosphorylated residue(s), but this requirement can be circumvented by FC. Plasma membranes from yeast expressing AHA2 or purified GST-C-terminal fusion protein treated or not treated with alkaline phosphatase (AP) were run on SDS-PAGE and polypeptides in the gel were stained with Coomassie R-250 (A). Arrows to the left indicate 97-kDa AHA2 (upper arrow), 47-kDa alkaline phosphatase (middle arrow), or 38-kDa AHA2 C-terminal domain/GST fusion protein (lower arrow). In parallel experiments the polypeptides were transferred to nitrocellulose membranes (B–D). Membranes were employed for either Western blotting with anti-H\(^\text{Y} \) antibody (B) or subjected to overlay with a 14-3-3-CFP fusion protein (C and D). C, binding of 14-3-3 protein at pH 6.3. D, pH dependence of 14-3-3 protein binding to AHA2. Values are given as “volume report” as calculated against an individual background value for each measurement. Data are typical for three independent experiments.

**Fig. 2.** Localization of AHA2 phosphorylation site at Thr\(^{947}\) by nanoelectrospray tandem mass spectrometry. Phosphorylated AHA2 expressed in yeast was isolated by SDS-PAGE. Phosphopeptides were purified from the AHA2 tryptic peptide mixture by nano-scale immobilized metal affinity chromatography and subsequently analyzed by MALDI-TOF mass spectrometry. A \( M_r \) 1410.6 phosphopeptide, assigned as phospho-GLDIETPSHYTV, was identified. This phosphopeptide, corresponding to the C-terminal peptide of AHA2, was sequenced by nanoelectrospray tandem mass spectrometry. The fragment ion signals in the tandem mass spectrum of the phosphopeptide confirmed the identity of the peptide GLDIETPSHYTV and localized the phosphorylation site at Thr\(^{947}\). No other phosphopeptides were detected by this approach.

Binding of 14-3-3 Protein to Dephosphorylated AHA2 Can Be Induced by FC—The fungal phytotoxin FC has been used as a tool to stabilize the binding of 14-3-3 protein to plant plasma membrane \( H^+ - \text{ATPase} \) (18, 19, 35). In the overlay assay, the addition of FC (10 \( \mu \text{M} \)) almost doubled the amount of 14-3-3 protein bound to AHA2 at pH 6.3 and had an even stronger effect at pH 7.3, where binding was increased several fold (Fig. 1, C, lane 5, and D). Interestingly, FC promoted binding of 14-3-3 protein to AHA2 even after phosphatase treatment (Fig. 1C, lane 6). Binding of 14-3-3 protein under these conditions was reduced compared with the degree of binding in the absence of phosphatase (Fig. 1, C, lane 1, and D).

Whereas the fusion between GST and the C-terminal domain of AHA2 did not bind any 14-3-3 protein at all in the absence of FC (see above), binding was strongly induced by FC and was not affected by phosphatase treatment (Fig. 1C, lanes 7 and 8, and D). The higher intensity of the band corresponding to the C-terminal domain compared with the full-length AHA2 (Fig. 1C, lane 1) reflects the higher molar amount of C-terminal protein loaded onto the gel (Fig. 1A). The high molecular weight band in panels B and C (lanes 7 and 8) most likely corresponds to an aggregate of the GST-AHA2 C-terminal fusion protein since it was recognized by anti-H\(^{\text{Y}} - \text{ATPase} \) antibodies (Fig. 1B) and was able to bind 14-3-3 protein (Fig. 1C). Quantification of the AHA2 band and the C-terminal domain
Interaction between Fusicoccin, 14-3-3, and AHA2

**Fig. 3. Summary of constructs used to identify a 14-3-3 binding site in the extreme end of the C terminus of AHA2.** The yeast two-hybrid system (A), overlay assays (B), and peptide binding studies (C) were employed. A, various parts of the C-terminal domain of AHA2 employed in two-hybrid screens. ah2(851-948), ah2(895-948), and ah2(851-895): numbers in parentheses indicate position in the AHA2 sequence; B, deletion mutants employed in overlay assays. AHA2, ah2Δ3, and ah2Δ10, intact AHA2 and the enzyme devoid of the 3 and 10 C-terminal residues, respectively. C, surface plasmon resonance was measured to study the interactions between peptides and 14-3-3 protein. BA(933-948), biotinylated peptide representing residues 933–948 of AHA2. BA(933-948)-P, the same peptide phosphorylated at the residue representing position Thr-947. Black filling, fusion proteins or peptide displayed strong 14-3-3 binding; no filling, no 14-3-3 binding. The shortest construct to bind 14-3-3 was the phosphorylated peptide BA(933–948)-P. Below, a model of the C-terminal regulatory domain of AHA2 plasma membrane H⁺-ATPase. The C-terminal end of the AHA2 sequence is shown. Open boxes indicate regions of importance for regulation. Regions I and II comprise amino acid residues that when altered result in an activated enzyme (24). 14-3-3, 14-3-3 protein binding site; FC, fusicoccin binding site.

from the Coomassie Blue-stained gels (Fig. 1A) did not reveal any significant difference between the apparent sizes of the full-length AHA2 and the C-terminal domain to bind 14-3-3 protein (data not shown).

**Ty**r**946**,**Thr**-Val in the C-terminal End of AHA2 Are Essential for 14-3-3 Protein Binding**—To demonstrate interaction between 14-3-3 and various regions of the AHA2 H⁺-ATPase, a two-hybrid screen was undertaken. Regions of AHA2, fused in frame to the GAL4 DNA binding domain vector pACT2, were cotransformed into yeast with an *Arabidopsis* 14-3-3 gene (*GF14-ψ*; Ref. 29) contained in the GAL4 transcriptional activator domain vector pAS1-CYH. The two-hybrid constructs employed are indicated in Fig. 3.

The yeast strain coexpressing the 14-3-3 clone and a construct containing the last 54 C-terminal amino acids of AHA2 (residues 895–948) was able to grow on a medium lacking histidine and expressed high levels of β-galactosidase activity (Table I), indicating a direct interaction between these two fusion proteins. In contrast, a region further upstream comprising residues 851–895 did not produce any signal in the two-hybrid system.

In order to narrow down the extent of the interacting region, the *in vitro* overlay assay described above was employed (Fig. 4). In these experiments, AHA2 expressed in yeast membranes was used as the binding partner. Following incubation with the recombinant 14-3-3 protein, 14-3-3 protein bound to the C terminus of AHA2 was detected. FC was used as a tool to stabilize the *in vitro* association between 14-3-3 protein and the AHA2 C terminus.

In the presence of FC, a strong interaction between full-length AHA2 and recombinant 14-3-3 protein could be detected (Fig. 4B). AHA2 H⁺-ATPases carrying deletions of 3 and 10 amino acid residues were tested for their ability to bind 14-3-3 protein and FC. Deletion of the three C-terminal amino acid residues (Ty**r**946,**Thr**-Val) was sufficient to generate an H⁺-ATPase that could not bind 14-3-3 protein (Fig. 4B). These data suggest that residues in the extreme C-terminal end of AHA2 are essential for binding of 14-3-3 protein in the overlay assay. Furthermore, the H⁺-ATPase activity of the deletion mutants devoid of either 3 or 10 C-terminal amino acid residues could not be activated by 14-3-3 and FC (Fig. 4D). This result is in agreement with the fact that these mutants bound neither 14-3-3 protein (Fig. 4B) nor FC (Fig. 4C).

the three C-terminal residues were changed individually into alanine residues in order to identify residues of particular importance for activation of AHA2 by 14-3-3 protein and FC. The alanine-scanning survey demonstrated that all three residues (Ty**r**946, Thr**947**, and Val**948**) are important for the FC-dependent activation of AHA2 by 14-3-3 protein (Fig. 4D). Altering amino acid residues further upstream (residues 939–945) individually into alanines did not affect the FC-dependent activation of AHA2 by 14-3-3 protein (data not shown).

**Phosphorylation of Thr**947** Is Required for Binding of 14-3-3 Protein**—Using surface plasmon resonance spectroscopy, we quantitatively studied 14-3-3 protein binding to BA(933–948), a synthetic 16-residue peptide derived from the C-terminal end of AHA2. However, this peptide did not give rise to a signal, even when FC was added (data not shown). Since binding of 14-3-3 protein to AHA2 in the absence of FC seemed to involve a phosphorylated residue (see above), the binding experiments were carried out with the corresponding phosphorylated peptide (Table II).

**Phosphorylation of Thr**947** in peptide BA(933–948)-P gave rise to a large signal (Table II). This interaction was not dependent on the presence of FC in the medium, but the affinity of binding was significantly enhanced by this compound. 14-3-3 protein bound reversibly to BA(933–948)-P in a concentration-dependent and saturable fashion (data not shown). From the
the dissociation was slower, with a rate constant for the dissociation phase: in the presence of FC the dissociation rate constant was about 15-fold lower than in the absence of the toxin. These data confirmed that the dissociation rate constant in the presence of FC was about 15-fold lower than in the absence of the toxin. These data confirmed the extreme C-terminal end of AHA2 contains a 14-3-3 binding site and that phosphorylation of Thr947 is essential for

analysis of the sensorgrams the kinetic rate constants $k_{on}$ and $k_{off}$ were obtained, and the equilibrium dissociation constant $K_{D}$ was calculated as $k_{off}/k_{on}$ (Table II). In the presence of FC, the $K_{D}$ constants were 88 and 7 nM, respectively. It is interesting to note that the difference in the affinity was mainly due to the dissociation phase: in the presence of FC the dissociation was slower, with a $k_{off}$ value about 15-fold lower than in the absence of the toxin. These data confirmed the extreme C-terminal end of AHA2 contains a 14-3-3 binding site and that phosphorylation of Thr947 is essential for

TABLE II

| Protein | $k_{on}$ | $k_{off}$ | $K_{D}$ (nM) |
|---------|---------|---------|-------------|
| BA-(933–948)-P | $1.6 \times 10^8$ | $1.4 \times 10^{-2}$ | 88 |
| BA-(933–948)-P + FC | $3.0 \times 10^7$ | $6.5 \times 10^{-4}$ | 7 |
| RSNSTpSTP (PS-Raf-259)$^{b,c}$ | | | 510$^c$ |
| RL-YHSLP$^{b,c}$ | 37.4$^b$ |

$^{a}$ $10^{-2}$ or $10^{-3}$ M FC.

$^{b}$ Ref. 32.

$^{c}$ A phosphopeptide representing an optimized sequence for 14-3-3 binding.

$^{d}$ A phosphopeptide representing a 14-3-3 binding sequence in Raf-1 kinase.

In the overlay assay, the BA-(933–948)-P phosphopeptide was found to inhibit binding of 14-3-3 protein to the AHA2 H$^+$-ATPase (Fig. 5A). This would suggest that BA-(933–948)-P competes with AHA2 in occupying a binding site in 14-3-3 protein. Furthermore, BA-(933–948)-P phosphopeptide abolished the FC-dependent activation by 14-3-3 protein of ATP hydrolytic activity of AHA2 (Fig. 5B). The effect of BA-(933–948)-P was compared with the effect of a peptide (phosphoserine-Raf-259) derived from Raf-1 kinase, representing the 14-3-3 binding sequence in this protein (17). BA-(933–948)-P was found to be at least an order of magnitude more potent than the phosphoserine-Raf-259 peptide in this assay.

The Phosphorylated C-terminal End of AHA2 Generates a FC Receptor in Concert with 14-3-3 Protein—The complex between 14-3-3 and peptide BA-(933–948)-P bound tritiated FC in a FC binding assay, whereas the corresponding unphosphorylated peptide did not (Fig. 4E). This result strongly suggested that the region covered by this phosphopeptide comprises the part of the H$^+$-ATPase contributing to the FC receptor.

The 14-3-3 Protein Recognition Site in AHA2 Is Separated from Autoinhibitory Residues—The C terminus of the plant plasma membrane H$^+$-ATPase contains putative autoinhibitory sequences (Fig. 3; Refs. 26, 29, and 37), and the 14-3-3 binding site identified could be part of such a sequence. To test this possibility, deletion mutants of AHA2 lacking 3 or 10 C-terminal residues were functionally expressed in yeast and characterized biochemically. Deletion of the last three residues, comprising the 14-3-3 binding site, did not affect biochemical parameters such as $K_{D}$ for ATP and pH optimum (data not shown). These results indicated that the 14-3-3 binding site is separated in space from sequences involved in controlling these kinetic parameters (Fig. 3).

Taken together, our data strongly suggest that: (i) Tyr946-Thr-Val forms the 14-3-3 binding site in AHA2, (ii) phosphorylation of Thr947 is essential for FC-independent binding of the 14-3-3 protein, (iii) the minimal part of AHA2 identified...
that contributes to the FC receptor corresponds to the 14-3-3 binding site, and (iv) the 14-3-3 binding site is separated from amino acid residues controlling ATP affinity and pH optimum.

**DISCUSSION**

In this study we have identified the sequence Tyr$^{946}$-Thr-Val in the extreme C-terminal end of Arabidopsis plasma membrane H$^+$-ATPase AHA2 as a recognition site for 14-3-3 proteins. Binding of 14-3-3 to AHA2 was dependent upon phosphorylation of the penultimate residue Thr$^{947}$. This conclusion is based on the following lines of evidence: (i) deletion of Tyr$^{946}$-Thr-Val from the C terminus of AHA2 expressed in yeast membranes abolishes interaction with 14-3-3 protein; (ii) Thr$^{947}$ was found to be phosphorylated in AHA2 expressed in yeast membranes; (iii) a phosphopeptide, BA-(933–948)-P, representing the 16 C-terminal residues of AHA2 and phosphorylated at a position corresponding to Thr$^{947}$ in AHA2, binds 14-3-3 protein; (iv) the peptide BA-(933–948)-P inhibits both binding of 14-3-3 protein to AHA2 as well as 14-3-3 protein-dependent activation of ATP hydrolytic activity of AHA2.

FC is the only compound known to affect specifically an interaction between 14-3-3 and a target protein. Our data suggest that the role of FC is to stabilize the association between ATPase and 14-3-3 protein, since dissociation of 14-3-3 from the peptide was decreased by an order of magnitude by FC, whereas association was not increased (Table II).

Whereas phosphorylation of Thr$^{947}$ apparently is a prerequisite for binding of 14-3-3 to full-length AHA2, our data suggest that FC circumvents this need. This conclusion is also corroborated by the effect of alkaline phosphatase on the native enzyme (Fig. 1). However, FC was not able to promote binding of 14-3-3 protein to the unphosphorylated peptide BA-(933–948). Apparently, with the short peptide BA-(933–948)-P, fusicon action is dependent upon phosphorylation of Thr$^{947}$, whereas this is not a prerequisite with longer C-terminal fragments of AHA2 (Fig. 1). These inconsistent results suggest that the binding site for FC and/or 14-3-3 on AHA2 might be more complex than just the three C-terminal amino acids.

Single point mutations in the C-terminal domain of AHA2 leading to an activated enzyme group in two regions, region I and region II, one or both of which may be part of the autoinhibitor of the enzyme (Fig. 3; Ref. 26). Interestingly, none of the putative autoinhibitors overlaps with the binding site(s) for 14-3-3 protein and FC (Fig. 3).

A number of 14-3-3 binding motifs in target proteins have been described. A peptide derived from the Raf-1 sequence was shown to bind to a 14-3-3 protein in vitro provided that a serine in the sequence was phosphorylated (17). The 14-3-3 binding motif in Raf-1 was found to be RSXpXSP (pS indicates a phosphorylated serine) and has been identified in several other 14-3-3 binding proteins (17). Screening of peptide libraries has identified another motif, RXY/FpXSP (38), which binds to 14-3-3 proteins. In c-Cbl, an RX$^p_{1-2}$SS$^p_{2-3}$ 14-3-3 binding motif has been reported (39). A systematic analysis of the 14-3-3 protein binding capacity of peptides derived from the glycoprotein Ib-IX-V complex revealed the presence of multiple sites for 14-3-3 protein binding, among which was GHSL situated in the extreme C-terminal end of this protein (40). The 14-3-3 binding site in AHA2 is also situated in the extreme C-terminal end of the enzyme. However, the 14-3-3 binding site identified in this study has no homology to other known motifs of 14-3-3 action.

Compared with the affinity of other 14-3-3 binding motifs, phosphopeptide BA-(933–948)-P was found to interact very strongly with 14-3-3 protein ($K_D = 88.0$ nM). Thus, binding was 1 order of magnitude higher than the binding of the 14-3-3 protein to the phosphoserine-Raf-259 peptide (RSRSTpSTP; Table I) covering the recognition site of 14-3-3 protein in the Raf-1 kinase (38), and the association was comparable to the binding of 14-3-3 to a peptide (RLYHpSLP; Table II) optimized for binding (38). Furthermore, in the presence of FC, binding increased by an order of magnitude ($K_D = 7$ nM). This might explain the toxic effect of FC on plant cells since association of 14-3-3 protein to plasma membrane H$^+$-ATPase becomes practically irreversible.

14-3-3 protein has been co-crystallized with a peptide representing a common 14-3-3 protein binding motif (RXpXSP). The peptide was found to interact with a groove in the 14-3-3 protein made up of extremely conserved amino acid residues, many of which are negatively charged (38). A non-phosphorylated peptide derived from the 14-3-3 protein binding site in exoenzyme S associates with hydrophobic residues and binds to a similar region of 14-3-3 protein (41, 42). It is not known whether other binding motifs bind to the same site of the 14-3-3 protein. It has recently been observed that glycoprotein Ib binds to 14-3-3 protein at a site distinct from the Raf-binding site (43). Whether the AHA2 recognition sequence binds to the sites in 14-3-3 occupied by Raf-1 or glycoprotein Ib remains to be determined.

Our results suggest that in vivo phosphorylation of Thr$^{947}$ might provide a mechanism for the regulation of 14-3-3 binding to the H$^+$-ATPase. Indeed, it has recently been demonstrated that Thr$^{947}$ of plasma membrane H$^+$-ATPase is phosphorylated in vivo in spinach (44), confirming the important role of this conserved residue. In addition, the data reported in this paper suggest a model for protein kinase-mediated activation of plasma membrane H$^+$-ATPase.

That new binding sites not resembling other 14-3-3 binding motifs can be found is surprising since the groove in 14-3-3 protein that accommodates phosphorylated peptides is extremely well conserved. This suggest that the number of 14-3-3 binding proteins might be much higher than previously thought.

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