Activation of the plant plasma membrane H\textsuperscript{+}-ATPase by phosphorylation and binding of 14-3-3 proteins converts a dimer into a hexamer

Justyna Kanczewska*, Sergio Marco*, Caroline Vandermeeren*, Olivier Maudoux*, Jean-Louis Rigaud†, and Marc Boutry**

*Unité de Biochimie Physiologique, Institut des Sciences de la Vie, University of Louvain, Croix du Sud, 2-20, B-1348 Louvain-la-Neuve, Belgium; and †Institut Curie, Unité Mixte de Recherche, Centre National de la Recherche Scientifique 168, and Laboratoire de Recherche, Commissariat à l’Energie Atomique 34V, 11 Rue Pierre et Marie Curie, 75231 Paris, France

Edited by Randy Schekman, University of California, Berkeley, CA, and approved June 30, 2005 (received for review May 31, 2005)

Plant plasma membrane H\textsuperscript{+}-ATPases (PMAs) can be activated by phosphorylation of their penultimate residue (a Thr) and the subsequent binding of regulatory 14-3-3 proteins. Although 14-3-3 proteins usually exist as dimers and can bind two targets, the in vivo effects of their binding on the quaternary structure of H\textsuperscript{+}-ATPases have never been examined. To address this question, we used a Nicotiana tabacum cell line expressing the Nicotiana plumbaginifolia PMA2 isoform with a 6-His tag. The purified PMA2 was mainly nonphosphorylated and 14-3-3-free, and it was shown by blue native gel electrophoresis and chemical cross-linking to exist as a dimer. Fusicoccin treatment of the cells resulted in a dramatic increase in Thr phosphorylation, 14-3-3 binding, and ATPase activity, showing that oligomerization is not necessary for ATPase activity. The PMA2 isoform with a 6-His tag. The purified PMA2 was baginifolia, which is expressed in yeast, also has a 14-3-3 binding were observed also when cells in stationary medium. When expressed in yeast, PMA2 was also phosphorylated and formed a complex with 14-3-3 proteins without requiring fusicoccin; no complex was observed when phosphorylation was prevented by mutagenesis. Single-particle analysis by cryoelectron microscopy showed that the PMA2–14-3-3 complex is a wheel-like structure with a 6-fold symmetry, suggesting that the activated complex consists of six H\textsuperscript{+}-ATPase molecules and six 14-3-3 molecules.

The plant plasma membrane H\textsuperscript{+}-ATPase (PMA) creates a proton electrochemical gradient across the membrane, providing a driving force for ion and metabolite transport by a large range of secondary transporters (for reviews, see refs. 1 and 2).

Although H\textsuperscript{+}-ATPases consist of a single type of polypeptide, in some cases, they exist as homooligomers. Radiation inactivation of red beet showed that the H\textsuperscript{+}-ATPase exists as a dimer in native plasma membranes and after reconstitution in vesicles, whereas the solubilized enzyme is monomeric; all forms have ATPase activity, showing that oligomerization is not necessary for activity (3). A more recent cryoelectron microscopy study showed that an Arabidopsis H\textsuperscript{+}-ATPase expressed in Saccharomyces cerevisiae exists as a dimer in reconstituted 2D crystals (4). The putative H\textsuperscript{+}-ATPase of the thermophilic bacterium, Methanococcus jannaschii, which is expressed in yeast, also has a dimeric structure (5).

The Neurospora crassa H\textsuperscript{+}-ATPase is active as a monomer but can form stable hexamers in the plasma membrane under certain conditions (6). Cryoelectron microscopy studies of this H\textsuperscript{+}-ATPase reconstituted into 2D crystals (7) or in detergent (8) also showed a hexamer.

The quaternary structure of the H\textsuperscript{+}-ATPase might also depend on the activation status of the enzyme. For example, the C-terminal region of the plant H\textsuperscript{+}-ATPase is known to be an autoinhibitory regulator (9), which can be phosphorylated on the penultimate residue (a Thr) and then bind regulatory 14-3-3 proteins, resulting in an activated enzyme (10–13). Unfortunately, this complex is not readily observed in plants under normal conditions. It is seen, together with H\textsuperscript{+}-ATPase phosphorylation, in guard cells after blue-light activation (14), but the difficulty in isolating large amounts of this material precludes detailed structural characterization. The H\textsuperscript{+}-ATPase–14-3-3 complex can be seen in plants treated with fusicoccin (FC), which is a toxin that is produced by the fungus Fusicoccum amygdali. FC binds to, and stabilizes, the H\textsuperscript{+}-ATPase–14-3-3 complex (15). This complex is formed in the absence of FC when the Nicotiana plumbaginifolia PMA2 isoform is expressed in yeast (13).

The 14-3-3 proteins usually exist as dimers with two binding sites (15), and therefore, it is possible that a 14-3-3 dimer links two H\textsuperscript{+}-ATPase molecules together, resulting in oligomerization. However, whether this oligomerization occurs and, if so, what the composition of the complex is are not known. Here, we show that the N. plumbaginifolia PMA2 exists as a dimer and, upon activation, is converted into a larger oligomer by phosphorylation and the binding of 14-3-3 proteins.

Experimental Procedures

Plant and Yeast Material. Nicotiana tabacum BY2-PMA2 (16) and the yeast strains YAKPMA2-6-His, YAKPMA2-E14D-6-His, YAKPMA2-E14D-T955A, YAKPMA2-E14D-C6-His (13), YAKPMA4 (17), and YAKPMA4-A129P (18) expressing plant H\textsuperscript{+}-ATPases have been described.

Preparation of Subcellular Fractions and Purification of 6-His-Tagged PMA2. The microsomal fraction from N. tabacum was prepared as described in ref. 16, except that urea was not included in the buffers. Yeast plasma membranes were prepared as described in ref. 19.

Solubilization and purification were performed as described in ref. 13, except that 0.06% polyoxyethylene 8-mistryl ether (C\textsubscript{14}E\textsubscript{8}) was used for stripping and 1.4% β-dodecyl maltoside (DDM) was used for solubilization.

For electron microscopy, PMA2 was further purified by size-exclusion chromatography using a Sephacryl S-300 column (Amersham Biosciences) eluted with 150 mM KCl/1 mM MgCl\textsubscript{2}/5% glycerol/10 mM imidazole, pH 7.0 (HCl)/0.016% DDM. Fractions containing the PMA2-14-3-3 complex were...
pooled, concentrated by Ni chromatography, and rapidly examined by electron microscopy.

**ATPase Assay.** ATPase assays were performed as described in ref. 19.

**Immunodetection.** SDS/PAGE and Western blotting were performed by using standard methods. Bound antibodies against H+–ATPase (13), 14-3-3 [N. tabacum isofrom T14–3c expressed in Escherichia coli (20) was purified and injected into rabbits], or phospho-Thr (Zymed) were detected by using the appropriate alkaline phosphatase-conjugated anti-IgG antibodies (Boehringer Mannheim) and chemiluminescence. We performed the 14-3-3 overlay as described in ref. 13, except that bound 14-3-3 was immunodetected.

**Blue Native PAGE.** Blue native PAGE was performed as described (21) by using a 5–18% polyacrylamide gradient, allowing separation of proteins from 50 to 800 kDa. Samples were solubilized in 1% DDM/50 mM EDTA/750 mM aminocaproic acid/50 mM Bistris, pH 7.0.

**Sucrose Density-Gradient Centrifugation.** Solubilized membrane proteins (1.5 mg) were centrifuged for 16 h at 210,000 × g (TST60; Kontron, Zurich) at 2°C on a discontinuous (18 layers) gradient of 10–24% sucrose in solubilization buffer, and 200-μl fractions were collected.

**Cross-Linking.** Cross-linking was performed by incubating doubling dilutions of purified PMA2 or microsomal fraction for 1 h at 20°C with 10 mM dimethyl suberimidate (TST60; Kontron, Zurich) at 2°C on a discontinuous (18 layers) gradient of 10–24% sucrose in solubilization buffer, and 200-μl fractions were collected.

**Electron Microscopy and Image Analysis.** Aliquots (5 μl) of freshly purified PMA2–14-3-3 complex were deposited on glow-discharged carbon-coated 300-mesh grids, and excess sample was removed with filter paper. The grids were then frozen in liquid ethane at −178°C by using a CPC station (Leica, Deerfield, IL) and transferred to a CM120 cryoelectron microscope (Philips, Eindhoven, the Netherlands). Electron micrographs were recorded at 120 kV, with a defocus of −1.5 μm and a nominal magnification of ×45,000, by using a low-dose system and a 1,024 × 1,024-pixel slow-scan charge-coupled device (sCCD) (Gatan, Pleasanton, CA). The pixel size, which was calibrated by using bacteriorhodopsin crystals, was 3.9 ± 0.1 Å (P = 0.99).

A total of 1,035 single projections of PMA2–14-3-3 complex were windowed and centered before classification. Self-organizing mapping (22), segmented by multivariate statistical analysis (23), was used to identify homogeneous groups of projections. Images belonging to the major class were aligned by using a free-reference algorithm (24) before computing an average image and performing rotational analysis. The resolution of the average image was estimated by using the spectral signal-to-noise ratio (SSNR) method (25). X-MIPP software (26) was used for image processing.

**Results**

**PMA2 Exists as a Dimer.** PMA2 with a 6-His tag between residues 3 and 4 was purified from the N. tabacum BY2-PMA2 cell line by Ni chromatography, as described in ref. 16; the tag does not affect the enzyme activity when expressed in yeast (13). On SDS gels, a major band of the expected size (∼100 kDa) was seen, which was recognized by anti-PMA antibodies (16) or by anti-6 His antibodies (data not shown).

When purified PMA2 was analyzed by blue native PAGE, a nondenaturing electrophoretic system, its apparent size was higher than that of the 158-kDa marker (i.e., greater than the expected size of 105 kDa and closer to the size of a dimer; Fig. 1). The minor bands detected by Coomassie blue staining on either side of the 669-kDa marker did not correspond with H+–ATPase because they did not bind anti-PMA antibodies (Fig. 1) and were also present in material that was mock-purified from nontransformed cells (data not shown). We then examined whether PMA2 existed as a dimer by chemical cross-linking using the bifunctional agent DMS. When purified PMA2 was incubated with DMS and analyzed by blue native PAGE and Western blotting, a significant amount of the material appeared as a dimer (Fig. 24). The dimer/monomer ratio was not reduced when the sample was diluted, showing that cross-linking stabilized a preexisting dimer. To rule out the possibility that dimerization was an artifact of solubilization, DMS cross-linking

![Fig. 1. Blue native gel electrophoresis of PMA2 and the PMA2–14-3-3 complex. BY2-PMA2 cells (100-ml, 7-day-old cultures) were left untreated (–) or treated for 30 min with 10 μM FC (+), and the tagged PMA2 was then purified as described in Experimental Procedures and analyzed by blue native PAGE, followed by either Coomassie blue staining or Western blotting with antibodies against PMA, phospho-Thr (P-Thr), or 14-3-3. Aldolase (158 kDa), catalase (232 kDa), ferritin (440 kDa), and thyroglobulin (669 kDa) were used as size markers.](image-url)

![Fig. 2. Chemical cross-linking of PMA2. Doubling dilutions of purified 6-His-tagged PMA2 (A) or BY2-PMA2 microsomal proteins (B) were incubated with 10 mM DMS, as described in Experimental Procedures, and then analyzed by SDS/PAGE and Western blotting using anti-PMA antibodies. The size markers are shown on the left. The nondiluted sample (1) corresponds to 100 μl of the Ni chromatography eluate (A) or 64 μg of microsomal proteins (B).](image-url)
Fig. 1. Transfer of cells to fresh medium activates PMA2 phosphorylation and 14-3-3 binding. A 400-ml, 7-day BY2-PMA2 cell culture was divided into two equal parts, one of which was left as it was and the other of which was centrifuged with the cells resuspended in 400 ml of fresh culture medium. Both samples were incubated for 24 h and then used for microsome preparation and H⁺-ATPase purification, as described in Experimental Procedures. Half of each sample was supplemented with 10 μM FC at the homogenization step, whereas the other half was processed in the presence of 7 M urea from homogenization onwards. One-fifth of the various samples was analyzed by SDS/PAGE and Western blotting.

was performed on a microsomal fraction, and most of the enzyme was found as a dimer (Fig. 2B).

**Fig. 2.** (A) BY2-PMA2 cells were incubated for 30 min with the indicated concentration of FC, and 6-His-tagged PMA2 was then purified and analyzed by SDS/PAGE and Western blotting. (B) BY2-PMA2 cells were incubated with 10 μM FC for the indicated time, and the 6-His tagged PMA2 was then purified and analyzed by SDS/PAGE and Western blotting. For time 0, the cells were collected immediately after FC addition. Signals were quantified by image analysis; 100% corresponds to the signal observed at 40 min. (C) Three cell cultures were grown in medium alone (control) or medium containing 10 μM FC (+ FC) or 10 μM FC plus 30 μM erythrosin B (+ FC + EB). The pH (mean ± SD of four experiments) of 1-ml aliquots was then recorded at the indicated times.

**Fig. 3.** Effect of FC on PMA2 phosphorylation and 14-3-3 binding and on acidification of the cell medium. (A) BY2-PMA2 cells were incubated for 30 min with the indicated concentration of FC, and 6-His-tagged PMA2 was then purified and analyzed by SDS/PAGE and Western blotting. (B) BY2-PMA2 cells were incubated with 10 μM FC for the indicated time, and the 6-His tagged PMA2 was then purified and analyzed by SDS/PAGE and Western blotting. For time 0, the cells were collected immediately after FC addition. Signals were quantified by image analysis; 100% corresponds to the signal observed at 40 min. (C) Three cell cultures were grown in medium alone (control) or medium containing 10 μM FC (+ FC) or 10 μM FC plus 30 μM erythrosin B (+ FC + EB). The pH (mean ± SD of four experiments) of 1-ml aliquots was then recorded at the indicated times.

**FC Treatment Induces Thr Phosphorylation and 14-3-3 Protein Binding and Stimulates Acidification of the Culture Medium.** The H⁺-ATPase–14-3-3 complex is barely detectable in plant tissues under normal conditions. Therefore, we tried to increase the amount of complex by using FC, which stabilizes this complex (15). BY2-PMA2 cells were treated with 0–10 μM FC, and PMA2 was then purified and analyzed by SDS/PAGE and Western blotting. FC treatment had no effect on the amount of PMA2 but caused a concentration-dependent increase in phospho-Thr-PMA2 and copurified 14-3-3 proteins (Fig. 3A).

The effect of treatment with 10 μM FC was also followed over time (Fig. 3B). When FC was added immediately before harvesting (time 0; Fig. 3B) like in the untreated sample (data not shown), a low level of Thr phosphorylation and 14-3-3 proteins was observed, showing that only a small fraction of PMA2 was activated. However, a clear increase in phosphorylation and 14-3-3 binding was seen at 5 min, reaching a plateau at 20 min. The amount of PMA2 in the purified fraction did not change over this incubation period (data not shown). This observation indicates that in vivo FC treatment induced PMA2 phosphorylation and 14-3-3 binding, rather than stabilizing a preexisting complex.

To correlate H⁺-ATPase–14-3-3 complex formation with a physiological property, the effect of FC on proton pumping was followed by recording the pH of the incubation medium of BY2-PMA2 cells. FC addition resulted in rapid acidification of the medium, which was blocked by addition of erythromycin B, a cell-permeating H⁺-ATPase inhibitor (Fig. 3C). This in vivo activation was correlated with an increase in the vanadate-sensitive ATPase activity of the plasma membrane from 1.02 ± 0.029 to 2.65 ± 0.054 μmol·min⁻¹·mg⁻¹ protein at 30 min after addition of FC.

These in vivo and in vitro data demonstrate that the FC-induced formation of the H⁺-ATPase–14-3-3 complex stimulates H⁺-ATPase activity in BY2-PMA2 cells.

**H⁺-ATPase Is Activated upon Cell Transfer to Fresh Culture Medium.** Although FC is an efficient tool for promoting H⁺-ATPase–14-3-3 complex formation, we wanted to know whether complex formation could occur in its absence. We noted that the PMA2 phosphorylation level was higher in early exponential growth phase than in stationary phase (data not shown), suggesting that transferring cells to fresh growth medium activates H⁺-ATPase. This hypothesis was tested by dividing a 7-day culture into two halves, one of which was left as it was and the other of which was transferred to fresh growth medium. Both halves were then collected 24 h later, when the ATPase activity of the microsomal fraction was found to be higher in the refreshed cells than in the starved cells (0.745 ± 0.064 and 0.531 ± 0.040 μmol·min⁻¹·mg⁻¹ protein, respectively). In one approach, because we found the H⁺-ATPase–14-3-3 complex to be labile during purification, FC was added at the homogenization step to stabilize the preexisting complex; this addition had no effect on the phosphorylation
level, nor did it increase the amount of bound 14-3-3 proteins (Fig. 3B). Transferring the cells to fresh medium markedly increased PMA2 phosphorylation and 14-3-3 binding (Fig. 4). By using another approach, we omitted FC but performed homogenization and all subsequent steps in the presence of urea to avoid any in vitro dephosphorylation of unprotected PMA2. We confirmed the increase in PMA2 phosphorylation in cells that were transferred to fresh medium (Fig. 4); 14-3-3 proteins could not be identified in this case because urea denatured the complex. Increased PMA2 phosphorylation was already observed after shorter periods of time (e.g., 2 h) but not consistently to the level at which it was observed after 24 h (data not shown).

Thr Phosphorylation and 14-3-3 Binding Modify the Oligomerization Status. Blue native PAGE analysis of PMA2 purified from untreated and in vivo FC-treated cells showed that the dimer band was less abundant after FC treatment and that a higher-molecular-mass band appeared (Fig. 1). Western blotting confirmed that both bands were H^+-ATPase and showed that only the higher-molecular-mass band reacted with anti-phospho-Thr and anti-14-3-3 antibodies (Fig. 1), indicating that it consisted of phosphorylated PMA2 complexed with 14-3-3 proteins. The apparent size of this complex was three to three and a half times that of the H^+-ATPase dimer, suggesting that, on interacting with 14-3-3 proteins, the dimer was converted into a hexamer.

To confirm the formation of this larger complex, the microsomal fraction from untransformed N. tabacum BY2 cells was solubilized with DDM and directly analyzed by sucrose density-gradient centrifugation, followed by Western blotting (Fig. 5A). This approach allowed us to study all H^+-ATPases, rather than a single isoform. For FC-untreated material, the H^+-ATPase peak was in fraction 8, no phospho-Thr was detected, and 14-3-3 proteins were found in the upper part of the gradient (fractions 9–11); because 14-3-3 proteins are soluble, these proteins must be associated with other microsomal proteins. For FC-treated cells, the H^+-ATPase peak was in fractions 5 and 6, in which 14-3-3 proteins and phospho-Thr were also detected, confirming that the complex has a larger size than the free H^+-ATPase. Addition of 14-3-3 proteins to the membranes from FC-untreated cells did not modify H^+-ATPase sedimentation, indicating that phosphorylation is required for complex formation.

Analysis of PMA2 and PMA4 Expressed in Yeast. We previously expressed PMA2 in the yeast S. cerevisiae and showed that phosphorylation of its own H^+-ATPase genes and that a small fraction of the PMA2 is phosphorylated on the penultimate Thr and binds regulatory 14-3-3 proteins. More complex is formed by using the activated PMA2 mutant E14D (with a single Glu-14 → Asp point mutation) than with wild-type PMA2 (13). To determine the size of the complex formed in yeast, we analyzed the mutant enzyme by sucrose gradient centrifugation of proteins solubilized from yeast plasma membranes; because the PMA2–14-3-3 complex forms spontaneously in yeast, FC was not used in these experiments (Fig. 5B). The PMA2–E14D peak was broad, the lighter fractions containing a nonphosphorylated and 14-3-3-free form and the heavier fractions containing phospho-Thr and 14-3-3 proteins. This distribution agrees with the previous separation by gel filtration of free H^+-ATPase and the larger H^+-ATPase–

![Fig. 5](https://www.pnas.org/cgi/doi/10.1073/pnas.0504498102)

**Fig. 5.** Sucrose density centrifugation and overlay analysis. *N. tabacum* BY2 microsomal fractions (A) or plasma membranes from the indicated S. cerevisiae PMA2 or PMA4 transformants (B) were solubilized and centrifuged on a 10–24% sucrose gradient, as described in Experimental Procedures, and fractions were then collected and analyzed by SDS/PAGE and Western blotting with the indicated antibodies. BY2: *N. tabacum* BY2 cells; BY2 + FC: 10 μM FC was added to *N. tabacum* BY2 cells 20 min before harvesting and homogenization; BY2 + 14-3-3: 0.225 mg of purified 14-3-3 proteins was added to 1.5 mg of microsomal fraction, and the mixture was incubated for 15 min at 21°C before solubilization. (C) Plasma membranes (2 μg of protein) from yeast cells expressing the indicated PMA2 isoforms were analyzed by SDS/PAGE and Western blotting for PMA and phospho-Thr and by overlay with 14-3-3 in the absence or presence of 10 μM FC.
14-3-3 complex (13). Upon mutation of the phosphorylatable Thr of PMA2-E14D into Ala (PMA2-E14D-T955A), H⁺-ATPase was found only in the lighter fraction (Fig. 5B). Similar results were obtained by using PMA2 E14D tagged with 6-His at the C terminus (PMA2-E14D-C), two positions downstream of the phosphorylatable Thr. Thus, preventing Thr phosphorylation also prevented 14-3-3 binding.

PMA4, another widely expressed N. plumbaginifolia H⁺-ATPase belonging to subfamily II, also permits transformed yeast to grow in the absence of yeast H⁺-ATPase (17). On sucrose gradient centrifugation, most of PMA4 sedimented in the light fractions (8–10), whereas a small part was found to be phosphorylated and complexed with 14-3-3 proteins in heavier fractions (PMA4, Fig. 5B). We then analyzed an activated PMA4 mutant, A129P, in which Asp-129 is mutated into Pro, that allows better yeast growth. This mutant is more phosphorylated and binds more 14-3-3 proteins than wild-type PMA4 (18). On sucrose gradient centrifugation, a larger fraction of PMA4-A129P sedimented in the heavier fractions; this enzyme was phosphorylated and associated with 14-3-3 proteins (Fig. 5B).

Therefore, we can conclude that H⁺-ATPase phosphorylation and 14-3-3 binding result in the formation of a larger complex. To confirm that 14-3-3 binding depends on H⁺-ATPase phosphorylation, we performed an overlay assay with the two PMA2 mutants described above. After SDS-PAGE, H⁺-ATPases were transferred to nitrocellulose membranes and allowed to react with 14-3-3 proteins, the binding of which was detected with antibodies (Fig. 5C). As described above, Thr mutation (PMA2-E14D-T955A) or 6-His C-terminal tagging (PMA2-E14D-C) prevented phosphorylation, and no 14-3-3 binding was found. Even in the presence of FC during the overlay assay, no 14-3-3 binding was observed for PMA2-E14D-T955A, and only a small 14-3-3 binding was observed for PMA2-E14D-C.

Electron Microscopy and Image Analysis. The exact degree of oligomerization of H⁺-ATPase–14-3-3 complexes cannot be determined accurately from the mobility on blue native PAGE or sucrose gradient centrifugation, especially in the sucrose gradient centrifugation, because detergents bound to the complex artificially lower its density. Therefore, we used cryo-electron microscopy and single-particle analysis to determine the oligomerization status of the PMA2–14-3-3 complex. The complexes formed with the activated PMA2-E14D mutant expressed in yeast in the absence of FC or in BY2-PMA2 after in vivo FC treatment both eluted in the same fraction on size-exclusion chromatography (data not shown), indicating that they were similar in size. On electron microscopic analysis, both complexes appeared to be monodisperse and of a similar size. We then analyzed the complex produced in yeast in more detail. A representative gallery of cryo-electron microscopy projections is shown in Fig. 6A. The averaging of the 685 projections belonging to the major class of self-organizing mapping segmentation (Fig. 6B) gave a wheel-like structure (diameter, 14.7 nm) with a “rim” (thickness, ~2.0 nm) containing six prominent “spokes” (~3.2 × 1.9 nm) meeting at a central lower density “hub” (radius, ~2.15 nm). Rotational power spectra analysis of the average image, after filtration at the estimated resolution of 26 Å, demonstrated a clear 6-fold symmetry (82% of the symmetry was found in harmonic 6).

Discussion

The plant PMA2 H⁺-ATPase exists in two forms, a basic low activity form and an activated form that is phosphorylated on the penultimate Thr and binds regulatory 14-3-3 proteins. In this study, we showed by blue native PAGE and chemical cross-linking that the basic form (both membrane-bound and solubilized) exists as a dimer. A dimeric structure has been reported for AHA2, an Arabidopsis H⁺-ATPase, when expressed in yeast and crystallized in 2D (4). These data contrast with the Neurospora crassa PMA, which is reported to form stable hexamers (7, 8).

By using three different experimental approaches (blue native PAGE, density-gradient centrifugation, and electron microscopy), we demonstrated that the size of the activated phosphorylated PMA2–14-3-3 complex is much greater than that expected for a PMA2 dimer plus a 14-3-3 protein dimer. Electron microscopy analysis showed the complex to be a hexamer.

What triggers the formation of the complex? Our data clearly show that phosphorylation is required. Indeed, we have never observed a nonphosphorylated H⁺-ATPase–14-3-3 complex. Moreover, preventing Thr phosphorylation of PMA2 by mutagenesis (Thr955Ala or a C-terminal His-tag) resulted in no complex being formed. Mutations (PMA2-E14D or PMA4-E14D-A129P) or culture conditions (new growth medium or FC treatment) that resulted in increased complex formation always showed increased Thr phosphorylation. This observation is in agreement with (i) the observation that, when BY2 cells were treated in vivo with FC, the increase in 14-3-3 binding paralleled that in Thr phosphorylation, suggesting that a stable complex does not form without phosphorylation and that FC, by stabilizing the complex, displaces the equilibrium toward complex formation; and (ii) previous overlay data showing that 14-3-3

Fig. 6. Electron microscopy analysis of the purified PMA2–14-3-3 complex. (A) Representative gallery of cryo-electron microscopy projections of PMA2–14-3-3 purified by Ni chromatography and gel filtration. (B) Average image of 685 particles, revealing a 6-fold symmetry wheel-like structure consisting of three density regions, a circular “rim” (thickness, ~2.0 nm), six prominent “spokes” (~3.2 × 1.9 nm), and a central “hub” of lower density (radius, ~2.15 nm). (Scale bar, 5 nm.)
proteins do not bind to H\textsuperscript{+}-ATPase that has been dephosphorylated (11–13, 20). The next question is whether phosphorylation is sufficient for the formation of the large complex or whether 14-3-3 proteins are required. Attempts to displace 14-3-3 proteins by using synthetic peptides mimicking the phosphorylated PMA2 C terminus were unsuccessful, thus precluding the biochemical characterization of the free phosphorylated H\textsuperscript{+}-ATPase. Also, binding (11) and structural (27) data have shown that the phosphorylated Thr and the surrounding residues have a major role in 14-3-3 binding, and therefore, it is difficult to prevent 14-3-3 binding (e.g., by mutagenesis) without altering Thr phosphorylation. However, a large complex without 14-3-3 proteins was never observed. We cannot exclude the possibility that such a complex exists in vivo, but in this case, it would have to be very labile. Also, we have never found any conditions that stimulate phosphorylation but not 14-3-3 binding. This failure is not due to artificial dephosphorylation during sample preparation, because no increase in phosphorylation was seen when the homogenization and subcellular fractionation were performed in the presence of urea. Therefore, it seems that phosphorylation results in the rapid formation of a complex between H\textsuperscript{+}-ATPase and 14-3-3 proteins.

The recent 3D structure obtained for a complex consisting of a 14-3-3 dimer and a synthetic phosphopeptide corresponding to the five C-terminal residues of PMA2 (27) sheds some light on this. However, a major difference between these preparations is that such a complex exists in yeast and in plant cells treated with FC. The solubilized hexameric PMA2–14-3-3 complex seen by cryoelectron microscopy has a wheel-like structure reminiscent of that seen for N. crassa H\textsuperscript{+}-ATPase in detergent (7, 8). However, a major difference between these preparations is that 14-3-3 proteins do not interact with the N. crassa H\textsuperscript{+}-ATPase. In the latter, the transmembrane domains appear as six separated prominent densities, which stand out from an undulating hollow dome made by the loosely packed cytoplasmic head regions (8). Assuming an analogous organization in the PMA2–14-3-3 complex, the PMA2 transmembrane domains should be located at the peripheral high-density “rim” (thickness, \(\approx 2.0 \text{ nm}\)) and the cytoplasmic domains should correspond to the “spokes” (\(\approx 3.2 \times 1.9 \text{ nm}\)). In this case, the central low-density “hub” (radius, \(\approx 2.15 \text{ nm}\)) should be built up from the 14-3-3 proteins. According to this model, the cytoplasmic domains and/or the 14-3-3 polypeptides are implicated in hexamer stabilization.

The 6-fold symmetry of the PMA2–14-3-3 complex observed by electron microscopy, its apparent size on blue native PAGE, and the 1:1 (PMA2/14-3-3) stoichiometry deduced from SDS/PAGE analysis of the PMA2–14-3-3 complex isolated from yeast (13) support the conclusion that the PMA2–14-3-3 complex consists of six ATPase molecules and six 14-3-3 molecules. Although PMA2 and 14-3-3 form dimers when analyzed separately, the resolution of the electron microscopy analysis was not high enough to identify such dimers. However, we cannot exclude the possibility that the dimeric organization of both partners is modified when they interact within the hexameric structure.

Apart from in cells treated with FC, H\textsuperscript{+}-ATPase activation by 14-3-3 proteins has been found only in guard cells upon blue-light induction (14). In this study, we showed that transferring stationary growth phase BY2-PMA2 cells to fresh culture medium induced H\textsuperscript{+}-ATPase phosphorylation and 14-3-3 binding, resulting in higher ATPase activity. This activation makes sense, because cell metabolism in the early culture stage is expected to rely on active transport. Also, this system of a single cell type available in large amounts will allow us to use material-intensive biochemical approaches that could lead, for example, to the identification of upstream factors, such as kinases, that are involved in H\textsuperscript{+}-ATPase activation.

We thank Pierre Gosselin for excellent technical help. This work was supported by grants from the Interuniversity Attraction Poles Program—Belgian Science Policy, the European Community (IHP-RTN), the Belgian Fund for Scientific Research, the Human Frontier Science Program, and the Commissariat Français à l’Énergie Atomique (Nuclear Toxicology Program).