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The 14-3-3 Protein Interacts Directly with the C-Terminal Region of the Plant Plasma Membrane H$^+$-ATPase

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Accumulating evidence suggests that 14-3-3 proteins are involved in the regulation of plant plasma membrane H$^+$-ATPase activity. However, it is not known whether the 14-3-3 protein interacts directly or indirectly with the H$^+$-ATPase. In this study, detergent-solubilized plasma membrane H$^+$-ATPase isolated from fusicoccin-treated maize shoots was copurified with the 14-3-3 protein (as determined by protein gel blotting), and the H$^+$-ATPase was recovered in an activated state. In the absence of fusicoccin treatment, H$^+$-ATPase and the 14-3-3 protein were well separated, and the H$^+$-ATPase was recovered in a nonactivated form. Trypsin treatment removed the 10-kD C-terminal region from the H$^+$-ATPase as well as the 14-3-3 protein. Using the yeast two-hybrid system, we could show a direct interaction between Arabidopsis 14-3-3 *GF14-*ϕ and the last 98 C-terminal amino acids of the Arabidopsis AHA2 plasma membrane H$^+$-ATPase. We propose that the 14-3-3 protein is a natural ligand of the plasma membrane H$^+$-ATPase, regulating proton pumping by displacing the C-terminal autoinhibitory domain of the H$^+$-ATPase.

INTRODUCTION

The plant plasma membrane H$^+$-ATPase generates the proton and electrical gradient that is the driving force for active secondary transport across the plasma membrane. This provides the H$^+$-ATPase with a key role in nutrient uptake and growth and makes it a primary target for regulation (reviewed in Michelet and Boutry, 1995; Palmgren, 1997). H$^+$ pumping may be regulated via an autoinhibitory domain in the C-terminal region of the enzyme (Palmgren et al., 1990, 1991). Deletion of this autoinhibitory domain results in an activated form of the H$^+$-ATPase with a higher V_{max}, a lower K_m for ATP, a changed pH dependence with higher activity at physiological pH, and an increased coupling of H$^+$ pumping to ATP hydrolysis (Palmgren et al., 1990, 1991; Johansson et al., 1993; Rasi-Caldogno et al., 1993; Lanfermeijer and Prins, 1994; Baunsgaard et al., 1996). Very similar results have been obtained after treatment of isolated plasma membranes with lysophosphatidylcholine (Palmgren et al., 1991; Johansson et al., 1994; Lanfermeijer and Prins, 1994) and after treatment of intact tissue with the fungal toxin fusicoccin (Johansson et al., 1993, 1995; Rasi-Caldogno et al., 1993; Lanfermeijer and Prins, 1994). These treatments are suggested to result in a displacement of the C-terminal autoinhibitory domain.

Fusicoccin is a phytotoxin produced by the fungus *Fusicoccum amygdali* (Graniti, 1962). Fusicoccin strongly stimulates H$^+$ secretion from plant tissue, and it has been suggested that fusicoccin activates the plasma membrane H$^+$-ATPase by direct interaction with the enzyme (Marrè, 1979). However, fusicoccin binding activity was later demonstrated to copurify with a plasma membrane protein different from the H$^+$-ATPase (Feyerabend and Weiler, 1988; de Boer et al., 1989; De Michelis et al., 1989; Meyer et al., 1989). This putative fusicoccin binding protein with a molecular mass of 30 to 33 kD was later shown to belong to the 14-3-3 protein family (Korthout and de Boer, 1994; Marra et al., 1994; Oecking et al., 1994). Members of this family are highly conserved hydrophilic proteins with multiple, often regulatory, functions in a wide range of organisms (reviewed in Aitken, 1996). Oecking et al. (1994) suggested, as one possible mechanism for the activation of the H$^+$-ATPase, a direct interaction between the 14-3-3 protein and the H$^+$-ATPase, whereas others have favored the view that fusicoccin interfered at an early stage in a common signal transduction pathway involving the G-protein, phospholipase A_2, and a protein kinase

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with the H^+ pump as the final target (de Boer et al., 1994; Aducci et al., 1995).

In this work, we show that the 14-3-3 protein copurifies with the plasma membrane H^+-ATPase, but only after the plant material used for the enzyme purification has been pretreated with fusicoccin. We also show that this 14-3-3 protein is removed from the H^+-ATPase upon proteolytic removal of the C-terminal region from the enzyme. Using the yeast two-hybrid system, we show that the C-terminal region of the plasma membrane H^+-ATPase interacts positively with the 14-3-3 protein. These data indicate that the 14-3-3 protein interacts directly with the H^+-ATPase and is bound to the C-terminal region of the enzyme harboring the autoinhibitory domain.

RESULTS

The H^+-ATPase Copurifies with the 14-3-3 Protein in an Activated Form

In this study, we partially purified the plasma membrane H^+-ATPase in the fusicoccin-activated state. We also followed the fate of the 14-3-3 protein to investigate whether its activation is due to a direct interaction between the 14-3-3 protein and the H^+-ATPase.

Plasma membranes were isolated from a maize shoot microsomal fraction by partitioning in an aqueous polymer two-phase system (Larsson et al., 1994). To obtain a protein fraction enriched in H^+-ATPase, the plasma membrane vesicles were solubilized with dodecyl β-D-maltoside (Johansson et al., 1994), and most of the H^+-ATPase was precipitated between 10 and 20% polyethylene glycol (PEG) 3350, as shown in Figure 1. The H^+-ATPase was retained in its fusicoccin-activated state during purification, as shown in Figures 2A and 2B by the much higher activities in the fractions obtained from fusicoccin-treated shoots compared with controls. This difference in activities was not due to a lower yield of H^+-ATPase in the fractions obtained from control material, as shown by the very similar activities obtained after activation of the H^+-ATPase with lysophosphatidylcholine (Figure 2A). The shapes of the curves (Figure 2B) suggested complex kinetics, in agreement with the data of Roberts et al. (1995), or the presence of a mixture of H^+-ATPase isoforms catalyzing the same reaction.

Plasma membrane preparations from both fusicoccin-treated shoots and control material contained the 14-3-3 protein, as demonstrated in Figure 3. The 14-3-3 protein appeared as two closely spaced bands at 30 to 32 kD, in agreement with data from other species (reviewed in Aducci et al., 1995), and the plasma membrane preparation from fusicoccin-treated material contained significantly more 14-3-3 protein than did the control, also in accordance with earlier reports (Korthout and de Boer, 1994; Oecking et al., 1994). The 14-3-3 protein was recovered in the dodecyl β-D-maltoside supernatant together with the H^+-ATPase (Figure 3) and was coprecipitated with the H^+-ATPase in 20% PEG, as shown in Figure 4 (left lane). More importantly, however, when the 20% PEG fraction obtained from the fusicoccin-treated material was subjected to gel filtration chromatography (separating proteins according to native molecular mass), most of the 14-3-3 protein eluted with the H^+-ATPase, as shown in Figure 4. In the corresponding control fraction, all of the eluted 14-3-3 protein was well separated from the H^+-ATPase (Figure 4). 14-3-3 proteins form dimers (reviewed in Aitken, 1996) with a molecular mass of 60 to 66 kD, whereas the H^+-ATPase has a molecular mass of ~100 kD in the monomeric form and has been reported to be everything from monomeric to hexameric (e.g., Briskin and Reynolds-Niesman, 1989; Huang and Berry, 1990). The maize shoot H^+-ATPase moved as a broad peak corresponding to molecular masses of 100 kD to several hundred kilodaltons (Figure 4). Coelution of the 14-3-3 protein and H^+-ATPase therefore strongly indicates the presence of a complex between these two proteins. The H^+-ATPase coeluting with the 14-3-3 protein was recovered in an activated form, as demonstrated by the small additional activation by lysophosphatidylcholine compared with that of a control (Figure 5).

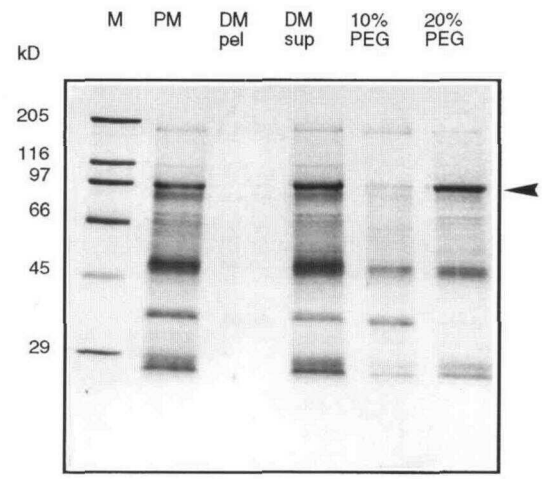

Figure 1. Polypeptide Patterns of Protein Fractions Obtained during Preparation of an H^+-ATPase–Enriched Fraction from Plasma Membrane Vesicles.

Aliquots of these protein fractions corresponding to 25 μg of the initial plasma membrane protein were loaded on the gel. Protein was stained with Coomassie blue. An arrowhead marks the position of the H^+-ATPase band. M, molecular mass markers, whose masses are given at left; PM, washed inside-out plasma membrane vesicles; DM pel, material pelleted after solubilization of the PM in dodecyl β-D-maltoside; DM sup, supernatant obtained after solubilization of the PM in dodecyl β-D-maltoside; 10% PEG, protein precipitated from the DM supernatant at 10% (w/v) PEG 3350; 20% PEG, protein precipitated from the DM supernatant between 10 and 20% PEG.

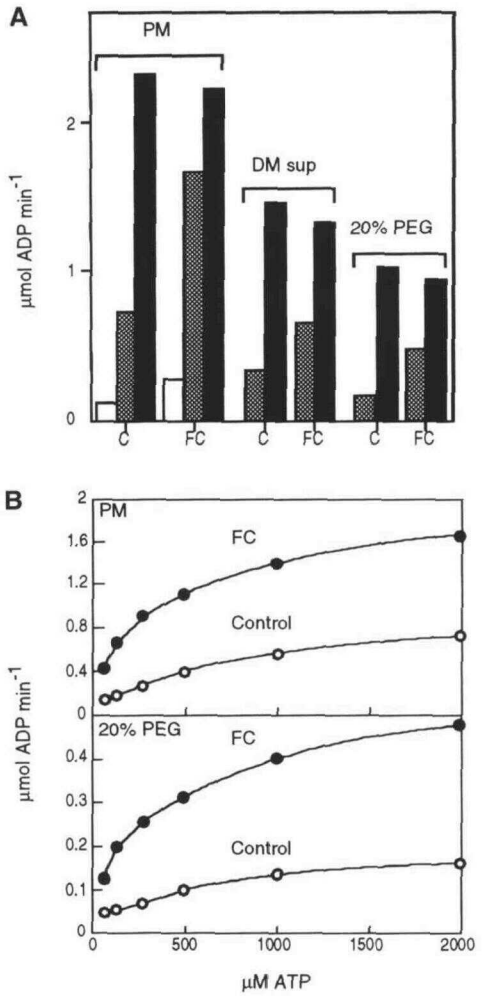

Figure 2. Effect of in Vivo Incubation with Fusicoccin on the ATP Hydrolytic Activity of Plasma Membrane Protein Fractions.

(A) ATP hydrolytic activity was measured with plasma membrane vesicles (PM), a dodecyl β-D-maltoside supernatant obtained from the plasma membranes (DM sup), and protein precipitated between 10 and 20% PEG (20% PEG). Activities are total activities and are calculated based on 1 mg of protein for the PM fraction, with representative recoveries from single experiments for the other fractions. Open bars represent activity measured without detergent (shows the activity of only the inside-out vesicles in the plasma membrane preparation); stippled bars, total activity (for the plasma membrane in the presence of 0.05% Brij 58, which produces 100% inside-out vesicles); black bars, activity in the presence of 0.02% lysophosphatidylcholine to fully activate the H^+-ATPase. All activities were assayed with 2 mM ATP. C, control; FC, fusicoccin.

(B) Activity was assessed as a function of ATP concentration measured with plasma membrane vesicles (in the presence of 0.05% Brij 58) and in an H^+-ATPase–enriched fraction (20% PEG). FC (●), fusicoccin; (○), control.

The Association between the 14-3-3 Protein and the H^+-ATPase Involves the C-Terminal Regulatory Domain of the H^+ Pump

To obtain additional evidence for the presence of an H^+-ATPase–14-3-3 protein complex, the 20% PEG fraction was subjected to native gel electrophoresis (dodecyl β-D-maltoside [DM]–PAGE) followed by SDS-PAGE. As shown in Figure 6, a substantial part of the 14-3-3 protein ran in the same position as the H^+-ATPase after DM-PAGE, but only when the plant material had been treated with fusicoccin. Similar results were obtained with Arabidopsis (data not shown).

Mild trypsin treatment removes a 7- to 10-kD C-terminal fragment containing a putative autoinhibitory domain from the H^+-ATPase, thus activating the enzyme (Palmgren et al., 1990, 1991). Fusicoccin activation of the H^+-ATPase was subsequently shown to involve this autoinhibitory domain (Johansson et al., 1993, 1995; Rasi-Caldogno et al., 1993; Lanfermeijer and Prins, 1994). To explore the possibility that the 14-3-3 protein interaction involves the C-terminal region of the H^+-ATPase, the 20% PEG fraction obtained from fusicoccin-treated material was incubated with trypsin, and the proteins were separated by two-dimensional PAGE. As shown

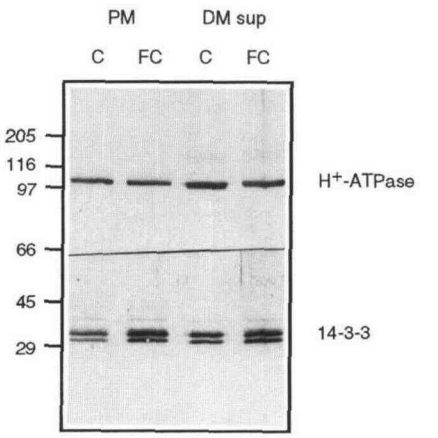

Figure 3. Protein Gel Blots Showing the Association of the 14-3-3 Protein with H^+-ATPase–Enriched Fractions as Dependent on Fusicoccin Treatment.

Plasma membranes were prepared from maize shoots treated for 1 hr with 5 µM fusicoccin (FC) and from control shoots (C). Aliquots of total plasma membrane (PM) and of protein solubilized by dodecyl β-D-maltoside (DM sup) were subjected to SDS-PAGE (20 µg of plasma membrane protein was loaded, and the other lanes received protein corresponding to the yield in the fraction). After transfer of the protein to a polyvinyldifluoride membrane, the membrane was cut into halves.

(Top) Upper half of the membrane that was probed with monoclonal antibody 46E5B11F6 raised against maize H^+-ATPase.

(Bottom) Lower half of the membrane that was probed with an antiserum raised against a barley 14-3-3 protein.

The positions of molecular mass marker proteins are indicated at left.

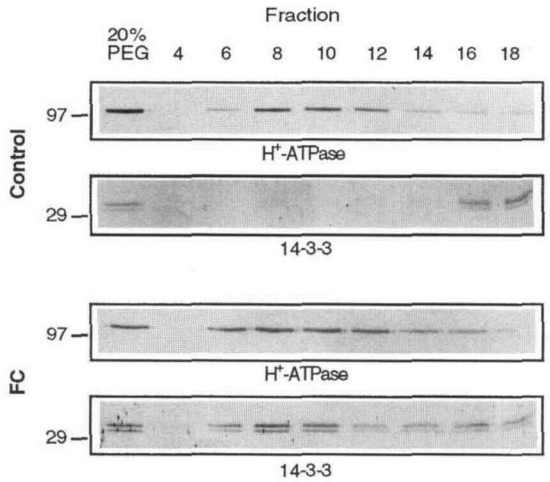

Figure 4. Protein Gel Blots Showing the H^+-ATPase and 14-3-3 Protein after Gel Filtration Chromatography of H^+-ATPase–Enriched Fractions.

Samples of the H^+-ATPase–enriched fraction (20% PEG) obtained either from control shoots or from shoots incubated with fusicoccin (FC) were run on a 10-cm Sephacryl S 300 HR (Pharmacia) column. Aliquots from eluted fractions (4 to 18) and from the sample loaded on the column (20% PEG) were subjected to SDS-PAGE. After transfer of the protein to a polyvinyldifluoride membrane, the membrane was cut in half; the upper half was probed with monoclonal antibody 46E5B11F6 raised against the maize H^+-ATPase, and the lower half was probed with an antiserum raised against a barley 14-3-3 protein. The positions of molecular mass marker proteins are indicated at left.

in Figures 7A and 7B, the 14-3-3 protein no longer ran in the same position as the H^+-ATPase in the DM-PAGE step (compare with Figures 6C and 6D), suggesting that the 14-3-3 protein was bound directly or indirectly to the C-terminal region of the H^+-ATPase removed by trypsin.

To confirm that trypsin treatment really removed the C-terminal region of the H^+-ATPase, we used an antiserum specific for this region. As demonstrated in Figure 7C, this antiserum stained only the H^+-ATPase band at ~100 kD and not the band at 90 kD (Figure 7A) expected to result from the removal of the C-terminal region (Palmgren et al., 1990, 1991). The band at 60 kD (Figure 7A) also was not stained. The 60-kD band, which is also obviously lacking the C-terminal region, is most likely the result of further proteolytic degradation of the 90-kD species. Notably, the staining pattern seen in Figures 7A and 7B shows that the removal of the C-terminal region from only about half of the H^+-ATPase molecules was sufficient for the removal of all of the 14-3-3 protein. This is in agreement with the data shown in Figure 2 for the 20% PEG fraction, indicating that the H^+-ATPase was only about half activated by fusicoccin compared with the complete activation achieved with lysophosphatidylcholine. Apparently, H^+-ATPase molecules with the bound 14-3-3 protein were degraded first, indicating that the C-terminal region is more susceptible to proteolysis when the H^+-ATPase is in the activated state.

The 14-3-3 Protein Interacts Directly with the C Terminus of AHA2

To ascertain that the 14-3-3 protein interacts directly with the C-terminal region of the plasma membrane H^+-ATPase, a yeast two-hybrid screen was undertaken. In the two-hybrid system, association between proteins fused to separate domains of the GAL4 transcription factor reconstitutes GAL4 activity in yeast. Two different regions of the AHA2 plasma membrane H^+-ATPase, fused in frame to the GAL4 DNA binding domain vector pAS1-CYH, were cotransformed into yeast with the Arabidopsis 14-3-3 *GF14-*ϕ gene (Lu et al., 1994) contained in the GAL4 transcriptional activation domain vector pACT2. In the yeast strain used, expression of the *lacZ* reporter gene and growth on a medium lacking histidine are dependent on functional GAL4 protein.

The fusion protein expressed from the 14-3-3 clone interacted sufficiently with the construct containing the AHA2

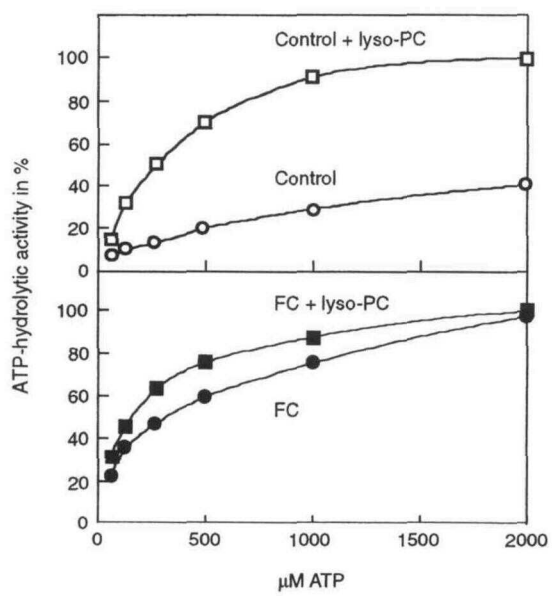

Figure 5. ATP Hydrolytic Activity of H^+-ATPase Purified by Gel Filtration Chromatography.

ATP hydrolytic activity was measured with peak fractions of ATPase activity obtained after gel filtration (Figure 4, fraction 9) of the H^+-ATPase obtained from either fusicoccin (FC)-treated or control shoots of maize. Activity was measured in the absence (○, control; ●, FC) or presence of 0.02% lysophosphatidylcholine (□, control + lyso-PC; ■, FC + lyso-PC) to fully activate the H^+-ATPase. Activities are expressed as the percentage of ATPase activity at 2 mM ATP in the presence of lysophosphatidylcholine.

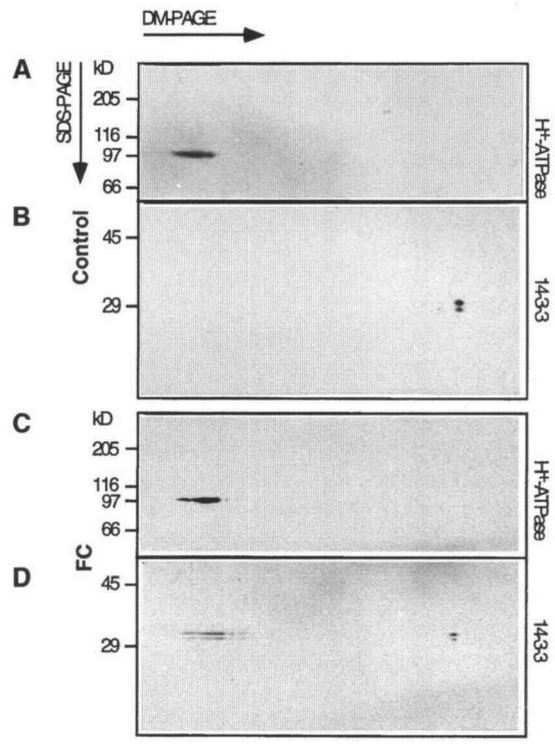

Figure 6. Protein Gel Blots Showing the H+-ATPase and 14-3-3 Protein after Two-Dimensional PAGE of the H+-ATPase–Enriched Fractions.

Aliquots of H+-ATPase–enriched fractions (the 20% PEG fraction) obtained from control and fusicoccin (FC)-treated shoots were separated first under nondenaturing conditions by DM-PAGE and then in the second dimension under denaturing conditions (SDS-PAGE). After transfer of the protein to a polyvinyldifluoride membrane, the membrane was halved.

(A) Control preparation, upper half of the transfer membrane.

(B) Control preparation, lower half of the transfer membrane.

(C) FC preparation, upper half of the transfer membrane.

(D) FC preparation, lower half of the transfer membrane.

In **(A)** and **(C)**, the H+-ATPase was detected by protein gel blotting using monoclonal antibody 46E5B11F6 raised against the maize H+-ATPase. In **(B)** and **(D)**, the 14-3-3 protein was detected by protein gel blotting using the anti-barley 14-3-3 protein antiserum. The positions of molecular mass marker proteins are indicated at left.

C-terminal region to allow the growth of the transformed host on media lacking histidine and to express high levels of β-galactosidase activity, as shown in Figure 8. The addition of fusicoccin had no effect on this interaction. Two proteins known to interact with each other in yeast, the SNF1 protein kinase and its activating subunit SNF4 (Celenza et al., 1989), gave a positive signal in the two-hybrid system. Under the same system, however, no interaction was observed between the 14-3-3 protein and part of the ATP-binding loop of AHA2. In addition, the empty vectors gave no signal.

DISCUSSION

We show here that when the plasma membrane H+-ATPase is purified in the fusicoccin-activated state, it copurifies with the 14-3-3 protein. In addition, we show that this 14-3-3 protein is removed from the H+-ATPase together with the C-terminal region of the H+-ATPase when treated with trypsin and that the 14-3-3 protein interacts with the last 98 C-terminal amino acids of the H+-ATPase when the yeast two-hybrid system is used. This demonstrates that the 14-3-3

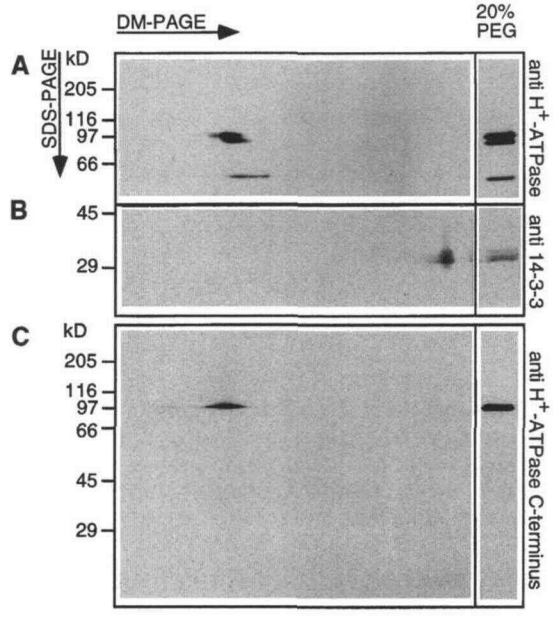

Figure 7. Protein Gel Blots Showing H+-ATPase Polypeptides and the 14-3-3 Protein after Two-Dimensional PAGE of an H+-ATPase–Enriched Fraction Subjected to Trypsin Treatment.

An H+-ATPase–enriched fraction (the 20% PEG fraction) from fusicoccin-treated shoots was incubated with trypsin to remove the C-terminal region of the H+-ATPase. Proteolysis was stopped by adding the Pefabloc SC protease inhibitor, and the protein was separated by nondenaturing DM-PAGE in the first dimension and by denaturing SDS-PAGE in the second dimension. After transfer of the protein to a polyvinyldifluoride membrane, the membrane was halved.

(A) Upper half of the membrane probed with monoclonal antibody 46E5B11F6 raised against the maize H+-ATPase (anti–H+-ATPase).

(B) Lower half of the same membrane as shown in **(A)**, probed with the anti–barley 14-3-3 protein antiserum (anti–14-3-3).

(C) The entire polyvinyldifluoride membrane probed with an antiserum raised against the C-terminal region of the Arabidopsis H+-ATPase isoform AHA3 (anti–H+-ATPase C terminus; No. 759).

The staining pattern for only the 20% PEG fraction after SDS-PAGE is shown at right. The positions of molecular mass marker proteins are indicated at left.

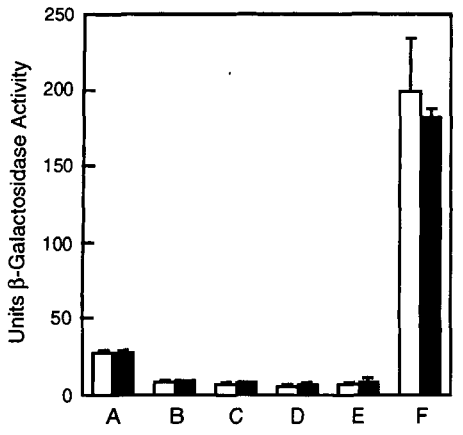

Figure 8. Results of the Two-Hybrid Screen Showing the Interaction between the C-Terminal Region of an Arabidopsis Plasma Membrane H^+-ATPase and a 14-3-3 Protein.

The two-hybrid vectors were cotransformed into yeast, and the activity of the β-galactosidase reporter gene was subsequently assayed as described in Methods. Positive interaction correlates with increased β-galactosidase activity. Open columns indicate no additions. Filled columns represent 5 μM fusicoccin added to the growth medium. Error bars indicate the standard error in three independent experiments. Bar A, positive control, pACT2/*SNF4* fusion versus pAS1/*SNF1* construct; bar B, pACT2 versus pAS1-CYH, empty vectors expressing the GAL4 transcriptional activation domain and the GAL4 DNA binding domain, respectively; bar C, pACT2 versus the pAS1-CYH1/14-3-3 *GF14-φ* fusion; bar D, pACT2/*AHA2* (amino acids 850 to 947) fusion involving the C-terminal 98 amino acids of AHA2 versus pAS1-CYH; bar E, pACT2/*AHA2* (amino acids 500 to 600) fusion involving part of the ATP binding loop of AHA2 versus pAS1-CYH1/14-3-3 *GF14-φ* construct; bar F, the pACT2/*AHA2* (amino acids 850 to 947) fusion versus the pAS1-CYH1/14-3-3 *GF14-φ* construct.

protein interacts directly with the C-terminal region of the H^+-ATPase.

The evidence presented here is supported by biochemical data on cosedimentation of H^+-ATPase and fusicoccin binding activity (Cocucci and Marrè, 1991), copurification of the H^+-ATPase and 14-3-3 protein that is abolished by trypsin treatment (Oecking et al., 1997), and recent data of De Michelis et al. (1996). Using antibodies specific to fusicoccin, De Michelis et al. (1996) showed a linear correlation between the amount of fusicoccin bound to the plasma membrane and activation of the H^+-ATPase and suggested that no amplification step was needed but that activation of H^+ pumping could be explained by direct interaction between the 14-3-3 protein and the H^+-ATPase.

Reversible phosphorylation of the plasma membrane H^+-ATPase has been suggested to be important for regulation of plant defense responses to fungal pathogens (Xing et al., 1996). Accordingly, Moorhead et al. (1996) have proposed a model in which the 14-3-3 protein acts as an inhibitor of the H^+-ATPase and in which the 14-3-3 protein is tightly bound to a phosphorylated site on the H^+-ATPase. When the 14-3-3 protein is released by dephosphorylation of the binding site or when the H^+-ATPase–14-3-3 protein complex is disrupted by fusicoccin, the H^+-ATPase is activated. However, this model does not seem to be compatible with the increased (not decreased) binding of the 14-3-3 protein to the plasma membrane observed when the H^+-ATPase is activated by fusicoccin (Korthout and de Boer, 1994; Oecking et al., 1994; Figure 3).

The model also seems to disagree with the direct correlation between the amount of fusicoccin bound to the plasma membrane and activation of the H^+-ATPase observed by De Michelis et al. (1996). In addition, the model of Moorhead et al. (1996) seems to be in conflict with our data and the data of Oecking et al. (1997), which shows that activation of the H^+-ATPase correlates with copurification of 14-3-3 protein with the enzyme. Moreover, in contrast to Moorhead et al. (1996), we could not increase plasma membrane H^+-ATPase activity by the addition of a peptide (phosphoserine–Raf-259) known to disrupt 14-3-3 binding to many target proteins. Peptide synthesized from two independent sources was used and was tested on plasma membranes isolated from yeast expressing Arabidopsis AHA2 H^+-ATPase (Baunsgaard et al., 1997), spinach leaves, and radish seedlings (data not shown). Both plasma membranes isolated from control and fusicoccin-activated plant material were used. We observed no effect of phosphoserine–Raf-259 on either H^+-ATPase activity or on the amount of 14-3-3 protein associated with the membranes (the latter only tested with fusicoccin-activated material). The 14-3-3 protein is therefore more likely to function as an activator protein of the H^+-ATPase rather than as an inhibitor protein. It is also possible, however, that the 14-3-3 protein may function as both an activator and inhibitor protein, depending on its mode of interaction with the H^+-ATPase (de Boer, 1997). This may be clarified by determining the binding site(s) for the 14-3-3 protein on the H^+-ATPase.

Recent data suggest that 14-3-3 proteins bind to a phosphorylated motif in their target proteins (Muslin et al., 1996). Aitken (1996) suggests that the sequence RVKPSPXP, where S is Ser-697 to Ser-703 (putatively phosphorylated) in a highly conserved domain located in a putative cytoplasmic loop of the H^+-ATPase, is one binding site for the 14-3-3 protein. Notably, the C-terminal region of the H^+-ATPase lacks the phosphorylated motif recognized by the 14-3-3 protein (Muslin et al., 1996), although it is implicated here in 14-3-3 protein binding (Figures 7 and 8). However, other so far uncharacterized motifs for binding are known to exist (reviewed in Aitken, 1996). Thus, the H^+-ATPase may have more than one binding site for the 14-3-3 protein, at least one of which is located in the C-terminal region (this work; Oecking et al., 1997) and one on a cytoplasmic loop (Aitken, 1996). The bridging of these sites by a 14-3-3 dimer or the binding of the 14-3-3 protein to any one site may thus modulate the activity of the H^+-ATPase, and binding to both

sites or to a single site may be affected by fusicoccin in a way not yet fully understood.

In the two-hybrid system, the association between the 14-3-3 protein and the C-terminal region of the plasma membrane H^+-ATPase was not affected by fusicoccin (Figure 8). This shows that fusicoccin is not required for binding of the 14-3-3 protein to the H^+-ATPase and suggests that the 14-3-3 protein is a natural ligand of the plasma membrane H^+-ATPase regulating H^+ pumping. The interaction between the 14-3-3 protein and H^+-ATPase is apparently reversible in the absence of fusicoccin. The increased association between the 14-3-3 protein and H^+-ATPase when treated with fusicoccin suggests that fusicoccin in some way stabilizes the H^+-ATPase–14-3-3 protein complex, thus rendering the association in practice irreversible, even in the presence of the competing phosphoserine–Raf-259 peptide. Therefore, application of fusicoccin strongly facilitates purification of the H^+-ATPase–14-3-3 protein complex.

METHODS

Plant Material

Maize (*Zea mays*) seeds were soaked overnight and sown in moistened vermiculite. Maize seedlings were cultivated for 4 days in darkness at 26°C. Entire shoots containing mesocotyls, primary leaves, and coleoptiles were collected. For fusicoccin stimulation, shoots were cut into small pieces with a razor blade and incubated for 1 hr with 5 μM fusicoccin in 0.1% ethanol.

Isolation of Plasma Membranes

Maize shoots (130 g) were homogenized in 130 mL of 250 mM Tris-HCl, pH 8.0, 300 mM sucrose, 25 mM EDTA, 5 mM DTT, 5 mM ascorbate, 0.6% (w/v) polyvinylpolypyrrolidone, and 1 mM phenylmethylsulfonyl fluoride. Plasma membranes were purified from the microsomal fraction (10,000 to 50,000*g* pellet) by partitioning at 4°C in an aqueous polymer two-phase system composed of 6.3% (w/w) dextran T500 (Pharmacia), 6.3% (w/w) polyethylene glycol (PEG) 1500 (British Petroleum Company, London, UK), 330 mM sucrose, 5 mM potassium phosphate, pH 7.8, 3 mM KCl, 0.1 mM EDTA, and 1 mM DTT (Larsson et al., 1994). The final plasma membrane pellet was suspended in 10 mM 2-(*N*-morpholino)ethanesulfonic acid (Mes)–1,3-*bis*(*tris*[hydroxymethyl]methylamino)propane (BTP), pH 7.0, 20% (v/v) glycerol, 5 mM EDTA, and 1 mM DTT (glycerol buffer).

Partial Purification of H^+-ATPase

Plasma membranes (4 mg of protein in 4 mL) were mixed with an equal volume of 1% (w/v) Brij 58 in glycerol buffer to turn all vesicles inside out (Johansson et al., 1995) and then were diluted fivefold in 0.5 M KCl in glycerol buffer to remove loosely bound protein. The plasma membranes were recovered by centrifugation at 100,000*g* for 45 min at 4°C and resuspended at room temperature in 4 mL of glycerol buffer containing 0.5 mM ATP and 5 mg mL^{-1} of the detergent dodecyl β-D-maltoside to solubilize the H^+-ATPase (Johansson et al., 1994). Unsolubilized material was removed by centrifugation at 40,000*g* for 20 min at 15°C. Protein was precipitated from the dodecyl β-D-maltoside supernatant by mixing with continuous stirring in PEG 3350 (Sigma; 60% [w/v] stock solution); protein precipitated in 10% PEG was collected as one fraction (10% PEG), and protein was precipitated between 10 and 20% PEG as another fraction (20% PEG). Precipitated protein was resuspended in 400 μL of 0.1% dodecyl β-D-maltoside in glycerol buffer. An aliquot (100 μL) of the 20% PEG fraction containing most of the H^+-ATPase (see above) was loaded on a 10-cm Sephacryl S-300 HR (Pharmacia) gel filtration column and eluted with detergent-free glycerol buffer. Fractions of five droplets corresponding to ~125 μL were collected.

H^+-ATPase Activity

ATP hydrolytic activity was measured essentially as described by Palmgren (1990). The assay medium contained 10 mM Mes-BTP, pH 7.0, 4 mM $MgCl_2$, 90 mM KCl, 50 mM KNO_3, 1 mM EDTA, 1 mM DTT, 0.25 mM NADH, 1 mM phosphoenolpyruvate, 50 μg of pyruvate kinase, 25 μg of lactate dehydrogenase, 10 to 50 μg of plasma membrane protein or an aliquot of purified H^+-ATPase, and different concentrations of ATP (as indicated) in a total volume of 1 mL. The assay was started by the addition of $MgCl_2$. In this assay, ATP hydrolysis is coupled enzymatically to the oxidation of NADH, and the rate of ATP hydrolysis is measured as the absorbance decrease at 340 nm. The detergents used in this study included 0.05% (w/v) Brij 58 to obtain 100% inside-out vesicles (Johansson et al., 1995) and 0.02% (w/v) lysophosphatidylcholine to obtain a fully activated H^+-ATPase (Palmgren et al., 1988; Johansson et al., 1993).

SDS-PAGE and Protein Gel Blotting

Protein was either precipitated with 10% (w/v) trichloroacetic acid before solubilization in the SDS cocktail or directly solubilized in the SDS cocktail at room temperature, and then it was subjected to SDS-PAGE, according to Laemmli (1970). Gels were stained with Coomassie Brilliant Blue R 250, or proteins were transferred electrophoretically to an Immobilone polyvinyldifluoride transfer membrane (Millipore) for immunostaining.

Antiserum 759, raised against a fusion protein containing the amino acid sequence 851 to 949 of AHA3 (*Arabidopsis thaliana* H^+-ATPase isoform 3; Pardo and Serrano, 1989), was a kind gift from R. Serrano (Instituto de Biologia Molecular y Celular de Plantas, Valencia, Spain). The monoclonal antibody 46E5B11F6, raised against maize H^+-ATPase (Palmgren and Christensen, 1994), was kindly provided by W. Michalke (Albert-Ludwigs-Universität, Freiburg, Germany). The anti–14-3-3 protein antiserum was raised against a barley 14-3-3 protein (Brandt et al., 1992). Nonfat milk powder (2%) in 140 mM NaCl, 10 mM Tris-HCl, pH 7.6, was used to block the transfer membrane, and this buffer was then used as incubation medium throughout the procedure. Antiserum 759 was diluted 1000-fold, whereas monoclonal antibody 46E5B11F6 and anti–14-3-3 protein antiserum were diluted 5000-fold. The secondary antibodies (alkaline phosphatase conjugated; Dianova, Hamburg, Germany) were diluted 8000-fold, and the protein gel blots were developed with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

Two-Dimensional PAGE

For the first dimension under nondenaturing conditions, aliquots (3 μL) of the 20% PEG fraction were mixed with 10 μL of glycerol buffer and loaded on a polyacrylamide gel containing 4.5% (w/v) acrylamide, 325 mM Tris-HCl, pH 8.8, 20% glycerol, and 0.05% dodecyl β-D-maltoside. For better handling, these gels were cast on transparencies. The electrophoresis buffer contained 0.192 M glycine-Tris, pH 8.3. After pre-electrophoresis at 6 mA for 5 min, samples were run under constant voltage at 180 V for 1 hr. This procedure is referred to as dodecyl β-D-maltoside (DM)–PAGE above. For the second dimension, the DM gel was cut into strips, and the strips were equilibrated in an SDS cocktail according to Laemmli (1970) and placed on top of an SDS gel before PAGE.

Cleavage of the H^+-ATPase with Trypsin

A sample (50 μL) of the 20% PEG fraction containing 4 mM ATP was mixed with an equal volume of 0.1 mg mL^{-1} trypsin in glycerol buffer and incubated for 5 min at room temperature. Proteolysis was stopped by the addition of 10 μL of the protease inhibitor Pefabloc SC (30 mg mL^{-1}; BIOMOL Research Laboratories, Plymouth Meeting, PA), and 10-μL aliquots were immediately subjected to DM-PAGE or solubilized in a SDS cocktail for SDS-PAGE.

Protein

Protein was measured essentially according to Bearden (1978), with BSA as a standard.

Two-Hybrid Screens

Saccharomyces cerevisiae strain Y190 (*MATa gal4 gal80 his3 trp1-901 ade2-101 ura3-52 leu2-3,-112*$^+$ URA3::*GAL→lacZ*, *LYS2*::*GAL→HIS3 cyh*^{r}) was used as a host for two-hybrid pACT2 and pAS1-CYH plasmid constructs (Durfee et al., 1993). These yeast expression vectors contain the 2-μm origin and the *LEU2* (pACT2) and *TRP1* (pAS1-CYH) genes for selection in yeast, and they have open reading frames encoding the GAL4 DNA binding domain (pAS1-CYH) and the GAL4 activation domain (pACT2), respectively, brought under the control of the alcohol dehydrogenase *ADH1* promoter (Durfee et al., 1993).

Yeast cells were transformed simultaneously with GAL4 DNA binding fusion plasmids and GAL4 transcriptional activation domain fusion plasmids. Transformants were plated on solid medium lacking tryptophan and leucine for selection of double transformants and on medium lacking tryptophan (30 μg mL^{-1}), leucine (30 μg mL^{-1}), and histidine (30 μg mL^{-1}) but containing 25 mM 3-aminotriazole (Sigma A8056) to determine whether the encoded proteins interacted. All yeast cultures were grown in liquid or on solid minimal medium composed of 0.7% yeast nitrogen base without amino acids (Difco) and 2% glucose, adenine (30 μg mL^{-1}), and, depending on the transforming plasmid, histidine, leucine, and tryptophan (30 μg mL^{-1}).

β-Galactosidase activity was measured on liquid cultures, using *O*-nitrophenyl β-D-galactopyranoside as a substrate. Quantitation was done under standard conditions (Guarente, 1983), except that the cells were permeabilized by incubating them in the presence of 0.2% sarcosine (Sigma L5125) for 30 min.

Construction of Plasmids

The cDNA encoding the C-terminal hydrophilic region (amino acids 850 to 947) of AHA2 (Arabidopsis H^+-ATPase isoform 2) was amplified by polymerase chain reaction (PCR), using full-length AHA2 as the template by using the primers 5′-GCGCATGCCATGGGGCTGAACTTGTTTGAG-3′, which introduced an NcoI site, and 5′-CGGGATCCTTACTACACAGTGTAGTGACTG-3′, which introduced a BamHI site downstream of the stop codon. The *AHA2* cDNA encoding amino acids 500 to 600 in the large cytoplasmic loop involved in ATP binding was PCR generated, using the primers 5′-CTAGCCATGGCTTTGAATCTTGGTGTT-3′, introducing an NcoI site, and 5′-GCGGATCCTAACCGATATCAGCTTTC-3′, introducing a stop codon followed by a BamHI site. The PCR products were cloned into pACT2. Arabidopsis 14-3-3 *GF14-φ* (Lu et al., 1994) was obtained by inserting a linker containing an NcoI site into the NdeI site in pCRII14-10 (a generous gift from R. Ferl, University of Florida, Gainesville, FL), and the 1.1-kb NcoI-BamHI fragment was cloned into pAS1-CYH.

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