Biochemical Evidence for the Requirement of 14-3-3 Protein Binding in Activation of the Guard-cell Plasma Membrane H⁺-ATPase by Blue Light

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Blue light (BL) activates the plasma membrane H⁺-ATPase via phosphorylation of the C-terminus with concomitant binding of 14-3-3 protein to the terminus in stomatal guard cells. However, the binding site and role of 14-3-3 protein in this physiological response have not been elucidated. We investigated the above using synthetic phosphopeptides designed from the C-terminus of Vicia H⁺-ATPase (isoform 1; VHA1). The presence of KGLIDTIQQHYphospho-Thr⁹⁵⁰ and other phosphopeptides, including typical phosphorylation sites in the C-terminus, had no effect on the binding. Incubation of BL-activated plasma membrane H⁺-ATPase with P-950 dissociated the 14-3-3 protein from the H⁺-ATPase without affecting phosphorylation levels and decreased the H⁺-ATPase activity. By contrast, incubation of P-950 with the activated H⁺-ATPase from fusco-cin-treated guard-cell protoplasts neither dissociated the 14-3-3 protein nor decreased the H⁺-ATPase activity. These results indicate that BL induces phosphorylation on threonine residue (Thr⁹⁵⁰) in the C-terminus of H⁺-ATPase, and that the binding of 14-3-3 to this site is required for the activation of H⁺-ATPase in stomatal guard cells.

Keywords: Blue light — 14-3-3 protein — Fusicoccin — Guard cells — H⁺-ATPase (EC 3.6.1.35) — Phosphothreonine residue — Stomata.

Abbreviations: BL, blue light; CNBr, cyanogen bromide; FC, fusicoccin; GCPs, guard-cell protoplasts; GST, glutathione-S-transferase; RL, red light, VHA; Vicia plasma membrane H⁺-ATPase.

Introduction

Blue light (BL) induces stomatal opening in higher plants, and the opening is initiated by the perception of BL through phot1 and phot2 (Briggs et al. 2001), which were recently identified as BL receptors in stomatal guard cells (Kinoshita et al. 2001). The opening is mediated by an accumulation of K⁺-salt in guard cells, and K⁺ uptake is driven by an inside-negative electrical potential across the plasma membrane (Assmann and Shimazaki 1999, Schroeder et al. 2001, Zeiger 1983). This electrical potential is created by a BL-activated H⁺ pump in the plasma membrane (Assmann et al. 1985, Shimazaki et al. 1986). Recent investigation has demonstrated that the H⁺ pump is the plasma membrane H⁺-ATPase, and that BL activates the H⁺-ATPase through phosphorylation of serine and threonine residues in the C-terminus (Kinoshita and Shimazaki 1999).

A 14-3-3 protein was co-precipitated with the H⁺-ATPase when guard-cell protoplasts (GCPs) were illuminated with BL, and a recombinant 14-3-3 protein was bound to the phosphorylated C-terminus of H⁺-ATPase in vitro. The amount of the 14-3-3 protein bound to the H⁺-ATPase was proportional to both the activity and phosphorylation levels of H⁺-ATPase, suggesting that the 14-3-3 protein plays a key role in the activation. An isoform vf14-3-3a specifically bound to the H⁺-ATPase in Vicia guard cells in vivo and had a higher affinity than that of vf14-3-3-b, although guard cells expressed at least four isoforms of 14-3-3-a, b, c, and d (Emi et al. 2001). However, the site of binding in the plasma membrane H⁺-ATPase and the role of the 14-3-3 protein in the BL response of stomata remains unknown.


The 14-3-3 proteins have been shown to bind to their target proteins in sequence-specific and phosphorylation-dependent manners, with consensus motifs containing phosphoserine residues of RSXPXSP and RXYPatXSP (Muslin et al. 1996, Yaffe et al. 1997). In plants, nitrate reductase and sucrose-phosphate synthase have similar motifs, and the binding of the 14-3-3 protein to these enzymes results in an inhibition of activity.

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**Results**

Amino acid sequence of C-terminus of the H'-ATPase

Fig. 1 shows the deduced amino acid sequences of the C-terminus of plasma membrane H'-ATPase, VHA1, and VHA2, which are major isoforms in *Vicia* guard cells (Nakajima et al. 1995, Hentzen et al. 1996). The 14-3-3a protein in guard cells was found to bind to the phosphorylated C-termini in response to BL, which could be obtained by cleavage of the H'-ATPases with cyanogen bromide (CNBr) (Kinoshita and Shimazaki 1999, Emi et al. 2001). Thus, the binding site of the 14-3-3 protein must be within CNBr-fragments of the C-terminus. To determine the site, we designed four synthetic phosphopeptides of 14–17 amino acids within the C-terminus of VHA1 (P-849, KFATRYFLphospho-S$_{849}$GKAWSNL; P-906, EKN-SYRELphospho-S$_{906}$EIAEQAK; P-933, HTLKGHVEphospho-S$_{933}$VVKLKGLD; P-950, KGLDIDTIQQHYphospho-T$_{950}$V). Protein motif analysis of the C-terminus of VHA1 revealed that

Bachmann et al. 1996, Toroster et al. 1998). The plasma membrane H'-ATPase, however, lacks such motifs. Recently, Ols-son et al. (1998) found that in vivo treatment of FC induces phosphorylation of C-terminal Thr$_{948}$ (numbering according to the Arabidopsis H'-ATPase isoform 1 [AHA1]) of H'-ATPase in spinach leaves. More recently, this phospho-Thr$_{948}$ residue and neighboring amino acids have been demonstrated to create a novel binding motif of YpT$_{948}$V for the 14-3-3 protein, and to regulate the physiological binding of 14-3-3 protein to the H'-ATPase (Fuglsang et al. 1999, Svennelid et al. 1999, Camoni et al. 2000, Maudoux et al. 2000).

In the present study, we determined the binding site of 14-3-3 protein in the C-terminus when the plasma membrane H'-ATPase is activated by BL, a physiological stimulus. Furthermore, we found that the phosphorylation is not sufficient and that the binding of 14-3-3 protein to the phosphorylation site is indispensable to the activation of plasma membrane H'-ATPase in stomatal guard cells.

![Fig. 1 Amino acid sequences of the C-terminus of H'-ATPase in *Vicia* guard cells. VHA1 [S79323] and VHA2 [AB022442] are isoforms of plasma membrane H'-ATPase, which is primarily expressed in *Vicia* guard cells. Boxes show regions of the synthetic phosphopeptides. Solid arrowheads indicate phosphorylated residues in each phosphopeptide. Asterisks show identical amino acids. Underlines indicate the methionine residues split by CNBr. Dotted-underlines indicate the proposed autoinhibitory domains (Axelsen et al. 1999, Palmgren 2001).](image)

![Fig. 2 Competition between the peptides and phosphorylated H'-ATPase for binding of the 14-3-3 protein. (A) Autoradiogram of immunoprecipitated H'-ATPase. The H'-ATPase was immunoprecipitated from $^{32}$P-labeled GCPs using the H'-ATPase antibodies under red background light (RL) and 2.5 min after the start of the BL pulse (BL). In each lane, the immunoprecipitated H'-ATPase obtained from 100 µg of guard cell proteins was subjected to SDS-PAGE. (B) Far Western blotting using recombinant GST–14-3-3 protein as a probe. The GST fusion protein was detected by anti-GST antibodies. GCPs were incubated under red background light for 40 min, then illuminated by a BL pulse for 30 s. GCPs were solubilized before (RL) and 2.5 min after the start of the BL pulse (BL). Guard-cell proteins (12.5 µg) were separated by SDS-PAGE. (C) Effect of peptides on the binding of 14-3-3 protein to phosphorylated H'-ATPase. First, 0.1 µg GST–14-3-3 protein was incubated with or without 10 µg peptides for 30 min at 24°C, after which the mixture was used as a probe for Far Western blotting. GCPs were solubilized for 2.5 min after the start of the BL pulse. Other conditions were the same as in (B). (D) Western blotting of H'-ATPase by the antibodies. Other conditions were the same as in (C). (E) Concentration-dependency of inhibition of 14-3-3 protein binding by P-950. GCPs were incubated under red background light for 40 min and then illuminated by a BL pulse for 30 s. GCPs were solubilized 2.5 min after the start of the BL pulse. Guard-cell proteins (12.5 µg) were separated by SDS-PAGE. GST–14-3-3 protein at 0.1 µM was incubated with P-950 at the indicated concentrations for 30 min at 24°C, after which the mixture was used as a probe for Far Western blotting. Solid arrowheads indicate the position of the H'-ATPase.](image)
Ser_949 and Ser_966 are amino acids that are putatively phosphorylatable by protein kinase C and cyclic nucleotide-dependent protein kinase, respectively. Ser_933 is a conserved serine residue in plant H^+–ATPases. Thr_950 is involved in the amino acid sequence of Yphospho-T_950V, which has been demonstrated to be a novel binding motif of H^+–ATPase (Fig. 2C). Thus, it may be possible that P-950 dissociates the 14-3-3 protein from the H^+–ATPase for the 14-3-3 protein in response to FC, a fungal toxin (Olsson et al. 1998, Fuglsang et al. 1999, Svennelid et al. 1999).

**P-950 inhibits the binding of 14-3-3 protein to phosphorylated H^+–ATPase**

Illumination of GCPs with BL was found to induce phosphorylation of the H^+–ATPase (Fig. 2A). Far Western blot analysis using the recombinant glutathione-S-transferase (GST)–14-3-3 protein as a probe confirmed the binding of 14-3-3 protein to phosphorylated H^+–ATPase (Fig. 2B). Fig. 2C shows the binding of 14-3-3 protein to the H^+–ATPase in the presence of synthetic phosphopeptides. The 14-3-3 protein did not bind to the phosphorylated H^+–ATPase when P-950 was present in the reaction mixture. Other phosphopeptides, including P-849, P-906, P-933, and dephosphorylated P-950 (DeP-950), had no effect on the binding. Prevention of the binding was not due to the degradation of H^+–ATPase, as the same amounts of H^+–ATPase proteins were present (Fig. 2D). P-950 inhibited the binding of 14-3-3 protein to the C-termini of both VHA1 and VHA2 isoforms (data not shown). These results suggest that P-950 suppresses the binding of 14-3-3 protein to the H^+–ATPase by competition, and that the phosphopeptide, P-950, includes the binding site for the 14-3-3 protein. The results also indicate that the phosphorylation occurs on the Thr_950 in the H^+–ATPase in response to BL. The prevention of this binding of the 14-3-3 protein to the H^+–ATPase was found to depend on the P-950 concentration (Fig. 2E). The half-inhibitory concentration of P-950 was 0.5 μM when the 14-3-3 protein was added at 0.1 μM.

**Inhibition of BL-dependent activation of the H^+–ATPase by P-950**

A phosphopeptide P-950 inhibited the binding of 14-3-3 protein to the phosphorylated plasma membrane H^+–ATPase (Fig. 2C). Thus, it may be possible that P-950 dissociates the 14-3-3 protein that was found to bind to the H^+–ATPase in vivo. We first investigated the effects of P-950 on the H^+–ATPase activity (Fig. 3A). ATP hydrolytic activity by the H^+–ATPase in guard-cell extract increased twofold after a pulse of BL (see None in Fig. 3). When P-950 was added to the guard-cell extract, the increase was completely eliminated. We immediately immunoprecipitated the H^+–ATPase from the guard-cell extract after the determination of ATP hydrolysis, and estimated the 14-3-3 protein by Western blotting after separation by SDS-PAGE. The 14-3-3 protein was co-precipitated with H^+–ATPase (in lane None), but disappeared completely when the guard-cell extract had been incubated with P-950 (in lane P-950) (Fig. 3B), suggesting that P-950 dissociated the 14-3-3 protein from the H^+–ATPase. In the same samples, the phosphorylation levels of H^+–ATPase did not change, as indirectly determined by Far Western blotting (Fig. 3C). These results indicate that incubation of the complex of H^+–ATPase and 14-3-3 protein with P-950 dissociates the 14-3-3 protein, and that the dissociation decreases the H^+–ATPase activity, although the phosphorylation levels remain unchanged. This result further suggests that activation of the H^+–ATPase by BL requires the binding of 14-3-3 protein to the phosphorylated C-terminus.

An addition of P-906 to the guard-cell extract had no effect on H^+–ATPase activity, while that of P-849 showed a slight inhibitory effect. Interestingly, P-933 suppressed an increase in H^+–ATPase activity without affecting the binding of
**Fig. 4** Effect of P-950 on the FC-induced activation of H\(^{-}\)-ATPase.
(A) ATP hydrolytic activity. GCPs were pre-incubated under red background light for 40 min at 24°C, after which FC at 10 µM was added to the GCPs. GCPs were disrupted before (-FC) and 5 min after the addition of FC (+FC). P-950 was added to guard-cell extract at 10 µM and kept for 5 min at 24°C. Other conditions were the same as in Fig. 3A. (B) Binding amount of 14-3-3 protein to the H\(^{-}\)-ATPase. The H\(^{-}\)-ATPase was immunoprecipitated from guard-cell extract after the end of ATP hydrolysis using the H\(^{-}\)-ATPase antibodies. Other conditions were the same as in Fig. 3B. (C) Phosphorylation levels of the H\(^{-}\)-ATPase. The guard-cell extract after the end of ATP hydrolysis was subjected to SDS-PAGE in 12.5% gels. Other conditions were the same as in Fig. 3C. 

14-3-3 protein or the phosphorylation levels of the H\(^{-}\)-ATPase (Fig. 3).

**Effect of P-950 on the FC-induced activation of the H\(^{-}\)-ATPase**

Fungal toxin FC activates H\(^{-}\)-ATPase through the accumulation of a complex of the phosphorylated H\(^{-}\)-ATPase and 14-3-3 protein in stomatal guard cells (Kinoshita and Shimazaki 2001) as well as in other plant tissues (Palmgren 2001). The binding of the 14-3-3 protein in the presence of FC seems to be irreversible (Olsson et al. 1998, Fuglsang et al. 1999). We examined the effects of P-950 on the H\(^{-}\)-ATPase in this system. The 14-3-3 protein bound to the H\(^{-}\)-ATPase was not released when guard-cell extracts from FC-treated GCPs were added by P-950 (Fig. 4B). Neither the activity nor the phosphorylation levels of the H\(^{-}\)-ATPase decreased in response to treatment with P-950 (Fig. 4A, C). Activity of the H\(^{-}\)-ATPase did not decrease by P-950 (Fig. 4A). The phosphorylation levels of the H\(^{-}\)-ATPase did not change by the treatment (Fig. 4C). These results indicate that FC irreversibly activates the H\(^{-}\)-ATPase by producing tight binding of 14-3-3 protein to the C-terminus of H\(^{-}\)-ATPase, which confirms the previous results (Olsson et al. 1998, Fuglsang et al. 1999, Svennelid et al. 1999, Kinoshita and Shimazaki 2001).

**Discussion**

In this study, we determined the binding site and role of 14-3-3 protein in BL-dependent activation of the H\(^{-}\)-ATPase in stomatal guard cells. Recent studies using FC-treated spinach leaves (Svennelid et al. 1999) and yeast-expressing recombinant plant H\(^{-}\)-ATPase (Fuglsang et al. 1999) have revealed that the sequence of Yphospho-TV in the C-terminal end of H\(^{-}\)-ATPase is a novel binding motif for the 14-3-3 protein. However, there is no biochemical evidence regarding the binding site under physiological conditions. To determine the binding site of 14-3-3 protein in the plasma membrane H\(^{-}\)-ATPase (VHA1) of guard cells, we designed the synthetic phosphopeptide (P-950) containing the sequence of Yphospho-T\(_{950}\)V of VHA1 (Fig. 1). The presence of P-950 prevented the binding of 14-3-3 protein to the phosphorylated H\(^{-}\)-ATPase (Fig. 2C), while dephosphorylated P-950 did not, indicating that the binding depends on phosphorylation of Thr\(_{950}\) in the H\(^{-}\)-ATPase. Other phosphopeptides including typical phosphorylated amino acid residues of well-conserved sites in the C-terminus of VHA1 (phospho-S\(_{849}\), phospho-S\(_{906}\), and phospho-S\(_{913}\)) neither prevented the binding of 14-3-3 protein (Fig. 2C) nor interacted with 14-3-3 protein, as analyzed by surface plasmon resonance (data not shown). These results indicate that Thr\(_{950}\) is a site of phosphorylation in the plasma membrane H\(^{-}\)-ATPase in response to BL, and that Yphospho-T\(_{950}\)V creates a binding site for 14-3-3 protein under physiological stimuli in stomatal guard cells.

The role of 14-3-3 protein binding in the activation process of the plasma membrane H\(^{-}\)-ATPase is not clear. One possibility is that the 14-3-3 protein maintains the phosphorylation state of the H\(^{-}\)-ATPase to prevent access of protein phosphatase by binding, while another is that the binding of 14-3-3 protein itself is absolutely required for the activation. To clarify this point, we determined both the activity and phosphorylation level of the H\(^{-}\)-ATPase in the same preparation. The phosphopeptide P-950 was found to dissociate 14-3-3 protein bound to the BL-activated, phosphorylated H\(^{-}\)-ATPase (Fig. 3). The H\(^{-}\)-ATPase activity decreased drastically, although the phosphorylation level remained unaltered. The biochemical evidence clearly indicates that phosphorylation of Thr\(_{950}\) is not sufficient for activation of the H\(^{-}\)-ATPase, and that activation requires binding of the 14-3-3 protein to the phosphorylated site under physiological conditions. Binding of the 14-3-3 protein to the phosphorylated C-terminus may play a key role in the displacement of the C-terminal inhibitory domain (Sze et al. 1999, Palmgren 2001), inducing the activation of the H\(^{-}\)-ATPase. Interestingly, P-933 was found to suppress an activated H\(^{-}\)-ATPase but had no effect on binding of the 14-3-3 protein to the H\(^{-}\)-ATPase (Fig. 3). Because displacement of the inhibitory domain might occur through 14-3-3 binding, this result suggests that P-933 itself may act as an autoinhibitor. Regenberg et al. (1995) have demonstrated that the removal of 38 C-terminal residues that include the P-933 region of plant H\(^{-}\)-ATPase produces a high-affinity state with a low K\(_{m}\) value for ATP of the H\(^{-}\)-ATPase expressed in yeast. It is possible that P-933 decreases the H\(^{-}\)-ATPase activity through a decrease in its affinity for ATP. In accord with this speculation, removal of 38 C-terminal residues had no effect on V\(_{m}\) further suggesting that there are other autoinhibitory regions upstream from the C-terminus.
Recent studies have demonstrated that FC activates the H\(^+-\)ATPase much more than a physiological stimulus of BL by decreasing \(K_m\) for ATP and increasing \(V_{\text{max}}\) in stomatal guard cells (Kinoshita and Shimazaki 1999, Kinoshita and Shimazaki 2001). The present study also revealed that the activity of FC-activated H\(^+-\)ATPase is seven-times higher than that of BL-activated H\(^+-\)ATPase in GCPs (Fig. 3, 4). By contrast, the phosphorylation levels of H\(^+-\)ATPase and the amount of binding 14-3-3 protein in response to FC and BL were almost the same. Such a difference in the activity is probably due to the complex formation of phosphorylated C-terminus and 14-3-3 protein in the presence of FC, which results in a non-physiological, extremely high-activity state.

Fuglsang et al. (1999) have demonstrated that FC enhances the affinity of 14-3-3 protein to the C-terminal phospho-Thr\(_{556}\) peptide, thus, decreasing the \(K_D\) constant from 88 nM to 7 nM. Consistent with these data, P-950 could dissociate 14-3-3 protein from the BL-dependent phosphorylated H\(^+-\)ATPase, but not from FC-dependent phosphorylated H\(^+-\)ATPase in GCPs (Fig. 3B, 4B). We must note, however, that several lines of evidence have indicated that binding of 14-3-3 protein to H\(^+-\)ATPase in the presence of FC does not require the phosphorylation of H\(^+-\)ATPase but, rather, involves the C-terminal motif YTV (Fuglsang et al. 1999, Fullone et al. 1998, Svennelid et al. 1999).

In conclusion, our results demonstrate that BL induces phosphorylation of Thr\(_{556}\) in H\(^+-\)ATPase, but that this phosphorylation is not sufficient for activation. Activation of the H\(^+-\)ATPase absolutely requires a concomitant binding of the 14-3-3 protein to the phosphorylated C-terminus. A schematic expression of this process is depicted in Fig. 5. A similar mechanism has been proposed by Palmgren (2001).

### Materials and Methods

**Plant materials and isolation of GCPs**

*Vicia faba* (cv. Ryoshi Issun) was cultured hydroponically in a green house as described previously (Shimazaki et al. 1992). GCPs were isolated enzymatically from the lower epidermis of 4- to 8-week-old leaves of *V. faba* according to a previously described method (Kinoshita and Shimazaki 1999). Isolated GCPs were stored in 0.4 M mannitol and 1 mM CaCl\(_2\), on ice until use in the dark. Protein was determined by the method of Bradford (1976) using BSA as a standard.

**Determination of phosphorylation levels of the plasma membrane H\(^+-\)ATPase**

Phosphorylation levels of the H\(^+-\)ATPase were determined using \(^{32}\)P-labeled GCPs as described previously (Kinoshita and Shimazaki 1999) with slight modifications. The GCP suspensions (0.5 mg protein ml\(^{-1}\)) were incubated with \(^{32}\)Porthophosphate (0.25 mCi (mg protein)\(^{-1}\)) under red background light for 80 min at 24°C, then illuminated with a 30-s pulse of BL. The reaction was terminated at 2.5 min after the pulse of BL by addition of an equal volume of the medium containing 100 mM MOPS-KOH pH 7.5, 5 mM EDTA, 200 mM NaCl, 1 mM PMSF, 20 \(\mu\)M leupeptin, 4 mM DTT, 20 mM NaF, 1 mM ammonium molybdate, 100 nM calyculin A, and 2% (w/v) Triton X-100 to the GCPs, followed by centrifugation for 1 min at 10,000g. The resulting supernatant was mixed with anti-serum against the plasma membrane H\(^+-\)ATPase at 0.5% (v/v). After incubation for 12 h with gentle mixing at 4°C, protein A-agarose (Santa Cruz Biotech., Inc., CA, U.S.A.) was added to the supernatant at 2.5%, and kept for 2 h at 4°C with gentle mixing. The sample was then centrifuged for 1 min at 10,000g, and the pellet was washed three times with 1 ml of Tris-based saline (TBS). The pellet was resuspended in the solubilizing medium (Laemmli 1970), and centrifuged at 10,000g for 1 min to remove agarose, and the supernatant was used for SDS-PAGE in 12.5% gel. Separated proteins on the gel were stained once with Coomassie Brilliant Blue for 1 h to visualize the protein profiles. The destained gel was dried on a gel drier. Autoradiography was carried out by exposing Fuji RX film (Fuji Film, Tokyo, Japan) to the dried gel for 2 d at room temperature.

**Filters**

Red background light at 600 \(\mu\)mol m\(^{-2}\) s\(^{-1}\) was obtained from a tungsten lamp (Philips EXR 300W) by passing the light through a red glass filter (Corning 2-61). BL at 100 \(\mu\)mol m\(^{-2}\) s\(^{-1}\) was obtained from a tungsten lamp (Sylvania EXR 150W) by passing the light through a glass filter (Corning 5-60). Photon flux density was measured with a quantum meter (Li-Cor, model 185A, Lincoln, NE, U.S.A.).

**Immunodetection**

The polyclonal antibodies used in this study have been described previously (Kinoshita and Shimazaki 1999). GCP proteins were solu-
bibilized according to the method of Tominaga et al. (2001). Following separation of proteins by SDS-PAGE, individual proteins were trans-
ferred to nitrocellulose membranes at 1 mA cm⁻² in transfer buffer (48 mM Tris, 39 mM glycine, and 20% methanol) by electroboothing (Trans-blot, Bio-Rad Lab., CA, U.S.A.). The membranes were pre-
incubated in blocking buffer for 1 h (0.05% Tween 20, 5% nonfat dry milk, 20 mM Tris-HCl, pH 7.4, 140 mM NaCl) and then incubated with polyclonal antibodies at a 1: 5,000 dilution in blocking buffer at room temperature for 2 h. The membrane was then rinsed three times for 5 min each in T-TBS (0.05% Tween 20, 20 mM Tris-HCl, pH 7.4, and 140 mM NaCl), and reacted with a goat anti-rabbit IgG secondary anti-
body conjugated to alkaline phosphatase (Bio-Rad Lab., CA, U.S.A.) at a dilution of 1 : 3,000 in blocking buffer at room temperature for 2 h. Development of the alkaline phosphate reaction was performed by 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazoium.

**Far Western blot analysis**

Far Western blotting was performed according to Fullone et al. (1998) with modifications. GCP proteins were subjected to SDS-
PAGE in 12.5% gel and blotted onto nitrocellulose. The membrane was incubated with incubation buffer (20 mM HEPES-KOH pH 7.7, 75 mM KCl, 0.1 mM EDTA, 5 mM MgCl₂, 1 mM dithiothreitol, 5% nonfat dry milk, and 0.04% Tween 20) for 1 h at room temperature. Incubation with 0.1 μM GST–14-3-3 or GST was performed for 16 h at 4°C in incubation buffer. After three washes with T-TBS, the mem-
brane was incubated with anti-GST antibodies (Pharmacia, Tokyo, Japan) at a dilution of 1: 2,000 for 2 h at room temperature in block-
ing buffer. The membrane was then washed three times for 5 min each in T-TBS, and reacted with anti-goat IgG secondary antibodies conjugated to alkaline phosphatase (Sigma) at a dilution of 1 : 5,000 for 2 h at room temperature in blocking buffer. Development of the alkaline phosphatase reaction was performed as described above. For the com-
petition experiment, after incubation of 0.1 μM GST–14-3-3 with syn-
thetic peptides corresponding to the C-terminus of VHA1 (P-849, KFATRYLphospho-SGKAKSEIAEQQAK; P-933, HTLKGHVEphospho-SVVKLGHLPSV; P-950, KGLDIDTIQQHYPHospho-TRIP; DeP-950, KGLDIDTIQQHYPHTYV) at the indicated concentration for 30 min at 24°C, this mixture was used for Far Western blotting. Dephosphorylated P-950 (DeP-950) was obtained by incubation of 1 mM P-950 with 20 units of alkaline phos-
phatase from calf intestine (Roche Molecular Biochemicals, Tokyo, Japan) in 50 μl of dephosphorylation buffer for 2 h at 37°C. The alka-
line phosphatase was then inactivated by heating at 65°C for 15 min.

**H'-ATPase activity**

ATP hydrolysis by H'-ATPase was measured according to the method of Kinoshita et al. (1995) with modifications. The superspension medium of GCPs (1.0 ml) consisted of 5 mM 2-(N-morpholino)ethanesulfonic acid-KOH, pH 6.0, 10 mM KCl, 1 mM CaCl₂, 0.4 M mannitol, and GCPs (0.5 mg protein). The GCPs in this mixture were incubated under backround red light for 40 min at 24°C, after which they were illuminated with BL for 30 or FC and was added at 10 μM. An aliquot of withdrawn GCP suspension (25 μl) was mixed with an equal volume (25 μl) of the medium containing 100 mM MOPS-KOH pH 7.5, 5 mM EDTA, 200 mM NaCl, 1 mM PMSF, 20 μM leupeptin, 2 mM DTT, and 0.05% (w/v) Triton X-100 to disrupt the GCPs. The disrupted GCPs were mixed with an equal volume of the reaction mix-
ture for the ATPase measurement. The reaction mixture consisted of 50 mM MOPS-KOH pH 7.0, 0.2 M mannitol, 100 mM NaCl, 100 mM KNO₃, 2.5 mM EDTA, 20 mM MgCl₂, 10 μg ml⁻¹ oligomycin, 1 mM ammonium molybdate, 0.5 mM PMSF, and 10 μM leupeptin. The reaction was then started by adding ATP at 2 mM and was run for 30 min at 24°C. The reaction was terminated by addition of 100 μl of 2 M HCl containing 25 mg ml⁻¹ ammonium molybdate. Released P₆ was developed by the addition of 10 μl of developing solution containing 1.25 mg ml⁻¹ l-amino-2-naphthol-4-sulfonic acid, 150 mg ml⁻¹ NaH₂SO₄, and 10 mg ml⁻¹ Na₂SO₄ for 30 min at 24°C, and was measured at 750 nm. The H'-ATPase activity was determined by the difference between the activities in the presence and absence of 100 μM vandate, an inhibitor of plasma membrane H'-ATPase.

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