

## Fusicoccin Effect on the *in Vitro* Interaction between Plant 14-3-3 Proteins and Plasma Membrane H<sup>+</sup>-ATPase\*

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A 17-amino acid peptide was selectively cleaved from the highly variant C terminus of the 33-kDa 14-3-3 isoform occurring in fusicoccin receptor preparations from maize and was sequenced. The determined C-terminal sequence was identical to that of the already known maize 14-3-3 homolog GF14-6, thus prompting the use of recombinant GF14-6 in an *in vitro* protein-protein interaction study. The cDNA of GF14-6 was expressed in *Escherichia coli* as a <sup>32</sup>P-phosphorylatable glutathione S-transferase fusion protein and was used as a probe in overlay experiments with H<sup>+</sup>-ATPase partially purified from maize roots. The results demonstrated that the recombinant protein specifically bound to H<sup>+</sup>-ATPase. The binding was dependent on Mg<sup>2+</sup> and was strongly increased by fusicoccin. Controlled trypsin digestion of H<sup>+</sup>-ATPase abolished the association with GF14-6, a finding that was suggestive of an interaction with the C terminus of the enzyme. To confirm this result, the C-terminal domain of H<sup>+</sup>-ATPase was expressed as a glutathione S-transferase fusion peptide and was used in overlay experiments. GF14-6 was also able to bind to the isolated C terminus, but only in the presence of fusicoccin.

The discovery that receptors of the fungal toxin fusicoccin (FC)<sup>1</sup> are members of the 14-3-3 family of eukaryotic regulatory proteins (1) has shed some light on the FC mechanism of activation of plasma membrane H<sup>+</sup>-ATPase (2–4). In fact, from animal research, it is known that a common property of 14-3-3 proteins is the capability to associate with interacting proteins (5, 6). Recently, it has been demonstrated that a similar mechanism of regulation occurs also for NADH: nitrate reductase from spinach leaves (7, 8). Nitrate reductase activity is inhibited upon binding of endogenous 14-3-3 proteins, which can occur only after the enzyme has been phosphorylated at a specific serine residue by a calcium-dependent protein kinase (9). It has also been shown that these endogenous 14-3-3 proteins are a mixture of different 14-3-3 homologs (7) and that multiple 14-3-3 isoforms are able to inhibit nitrate reductase *in*

*vitro* (10). The phosphorylated motif of the nitrate reductase involved in the interaction has been identified, and it is similar to consensus sequences for 14-3-3 binding, occurring in most animal 14-3-3-interacting proteins (10). Furthermore, it has been shown that FC is able to disrupt *in vitro* complexes between nitrate reductase and 14-3-3 proteins, thereby releasing their inhibitory effect. A speculative model for the regulation of plasma membrane H<sup>+</sup>-ATPase based on that worked out for nitrate reductase has been proposed by Moorhead *et al.* (8). According to it, the stimulatory effect of FC would result from its capability to dissociate 14-3-3 proteins from H<sup>+</sup>-ATPase. The *in vitro* evidence supporting this model relies on the activating effects of the phosphoserine 259-Raf1 peptide and protein phosphatase 2A on plasma membrane-bound H<sup>+</sup>-ATPase activity, which parallels that of FC.

However, results from other groups are in contrast with those presented by Moorhead *et al.* (8). In fact, it has been demonstrated that FC *in vivo* treatment of plant tissues, which produces a strong stimulation of H<sup>+</sup>-ATPase activity, concomitantly brings about a marked increase in plasma membrane-associated 14-3-3 proteins (4, 11). Also, the amount of FC bound to plasma membrane appears to be strictly correlated with stimulation of H<sup>+</sup>-ATPase activity (12). More recent evidence indicates that administration of FC *in vivo* or *in vitro* stabilizes (13) or promotes (14) the interaction between H<sup>+</sup>-ATPase and 14-3-3 proteins.

The data reported here demonstrate that recombinant GF14-6, a 14-3-3 homolog already identified in maize as a component of the G-box-binding complex (15), is able to associate *in vitro* with H<sup>+</sup>-ATPase and, remarkably, that this interaction is modulated by FC. By trypsin treatment of H<sup>+</sup>-ATPase and expression of the isolated C terminus as a GST fusion peptide, we also demonstrate that the 14-3-3 probe interacts with the C-terminal domain of H<sup>+</sup>-ATPase in a FC-dependent manner. Structural data, obtained by sequencing the hypervariable C-terminal domain of the 33-kDa 14-3-3 protein present in purified preparations of FC receptors from maize, strongly suggest that this isoform is highly homologous to GF14-6, thus prompting its use in the *in vitro* protein-protein interaction study.

### EXPERIMENTAL PROCEDURES

**Chemicals**—FC was prepared according to Ballio *et al.* (16). [<sup>32</sup>P]ATP (specific activity of 3000 Ci/mmol) was purchased from Amersham Pharmacia Biotech, and chemicals for gel electrophoresis were from Bio-Rad. Dodecyl β-D-maltoside was purchased from Sigma. All other reagents were analytical grade.

**Plant Material**—Maize seeds (*Zea mays* L. var. Paolo; Dekalb, Mestre, Italy) were grown for 6 days in the dark under previously described conditions (17).

**Purification of FC Receptors**—Partially purified FC-binding proteins

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<sup>1</sup> The abbreviations used are: FC, fusicoccin; GST, glutathione S-transferase; HPLC, high pressure liquid chromatography; PAGE, polyacrylamide gel electrophoresis.

were obtained as described previously (17). Briefly, crude solubilized receptors were prepared from maize shoot microsomes according to the method described by Aducci *et al.* (18). Solubilized receptors were pre-incubated for 30 min with 10 nM tritiated dihydrofusicocin at 27 °C and fractionated on a hydroxylapatite column. Radioactive fractions were pooled and further purified by anion-exchange HPLC using a DEAE TSK-5-PW column (Bio-Rad) and System Gold equipment (Beckman Instruments).

**Purification of H<sup>+</sup>-ATPase**—H<sup>+</sup>-ATPase from maize roots was solubilized from two-phase partitioned plasma membranes and purified by anion-exchange HPLC as previously described (11). To obtain C-terminally truncated H<sup>+</sup>-ATPase, before purification, plasma membranes were treated with trypsin under the conditions described by Rasi-Caldogno *et al.* (19). Where indicated, H<sup>+</sup>-ATPase was purified from maize roots incubated *in vivo* with 10 μM FC according to Johansson *et al.* (20).

**C-terminal Peptide Sequence**—Partially purified FC receptor preparations were subjected to hydrolysis with 70% formic acid for 48 h at 40 °C and then diluted 20-fold, freeze-dried, and blotted according to Marra *et al.* (2). 14-3-3 proteins were detected using rabbit anti-14-3-3 polyclonal antibodies at 1:5000 dilution and horseradish peroxidase-conjugated secondary antibodies (Bio-Rad) with the enhanced chemiluminescence detection system (ECL, Amersham Pharmacia Biotech) following the manufacturer's instructions.

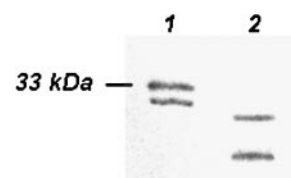
For determination of the C-terminal peptide sequence, partially purified FC receptor preparations were subjected to SDS-PAGE. The Coomassie Blue-stained 33-kDa band was excised and treated with 70% formic acid under the conditions described above. Oligopeptides were selectively extracted from the gel using the procedure described by Stone and Williams (21), treated with *o*-phthalaldehyde, and subjected to Edman degradation. Hydrolysis, isolation of peptides, and sequence analysis were performed by Eurosequence (Groningen, The Netherlands).

**Antiserum Production and Western Blotting**—The 19-amino acid C-terminal peptide of the 33-kDa 14-3-3 protein (YDPAEEIREAPKRDSSSEGQ) and peptide YKGVHVESVVKLKG (corresponding to residues 927–938 of the C-terminal sequence of *AHA2* H<sup>+</sup>-ATPase (*Arabidopsis* H<sup>+</sup>-ATPase isoform 2)) were synthesized by Neosystem (Strasbourg, France). The Tyr residues at the N termini were added to allow coupling to bovine serum albumin using bisdiazobenzidine.

Antigens (1 mg) were mixed with Freund's complete adjuvant (1:1, v/v) and injected into two rabbits. Two booster injections were given at 2-week intervals. Antisera were used for Western blot analysis as described by Marra *et al.* (2) at 1:5000 dilution. Immunoreactive proteins were revealed using the ECL system.

**Production of <sup>32</sup>P-Labeled GF14-6**—The cDNA of GF14-6, previously obtained in our laboratory by polymerase chain reaction and cloned into pTrc99A (data not shown), was moved from the vector, made blunt with Klenow enzyme, and subcloned into the *Sma*I site of the pGEX-2TK vector to express it as a GST fusion protein. JM105 cells, transformed with the expression vector, were grown at 37 °C in SOB broth (20 g/liter bactotryptone, 5 g/liter yeast extract, 8.5 mM NaCl, 2.5 mM KCl, 10 mM MgCl<sub>2</sub>, pH 7) with 100 μg/ml ampicillin up to A<sub>600</sub> = 0.6. After 6 h of induction with 0.5 mM isopropyl-β-D-thiogalactopyranoside, cells were harvested by centrifugation and lysed. 1 ml of cell lysate (40 μg of GST fusion protein) was incubated with 30 μl of glutathione-Sepharose 4B beads at room temperature for 30 min. After two washes with phosphate-buffered saline (140 mM NaCl, 2.7 mM KCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, and 1.8 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.3) and one wash with buffer A (20 mM Tris-HCl, 12 mM MgCl<sub>2</sub>, and 100 mM NaCl, pH 7.5), the beads were incubated for 30 min at 30 °C in 30 μl of buffer A containing 1 mM dithiothreitol, 30 μCi of [γ-<sup>32</sup>P]ATP (3000 Ci/mmol), and 10 units of the catalytic subunit of protein kinase A (Sigma). The reaction was stopped by the addition of 1 ml of phosphate-buffered saline containing 10 mM EDTA. The beads were washed three times with phosphate-buffered saline and then incubated with 1 unit of thrombin (Amersham Pharmacia Biotech) in 100 μl of phosphate-buffered saline overnight at 22 °C. The mixture was centrifuged, and the supernatant was collected. <sup>32</sup>P incorporation was measured with a Wallac 1410 β-counter.

**Expression of the H<sup>+</sup>-ATPase C-terminal Domain in *E. coli***—The cDNA fragment, corresponding to peptide 845–947 of the *mha2* (maize H<sup>+</sup>-ATPase isoform 2) isoform sequence of the H<sup>+</sup>-ATPase from maize (22), was amplified on total RNA from maize roots by reverse transcriptase polymerase chain reaction using specific oligonucleotides based on sequences 2737–2752 and 3034–3048 of the *mha2* cDNA. The resulting fragment was cloned into *Bam*HI-*Eco*RI sites of the pGEX-2TK vector. The expression and purification of the GST-fused C termi-



**FIG. 1. Formic acid treatment of 14-3-3 proteins from FC receptor preparations.** HPLC-purified FC receptor preparations from maize shoots (10 μg of total protein) were incubated with 70% formic acid for 48 h at 40 °C and then subjected to SDS-PAGE and blotted. 14-3-3 proteins were detected with antibodies raised against a plant 14-3-3 conserved region (2). Lane 1, untreated sample; lane 2, sample treated with formic acid.

nus were carried out under the same conditions adopted for GST-GF14-6 production.

**Far Western Blotting**—HPLC-purified H<sup>+</sup>-ATPase-containing fractions (5 μg of total protein) or the GST-fused C terminus (5 μg of protein) was subjected to SDS-PAGE and blotted onto nitrocellulose in transfer buffer (25 mM Tris, 192 mM glycine, and 0.01% SDS, pH 8.3). After denaturation-renaturation in 6 to 0.3 M guanidine HCl in buffer B (25 mM Hepes-NaOH, 25 mM NaCl, 5 mM MgCl<sub>2</sub>, and 1 mM dithiothreitol, pH 7.7), the membrane was saturated at 4 °C in buffer B containing 5% fatty acid-free milk and 0.04% Tween 20. Incubation with <sup>32</sup>P-labeled GF14-6 (600,000 cpm/ml) was performed overnight at 4 °C in buffer C (20 mM Hepes-KOH, 75 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, 2% fatty acid-free milk, and 0.04% Tween 20, pH 7.7) in the presence of FC and MgCl<sub>2</sub> when specified. After three washes with buffer C, the membrane was dried and subjected to autoradiography at –70 °C.

**Analytical Methods**—Protein concentration was determined by the method of Bradford (23) using bovine serum albumin as a standard. H<sup>+</sup>-ATPase activity was assayed according to Serrano (24).

## RESULTS

**C-terminal Sequence of the 33-kDa 14-3-3 Protein Present in FC Receptor Preparations**—Sequence data obtained on purified preparations of FC receptors from different sources demonstrated that they are members of the 14-3-3 protein family (2–4). Since all of the determined sequences were from highly invariant regions, no indication about isoform identity was obtained. Comparison of the known sequences of plant 14-3-3 proteins showed that the 20-amino acid carboxyl-terminal peptide is highly divergent, representing the major determinant of isoform diversity. The two maize isoforms identified up to now, GF14-6 and GF14-12, contain an acid-labile Asp-Pro bond (between residues 244 and 245) (25) close to the C terminus (15). This motif appears to be a peculiar feature of these homologs, as it does not occur in all other known plant 14-3-3 proteins.

To ascertain the occurrence of Asp-Pro bonds in the 14-3-3 proteins present in purified FC receptor preparations from maize shoots, a mild hydrolysis in 70% formic acid was performed. Samples were then subjected to Western blotting using anti-14-3-3 antibodies for immunodecoration (2). As shown in Fig. 1, the results demonstrated that both the 31- and 33-kDa 14-3-3 proteins, which are usually present in purified FC receptor preparations, were down-shifted. The electrophoresis mobility shift of the 33-kDa band appears to be compatible with the expected position of the Asp-Pro bond within the amino acid sequence.

Hence, to determine the sequence of the C-terminal acid-cleavable peptide, formic acid hydrolysis was performed on the 33-kDa band excised from the gel. Oligopeptides released by this treatment were selectively extracted (21) and subjected to Edman degradation. The determined sequence (PAEEIREAPKRDSSSEGQ) was 100% homologous to that of GF14-6.

The identity of the two C termini suggested a very high degree of homology between the whole proteins. In fact, as

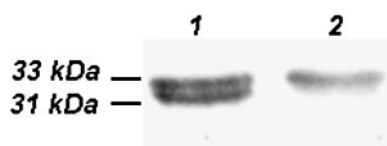


FIG. 2. Recognition of the 33-kDa 14-3-3 isoform from FC receptor preparations by anti-C terminus antibodies. Anti-C terminus antibodies were raised against the determined C-terminal sequence of the 33-kDa isoform. HPLC-purified FC receptor preparations were subjected to Western blotting (10  $\mu$ g of total protein) and probed with the anti-plant 14-3-3 conserved region antibodies (lane 1) and with the anti-C terminus antibodies (this work) (lane 2).

already mentioned, this hypervariable region represents the major determinant of plant isoform diversity (26). Attempts to determine the C-terminal sequence of the 31-kDa band were unsuccessful since only a tentative assignment of the first five residues was achieved.

**FC in Vivo Treatment Increases the Amount of Plasma Membrane-associated 33-kDa 14-3-3 Protein**—Polyclonal antibodies were raised against the determined C-terminal sequence of the 33-kDa protein. The specificity of the antiserum was assayed in Western blotting by testing the cross-reactivity toward 14-3-3 proteins partially purified from membrane preparations of radish seedlings and tobacco and spinach leaves. The antibodies were unable to recognize 14-3-3 proteins from these sources (data not shown), whereas in maize, only the 33-kDa isoform was recognized (Fig. 2, lane 2). It has been demonstrated that FC *in vivo* treatment brings about stimulation of H<sup>+</sup>-ATPase activity and concomitantly increases the amount of plasma membrane-associated 14-3-3 proteins (4, 11). Hence, the anti-C terminus antibodies were used to investigate whether FC *in vivo* incubation could increase the association of the 33-kDa isoform with plasma membrane. 14-3-3 proteins were HPLC-purified (11) from control or FC-preincubated maize roots and subjected to immunoblotting with the anti-C terminus antibodies. The results shown in Fig. 3 demonstrated that the 33-kDa 14-3-3 isoform was more abundant in fractions obtained from FC-treated samples.

**Recombinant 14-3-3 Binds to H<sup>+</sup>-ATPase: Binding Is Modulated by Mg<sup>2+</sup> and FC**—The structural and immunological pieces of evidence reported above led us to choose the GF14-6 isoform to perform protein-protein interaction studies between 14-3-3 and H<sup>+</sup>-ATPase. The cDNA of GF14-6 was cloned into pGEX-2TK and expressed as a GST fusion protein. The expression system produced a 14-3-3 protein with a cAMP-dependent kinase phosphorylation site at the N-terminal end. GST-GF14-6 was <sup>32</sup>P-labeled by incubation with [ $\gamma$ -<sup>32</sup>P]ATP and protein kinase A and digested with thrombin to remove GST. Partially HPLC-purified fractions of H<sup>+</sup>-ATPase from maize roots (11) were subjected to SDS-PAGE, blotted onto nitrocellulose, renatured, incubated under different conditions with radioactive GF14-6, and autoradiographed. The results showed that, among other bands present in the partially purified fractions (Fig. 4, lane A), only a 100-kDa polypeptide, which was recognized by the anti-H<sup>+</sup>-ATPase C terminus antibodies in Western blotting (Fig. 4, lane B), was labeled by the radioactive 14-3-3 probe in the presence of 5 mM Mg<sup>2+</sup> (Fig. 4, lane C).

Fig. 5 shows that the addition of 100  $\mu$ M FC to the incubation medium strongly enhanced the labeling of H<sup>+</sup>-ATPase. The effect of FC was still detectable, even though to a reduced extent, at a concentration as low as 5  $\mu$ M. The binding of GF14-6 to H<sup>+</sup>-ATPase was abolished by omitting Mg<sup>2+</sup> from the incubation medium, whereas the addition of 100  $\mu$ M FC in the absence of Mg<sup>2+</sup> partially restored the binding.

**Binding of Recombinant 14-3-3 Is Affected by Trypsin Treatment of H<sup>+</sup>-ATPase**—It is known that the C terminus of H<sup>+</sup>-

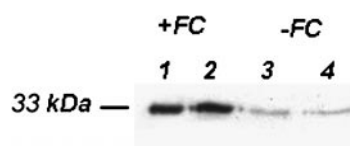


FIG. 3. Effect of FC *in vivo* treatment on the association of the 33-kDa 14-3-3 isoform with the plasma membrane. 14-3-3 proteins from untreated and FC-treated maize roots were solubilized from plasma membranes and purified by HPLC. Aliquots of 14-3-3 protein-containing fractions (10  $\mu$ g of total protein) were blotted and immunodecorated with anti-C terminus antibodies. Lanes 1 and 2, FC-treated samples; lanes 3 and 4, untreated samples.

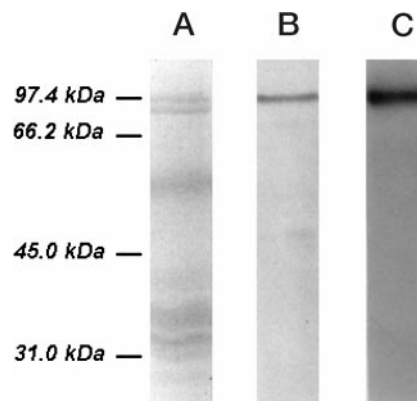


FIG. 4. Interaction between 14-3-3 and H<sup>+</sup>-ATPase. Lane A, 10% SDS-PAGE and Coomassie Blue staining of partially HPLC-purified H<sup>+</sup>-ATPase (5  $\mu$ g of total protein); lane B, immunoblotting of partially HPLC-purified H<sup>+</sup>-ATPase (5  $\mu$ g of total protein) using anti-H<sup>+</sup>-ATPase C terminus antibodies; lane C, far Western blotting using <sup>32</sup>P-labeled GF14-6 as a probe. Partially HPLC-purified H<sup>+</sup>-ATPase (5  $\mu$ g of total protein) was subjected to SDS-PAGE and blotted onto nitrocellulose. After renaturation, the membrane was incubated with <sup>32</sup>P-labeled GF14-6 (600,000 cpm/ml) overnight at 4 °C. The membrane was then washed three times with buffer C, dried, and subjected to autoradiography at -70 °C.

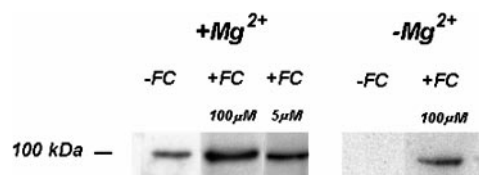


FIG. 5. Effect of Mg<sup>2+</sup> and FC on the interaction between 14-3-3 and H<sup>+</sup>-ATPase. The far Western blotting experiment was run under the same conditions described in the legend to Fig. 4. The concentration of MgCl<sub>2</sub> was 5 mM.

ATPase is an autoinhibitory domain, whose proteolytic removal, or displacement by FC and other effectors, activates the enzyme (19, 27). Therefore, the C terminus could represent a potential target site for binding of 14-3-3 proteins. To test this possibility with our *in vitro* system, plasma membrane-bound H<sup>+</sup>-ATPase was treated with trypsin under the conditions described by Rasi-Caldogno *et al.* (19) to remove the C-terminal region. As expected, this treatment brought about a stimulation (50% at pH 7.4) of the ATP hydrolytic activity of the enzyme (data not shown). The trypsin-cleaved H<sup>+</sup>-ATPase was then solubilized from the plasma membrane, HPLC-fractionated, and subjected to SDS-PAGE followed by Western blotting with anti-C terminus antibodies or to far Western blotting with the radioactive 14-3-3 probe. The results demonstrated that the trypsin-cleaved H<sup>+</sup>-ATPase was recognized neither by the labeled 14-3-3 probe (Fig. 6A) nor by the anti-H<sup>+</sup>-ATPase C terminus antibodies (Fig. 6B). Coomassie Blue staining (Fig. 6C) confirmed that the trypsin treatment caused a decrease in the molecular mass of the enzyme similar to that observed



**FIG. 6. Effect of trypsin treatment on the interaction between 14-3-3 and H<sup>+</sup>-ATPase.** To obtain C-terminally truncated H<sup>+</sup>-ATPase, plasma membranes from maize roots were treated with trypsin under the conditions described by Rasi-Caldogno *et al.* (19) and then solubilized and HPLC-fractionated (11). The HPLC-purified H<sup>+</sup>-ATPase from control or trypsin-treated plasma membranes (5  $\mu$ g of total protein) was subjected to far Western blotting under the same conditions described in the legend to Fig. 4. *A*, far Western blotting; *B*, immunoblotting with anti-H<sup>+</sup>-ATPase C terminus antibodies; *C*, SDS-PAGE and Coomassie Blue staining.

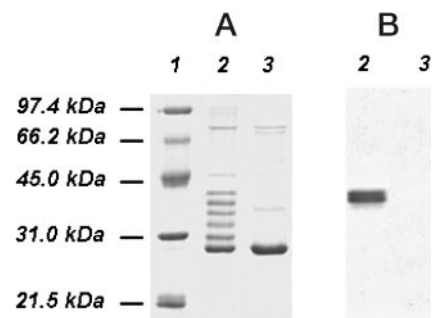
previously (19).

**Recombinant 14-3-3 Binds to the C-terminal Domain of H<sup>+</sup>-ATPase**—The C-terminal domain of the *mha2* isoform of maize H<sup>+</sup>-ATPase, corresponding to the last 103 amino acids of the sequence (residues 845–947), was expressed as a GST fusion peptide and purified by glutathione affinity chromatography. SDS-PAGE showed that multiple bands (in the range 26–36 kDa) were present in the purified fraction (Fig. 7*A*, lane 2). This heterogeneity was due, very likely, to partial proteolysis of the C-terminal domain occurring during purification. In fact, by incubation with thrombin, which completely removed the C-terminal peptide from GST fusion protein, only a single major band at 26 kDa was visible (Fig. 7*A*, lane 3). Immunoblotting of the purified GST-fused C terminus with anti-H<sup>+</sup>-ATPase C terminus antibodies (raised against a peptide corresponding to residues 927–938 of the *AHA2* H<sup>+</sup>-ATPase sequence) resulted in the immunodecoration of the two highest molecular mass bands (Fig. 7*B*, lane 2), whereas no bands were immunodecorated in the thrombin-treated sample (lane 3).

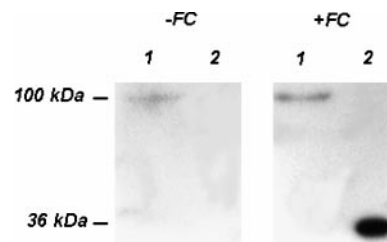
The affinity-purified GST-fused C terminus was blotted and incubated with the 14-3-3 probe under the standard conditions. H<sup>+</sup>-ATPase was also blotted in the same experiment as a control. As shown in Fig. 8 (left panel), in the absence of FC, the 14-3-3 probe labeled H<sup>+</sup>-ATPase (lane 1), whereas it did not interact with the GST-fused C terminus (lane 2). The administration of 100  $\mu$ M FC increased, as expected, the labeling of H<sup>+</sup>-ATPase (Fig. 8, right panel, lane 1) and, more remarkably, brought about a strong labeling of the highest molecular mass band (36 kDa) present in the C-terminal fraction, corresponding to the complete GST-fused C terminus (lane 2).

#### DISCUSSION

Recently, it has been shown by different groups that FC receptors are members of the 14-3-3 eukaryotic protein family (2–4). Yet, no information is currently available about the identity of the 14-3-3 isoform(s) competent in FC perception since all of the sequences determined thus far have been obtained from conserved regions of the proteins. In fact, comparison of known sequences indicates that plant 14-3-3 isoforms share a very high degree of homology, with differences being concentrated almost exclusively in the hypervariable 20-amino acid C-terminal peptide, which therefore represents the major determinant of isoform diversity (26). Hence, to obtain isoform-specific sequence information, we determined the sequence of the 17-amino acid C-terminal peptide of the 33-kDa 14-3-3 protein occurring in purified FC receptor preparations from maize shoots. There was a complete homology to the C terminus of GF14-6, a 14-3-3 homolog already identified in maize as a component of the G-box-binding complex (15). This finding, which was suggestive of the occurrence of a significant homology between the two proteins, prompted us to carry out an *in*



**FIG. 7. Expression of the GST-fused C-terminal domain of H<sup>+</sup>-ATPase.** *A*, SDS-PAGE and Coomassie Blue staining. Lane 1, molecular mass markers; lane 2, glutathione affinity-purified GST-fused C-terminal domain of H<sup>+</sup>-ATPase; lane 3, thrombin-digested GST-fused C-terminal domain of H<sup>+</sup>-ATPase. *B*, immunoblotting with anti-H<sup>+</sup>-ATPase C terminus antibodies. Lane 2, glutathione affinity-purified GST-fused C-terminal domain of H<sup>+</sup>-ATPase; lane 3, thrombin-digested GST-fused C-terminal domain of H<sup>+</sup>-ATPase. 8  $\mu$ g of total protein were loaded for each lane.



**FIG. 8. Interaction between 14-3-3 and the GST-fused C-terminal domain of H<sup>+</sup>-ATPase.** The far Western blotting experiment was run under the same conditions described in the legend to Fig. 4. Incubation with the 14-3-3 probe was performed both in the absence (–FC) and presence (+FC) of 100  $\mu$ M FC. Lane 1, partially HPLC-purified H<sup>+</sup>-ATPase (5  $\mu$ g of total protein); lane 2, glutathione affinity-purified GST-fused C-terminal domain of H<sup>+</sup>-ATPase (8  $\mu$ g of total protein).

*vitro* investigation of the functional properties of GF14-6. To this purpose, the cDNA of GF14-6 was cloned and expressed as a <sup>32</sup>P-phosphorylatable GST fusion protein, which was used as a probe for a partially purified H<sup>+</sup>-ATPase from maize roots in overlay experiments. The results demonstrated that GF14-6 is able to bind specifically to H<sup>+</sup>-ATPase and that different treatments or effectors are able to modulate the interaction between the two proteins. In fact, the binding was dependent on the presence of millimolar concentrations of Mg<sup>2+</sup>, a result reminiscent of that observed for binding of spinach 14-3-3 homologs to nitrate reductase (7) and in accordance with the emerging notion that 14-3-3 proteins bind to phosphorylated serines on target proteins (28). More important, the binding was strongly increased by FC. The effect was better seen at 100  $\mu$ M FC, but was also evident at physiological concentrations of the toxin, such as 5  $\mu$ M. Interestingly, FC was also effective in the absence of Mg<sup>2+</sup>, a result that suggests that Mg<sup>2+</sup> and FC may act by different mechanisms. Trypsin treatment of H<sup>+</sup>-ATPase under conditions known to bring about excision of the C-terminal domain abolished the interaction with the 14-3-3 probe and the FC effect as well. This finding strongly suggests that the binding site for 14-3-3 is located in the C-terminal domain, a region that appears to be also essential for FC action. This result was confirmed by using, in the overlay assay, a recombinant GST-fused C-terminal domain of H<sup>+</sup>-ATPase. In fact, it was found that GF14-6 was also able to associate with the isolated C terminus, but interestingly, in this case, the interaction was completely dependent on the presence of FC. Moreover, it was shown that only the complete C terminus was labeled by GF14-6, whereas the partially proteolyzed C-terminal fragments were unable to interact with the probe. Among

them, the highest molecular proteolyzed fragment is only slightly shorter than the full-length C terminus (amino acids 845–947 of the H<sup>+</sup>-ATPase sequence). In fact, it is recognized by the anti-C terminus antibodies raised against amino acids 927–938 of the H<sup>+</sup>-ATPase sequence (Fig. 7B, lane 2). These pieces of evidence suggest that the FC-induced interaction of 14-3-3 with H<sup>+</sup>-ATPase requires C-terminal integrity and may therefore underlie the involvement of tertiary structure constraints. The very last portion of the C terminus appears to be strictly involved in the FC-stimulated interaction with 14-3-3 proteins since the truncation of a few residues is sufficient to abolish it. This region might hence be crucial for the fulfillment of conformational requirements or, alternatively, directly involved in the binding of FC.

In contrast to binding to the whole H<sup>+</sup>-ATPase, the binding of GF14-6 to the GST-fused C terminus is totally dependent on FC. Since a large body of evidence indicates that 14-3-3 proteins interact physiologically with target proteins by binding to phosphorylated serines within consensus motifs (28), a rationale for this discrepancy could be that the recombinant and native C termini differ in their phosphorylation state, with the former being unphosphorylated and the latter being phosphorylated by regulatory endogenous protein kinases. This would also imply that physiological and FC-induced binding of 14-3-3 proteins to H<sup>+</sup>-ATPase proceeds through different mechanisms. To test this hypothesis, phosphorylation studies of the expressed C terminus with plant endogenous protein kinases are presently in progress.

In conclusion, the data reported here provide direct evidence of the capability of 14-3-3 proteins to interact with H<sup>+</sup>-ATPase and provide a better insight into the mechanism of H<sup>+</sup>-ATPase activation by FC. In fact, whereas contrasting models have been put forward (8, 13), our data appear to be fully compatible with that proposed by Oecking *et al.* (13), according to which FC strengthens the interaction between 14-3-3 and H<sup>+</sup>-ATPase, thus shifting cytosolic isoforms to the plasma membrane and locking H<sup>+</sup>-ATPase in its activated state. The identification of the C terminus as the 14-3-3-interacting region appears to be in accordance with the well documented regulatory function of this domain and with recent evidence indicating its direct interaction with 14-3-3 proteins (13, 29).

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**Fusicoccin Effect on the *in Vitro* Interaction between Plant 14-3-3 Proteins and Plasma Membrane H<sup>+</sup>-ATPase**

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