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Regulation of Plant Plasma Membrane H^+- and Ca^{2+}-ATPases by Terminal Domains

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In the last few years, major progress has been made to elucidate the structure, function, and regulation of P-type plasma membrane H^+- and Ca^{2+}-ATPases. Even though a number of regulatory proteins have been identified, many pieces are still lacking in order to understand the complete regulatory mechanisms of these pumps. In plant plasma membrane H^+- and Ca^{2+}-ATPases, autoinhibitory domains are situated in the C- and N-terminal domains, respectively. A model for a common mechanism of autoinhibition is discussed.

KEY WORDS: Plasma membrane H^+-ATPase; plasma membrane Ca^{2+}-ATPase; AHA2; ACA8; plant; Arabidopsis; autoinhibitory domain.

INTRODUCTION

P-type ATPases have been divided phylogenetically into five major subfamilies, each transporting a specific set of ions (Axelsen and Palmgren, 1998). Plasma membrane H^+-ATPases (P_{3A} pumps) are found in plants, fungi, protistae and archaea. They control the electrochemical gradient across the plasma membrane, which is of importance for a broad range of secondary transporters that are energized by the transmembrane chemical gradient and/or the membrane potential. Thus H^+-ATPases play the same crucial role as Na^+/K^+-ATPases (P_{2C} pumps) found only in animal cells. In all eukaryotes, Ca^{2+}-ATPases are important for cellular signaling by maintaining a low cytosolic Ca^{2+} concentration (Axelsen and Palmgren, 2001; Geisler *et al.*, 2000a). In contrast to proton pumps, plasma membrane localized Ca^{2+}-ATPases (P_{2B} pumps) are present in both plants and animals. In animals, P_{2B} Ca^{2+}-ATPases are exclusively found at the plasma membrane, whereas in plant they are located to endomembranes as well (Bonza *et al.*, 2000; Geisler *et al.*, 2000b; Hong *et al.*, 1999; Huang *et al.*, 1993; Schiøtt *et al.*, 2004).

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Plant plasma membrane H^+- and Ca^{2+}-ATPases are characterized by having regulatory domains (R-domains). These serve as autoinhibitors and are located either in the C-terminal (H^+-ATPases) or in the N-terminal cytoplasmic parts (Ca^{2+}-ATPases) (Axelsen and Palmgren, 2001). The R-domains contain both autoinhibitory regions, thought to interact intramolecularly with the pump protein, as well as regulatory regions, which interact with activating proteins that neutralize the constraint exerted by the autoinhibitor. The two different regions are separated in plasma membrane H^+ pumps, but at least partly overlapping in plasma membrane Ca^{2+} pumps.

REGULATION OF PLANT PLASMA MEMBRANE H^+-ATPases

The R-domain of plant plasma membrane H^+-ATPase consists of approximately 100 amino acid residues and has been the subject of intense studies. Originally, it was demonstrated that removal of the C-terminus by trypsin treatment resulted in an activated form of the H^+ pump and it was suggested that the C-terminus acts as an autoinhibitor by locking the pump in a low-activity state (Palmgren *et al.*, 1990, 1991). Mutagenesis experiments showed that autoinhibition could be impaired by mutations in either the C-terminus or within the central

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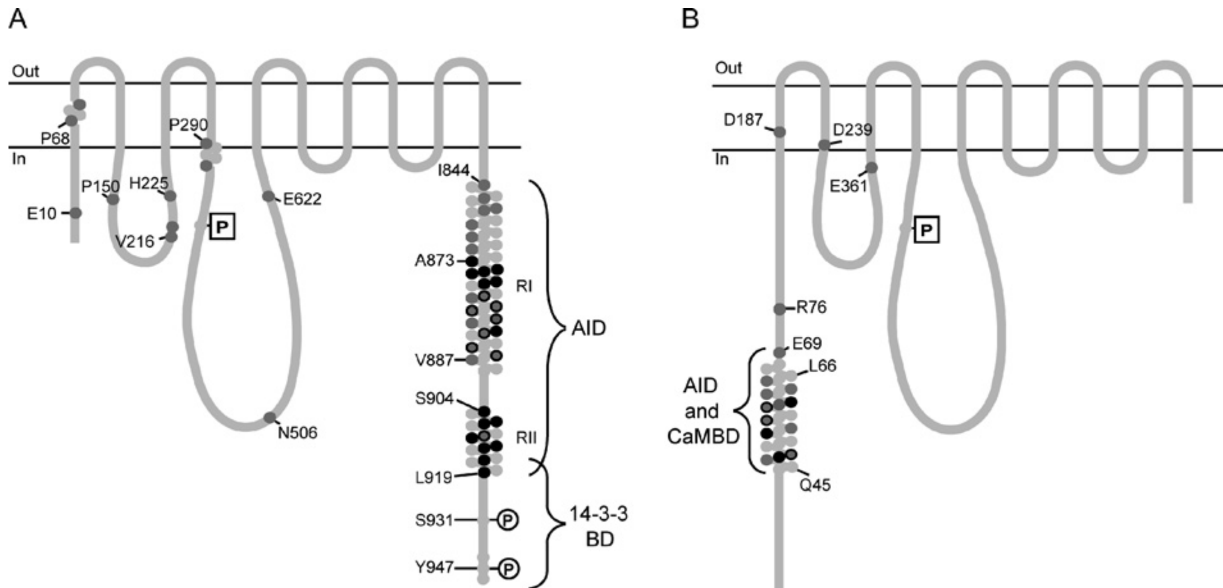

Fig. 1. Localization of amino acid residues involved in regulation of (A) plant plasma membrane H^+-ATPase and (B) plant plasma membrane Ca^{2+}-ATPase. (A) A schematic presentation of single residues that are involved in autoinhibition of the plasma membrane proton pump AHA2 from Arabidopsis (Axelsen *et al.*, 1999) or the plasma membrane proton pump PMA2 from *Nicotiana plumbaginifolia* (Morsomme *et al.*, 1996, 1998). For PMA2, the corresponding residues in AHA2 have been mapped after alignment of the two sequences by ClustalW using default parameters. (B) A schematic presentation of single residues involved in autoinhibition of the plasma membrane Ca^{2+}-ATPase ACA8 from Arabidopsis (Bækgaard *et al.*, 2006) or in the Arabidopsis endomembrane P_{2B} Ca^{2+} pump ACA2 (Curran *et al.*, 2000). For ACA2, the corresponding residues in ACA8 have been mapped after alignment of the two sequences by ClustalW using default parameters. *Black dots* mark the residues involved in autoinhibition of AHA2 (A) and ACA8 (B). *Dark grey dots* show the position of residues in AHA2 (A) and ACA8 (B), in which the corresponding residues in PMA2 and ACA2, respectively, have been shown to be involved in autoinhibition. *Encircled grey dots* represent residues that are involved in autoinhibition in both AHA2 and PMA2 (A) and in ACA8 and ACA2 (B). A *boxed P* marks the conserved aspartate residue that becomes phosphorylated during the pump reaction cycle, whereas an *encircled P* marks residues that are subject for regulatory phosphorylation. R-I and R-II, regulatory region I and II, respectively. AID, autoinhibitory domain; 14-3-3 BD, region interacting with activating 14-3-3 protein; CaMBD, calmodulin binding domain.

part of the pump (Fig. 1(A), Baunsgaard *et al.*, 1996; Morsomme *et al.*, 1998). A systematic alanine scanning of the C-terminus revealed two regions (I and II) being important for the autoinhibitory role of the C-terminus (Axelsen *et al.*, 1999). In the Arabidopsis proton pump, AHA2, these domains are located between Lys-863 to Leu-885 (I) and Ser-904 to Leu-919 (II). Several mutations within the central part of the pump can abolish the autoinhibitory effect of the C-terminus, but the intramolecular receptor for the C-terminal R-domain has not yet been identified.

The plasma membrane H^+ pump is regulated by activating 14-3-3 proteins at a unique site in the extreme end of the C-terminus. Interaction with 14-3-3 protein involves several residues in the C-terminal end of the H^+ pump molecule (Fuglsang *et al.*, 2003; Jelich-Ottmann *et al.*, 2001), but phosphorylation of a penultimate threonine residue in the C-terminus (Thr-947 in AHA2) is required in order to stabilize 14-3-3 protein binding (Fuglsang *et al.*, 1999; Maudoux *et al.*, 2000; Svennelid *et al.*, 1999). In stomatal guard cells, phosphorylation of this residue takes place *in vivo* in response to blue light (Kinoshita and Shimazaki, 1999). Two blue light photoreceptors, phototropins (phot1 and phot2), are identified in Arabidopsis; these are serine/threonine kinases containing light, oxygen, and voltage domains. The photoreceptors do not phosphorylate the H^+ pump themselves but mediate the signal from perception of blue light to phosphorylation and activation of the H^+ pump through a yet unidentified signal transduction cascade (Kinoshita *et al.*, 2001). The hormone abscisic acid (ABA) induces closure of stomatal aperture by utilizing the same signal cascade as blue light, since the blue light stimulated activation can be inhibited by ABA (Zhang *et al.*, 2004). Regulation of the H^+ pump is also found in relation to the transport of ions and solutes across the plasma membrane in roots. One example is the increased phosphorylation of the penultimate threonine residue in response to aluminum stress (Shen

et al., 2005). Thus activation of the proton pump is a prerequisite for secretion of citrate working as a chelator of Al^{3+} ions.

The protein kinase responsible for phosphorylation of the penultimate threonine residue has not yet been identified. However, the involvement of another kinase in regulation of the H^+ pump has recently been demonstrated (Fuglsang *et al.*, submitted). This kinase, PKS5, belongs to a family of kinases (PKS/CIPK) that are regulated by a Ca^{2+}-binding protein with homology to calcineurin B (CBL/SCaBP). PKS5 phosphorylates a serine residue upstream of the 14-3-3 binding site. Phosphorylation of the serine residue (Ser-931 in AHA2) results in a decrease of 14-3-3 binding to the H^+ pump shifting the pump into its low-activity state. The serine residue is highly conserved among plant H^+ pumps and is located between the two autoinhibitory domains and the terminal 14-3-3 binding site.

Despite the fact that proteins with homology to calcineurin B are involved in regulation of the H^+ pump, no protein phosphatases with homology to the catalytic subunit of calcineurin have been found in the plant genome. *In vitro*, other types of phosphatases can inhibit the interaction between the H^+ pump and the 14-3-3 proteins. This includes alkaline phosphatases (Fuglsang *et al.*, 1999) and a type 2A phosphatase, PP2A (Camoni *et al.*, 2000).

REGULATION OF PLANT PLASMA MEMBRANE Ca^{2+}-ATPases

Upon binding of Ca^{2+} ions, calmodulin (CaM) binds to and activates P_{2B} Ca^{2+} pumps. Although there does not exist a consensus CaM-binding domain (CaMBD), they are usually 15–30 amino acids long and have a tendency to form an α-helix containing two bulky hydrophobic residues that function as anchors for CaM binding (Crivici and Ikura, 1995; Yap *et al.*, 2000). An N-terminal CaMBD in a plant P_{2B} Ca^{2+} pump was first identified in the vacuolar Ca^{2+}-ATPase, BCA1, isolated from cauliflower (Malmström *et al.*, 1997), but since then it has been identified in several plant P_{2B} Ca^{2+} pumps including the plasma membrane located pumps, ACA8 and ACA9 from Arabidopsis (Bonza *et al.*, 2000; Schiøtt *et al.*, 2004).

An alanine mutagenesis scanning analysis has revealed that the CaMBD of ACA8 comprises a stretch of residues from Arg-43 to Lys-68. In this region, Trp-47 and Phe-60, conserved in Arabidopsis P_{2B} Ca^{2+}-ATPases, were shown to function as anchor residues for CaM binding. In addition to the hydrophobic anchor residues, 11 hydrophobic or basic residues were found to be important for the stability of the complex between CaM and the CaMBD of ACA8 (Bækgaard *et al.*, 2006). This confirms the importance of hydrophobic and electrostatic interactions between CaM and target proteins, observed in crystal structures of CaM complexed with other CaMBDs (Vetter and Leclerc, 2003).

When expressed in a Ca^{2+}-ATPase deficient yeast strain (K616) (Cunningham and Fink, 1994), plant P_{2B} Ca^{2+}-ATPases can functionally complement the yeast mutant provided that the N-terminus is deleted (Bonza *et al.*, 2004; Chung *et al.*, 2000; Geisler *et al.*, 2000b; Harper *et al.*, 1998; Schiøtt *et al.*, 2004; Schiøtt and Palmgren, 2005) or if single amino acid residues important for pump autoinhibition have been mutated (Fig. 1(B), Bækgaard *et al.*, 2006; Curran *et al.*, 2000). In the CaMBD of ACA8, six residues were identified to be important for pump autoinhibition. These included the hydrophobic anchor residues, Trp-43 and Phe-60, giving these residues a dual function in both autoinhibition and CaM recognition (Bækgaard *et al.*, 2006).

Regions outside of the CaMBD of plant P_{2B} Ca^{2+}-ATPase might also be involved in autoinhibition (Fig. 1(B)). First, the peptide Met-1 to Ile-116 in ACA8 inhibits an activated form of the pump better than the peptide Ile-41 to Thr-63 corresponding to the CaMBD (Luoni *et al.*, 2004).

Second, in the endoplasmic reticulum located Arabidopsis P_{2B} Ca^{2+}-ATPase, ACA2, residues that connect the catalytic domain of the ATPase with transmembrane domains are also involved in pump inhibition and could make interactions with the N-terminal autoinhibitory domain (Curran *et al.*, 2000). In addition, the intramolecular receptor involves the small cytoplasmic loop of ACA8 (Luoni *et al.*, 2004). This loop is part of the A-domain and includes the conserved TGES motif, in which the glutamate residue of the animal P_{2A} Ca^{2+}-ATPase, SERCA1, has been shown to be important for dephosphorylation of the phosphorylated reaction cycle intermediate during catalysis (Clausen *et al.*, 2004). Hence, in the autoinhibitory state of ACA8, the regulatory N-terminus may hinder the movement of the A-domain, thereby inhibiting the pump.

Phosphorylation and lipids may also affect the activity of plant plasma membrane Ca^{2+} pumps. Acidic phospholipids activate a plasma membrane Ca^{2+} pump from radish by a mechanism different from CaM stimulation (Bonza *et al.*, 2001). In contrast, phosphorylation has so far only been reported to regulate the endomembrane P_{2B} Ca^{2+} pump, ACA2. The pump is phosphorylated by a calcium-dependent protein kinase (CDPK) at Ser-45 near the CaMBD, which results in inhibition

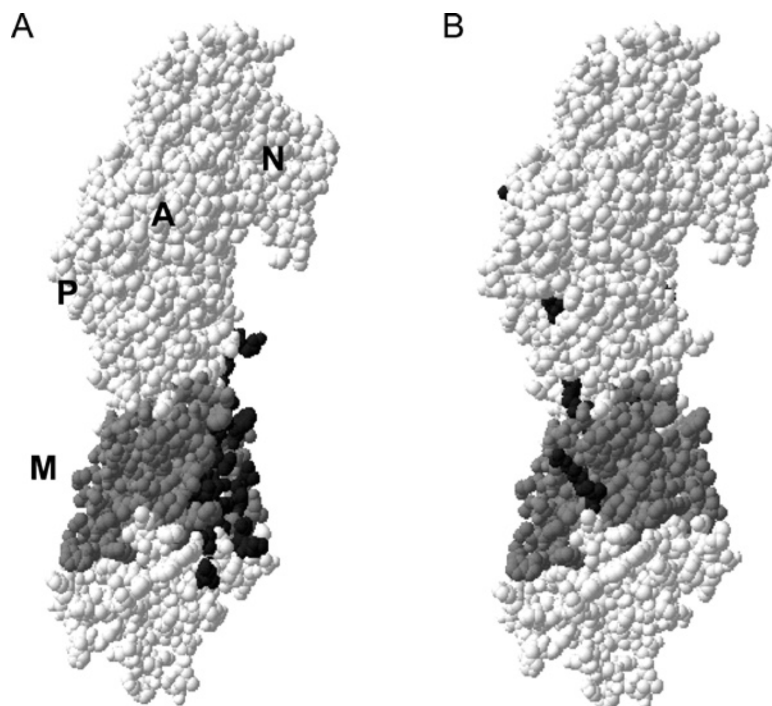

Fig. 2. Space-filling model of the three-dimensional structure of the P_{2A} Ca^{2+}-ATPase SERCA1a from rabbit with indicated regulatory residues from different plant P-type ATPases. The crystal structure is based on SERCA1a in the E_2 conformation (Toyoshima and Nomura, 2002) and visualized with Swiss-PdbViewer v3.7. (A) Residues that are suggested to bind phospholamban by the model of Toyoshima and coworkers are marked in *dark grey* (Toyoshima *et al*., 2003). (B) Residues in SERCA1 that correspond to residues in *N. plumbaginifolia* plasma membrane H^+-ATPase PMA2 (Morsomme *et al*., 1996, 1998) and Arabidopsis P_{2B} Ca^{2+}-ATPase ACA2 (Curran *et al*., 2000), that give rise to activated mutant pumps when substituted, are indicated in *dark grey*. Corresponding residues were mapped after alignment of the three sequences by ClustalW using default parameters. Residues in PMA2 correspond to residues in SERCA1a as follows: $E14_{PMA2}/K7_{SERCA}$, $P72_{PMA2}/R63_{SERCA}$, $W75_{PMA2}/L66_{SERCA}$, $P154_{PMA2}/P147_{SERCA}$, $V220_{PMA2}/V228_{SERCA}$, $H221_{PMA2}/S229_{SERCA}$, $H229_{PMA2}/D237_{SERCA}$, $P294_{PMA2}/P312_{SERCA}$, $S298_{PMA2}/T316_{SERCA}$, $N510_{PMA2}/R620_{SERCA}$, and $E626_{PMA2}/D737_{SERCA}$. The indicated ACA2 residues correspond to the following in SERCA1a residues: $E167_{ACA2}/E55_{SERCA}$, $D219_{ACA2}/Q108_{SERCA}$, and $E341_{ACA2}/K246_{SERCA}$. A, Actuator domain; N, nucleotide binding domain; M, transmembrane region; P, phosphorylation domain.

of CaM stimulation and of basal activity (Hwang *et al*., 2000a).

COULD THERE BE A GENERAL REGULATORY MECHANISM FOR P-TYPE ATPases?

Regulation of P-type ATPases, although different, do share some common characteristics, which could suggest a general mechanism for pump inhibition.

P_{3A} H^+-ATPases and P_{2B} Ca^{2+}-ATPases are both autoinhibitory proteins, inhibited by a C-terminal or N-terminal R-domain. The position of the R-domain to either termini might not be important for autoinhibition, as P_{2B} Ca^{2+}-ATPases from animals (PMCAs) and plants seem to be regulated similarly, despite of the different localization of the R-domain (Geisler *et al.,* 2000a). Furthermore, relocation of the C-terminal R-domain in PMCA4b to the N-terminus had only minor effects on autoinhibition and CaM regulation (Adamo and Grimaldi, 1998). Autoinhibitory domains of both plant and animal P_{2B} Ca^{2+} pumps inhibit P_{2A} Ca^{2+}-ATPases (Chiesi *et al*., 1991; Hwang *et al*., 2000b), which would suggest that an intramolecular receptor is conserved between P_{2A} and P_{2B} Ca^{2+}-ATPases.

Animal P_{2A} Ca^{2+}-ATPases, SERCAs that lack a built-in autoinhibitor, are regulated by binding of phospholamban (PLN), a member of low-molecular-mass proteins with a single transmembrane domain (Simmerman and Jones, 1998). Recently, a structural model has been proposed for the interaction between PLN and SERCA1a (Toyoshima *et al.*, 2003). The structure suggest that PLN binds into a groove formed by the transmembrane helices, M2, M4, M6, and M9, as it traverses the membrane and extends through the cytosol toward a binding site in the N-domain in SERCA1a (Fig. 2(A)). Cross-linking experiments of PLN and SERCA2a have shown that the same pump residues interact with PLN, although they may bind to other residues in PLN than suggested in the model (Chen *et al.*, 2003, 2005). The Na^{+}/K^{+}-ATPase seems to be regulated similarly to SERCA by several members of the FXYD protein family that includes phospholemman (FXYD1) and the α-subunit of Na^{+}/K^{+}-ATPase (FXYD2) (Sweadner and Rael, 2000). Following mutagenic studies and analysis of homology models based on the SERCA1a structure, the FXYD proteins are likewise suggested to interact with the transmembrane helices M2, M4, M6, and M9 (Füzesi *et al.*, 2005; Li *et al.*, 2004).

Mutagenic studies of plant plasma membrane H^{+}-ATPases and P_{2B} Ca^{2+}-ATPases have identified several amino acid residues in or near the transmembrane helices important for autoinhibition (Fig. 1; Curran *et al.*, 2000; Morsomme *et al.*, 1996, 1998), which could be important for intramolecular recognition of the autoinhibitory domain. In Fig. 2(B), autoinhibitory residues in the plant H^{+} and Ca^{2+} pumps have been aligned with the SERCA1a sequence and subsequently mapped into the structure of SERCA1a in the E_2 conformation (Toyoshima and Nomura, 2002). This allows for a comparison with the residues in SERCA1a that are suggested to interact with PLN (Fig. 2(A)). In both structures, the residues form a long stretch on the pump surface close to transmembrane regions. Whereas PLN interacting residues are situated on M2, M4, M6, and M9, autoinhibitory residues in plant H^{+} and Ca^{2+} pumps are located to M1, M2, M3, and M4. Even though the residues are not identical, this could indicate that plasma membrane H^{+}-ATPases and P_{2B} Ca^{2+}-ATPases are inhibited by an internal domain in a similar way as the external PLN inhibits SERCA.

If autoinhibitory domains of P-type pumps inhibit pump activity by interacting with residues in the vicinity of transmembrane helices, some physical constraints have to be considered. First, the region of PLN suggested to bind to the transmembrane helices in SERCA1a is itself a hydrophobic transmembrane helix (Toyoshima *et al.*, 2003). Similarly, FXYD proteins contain a transmembrane helix that promotes interaction with Na^{+}/K^{+}-ATPase (Sweadner and Rael, 2000). However, neither the plasma membrane H^{+}-ATPases nor the P_{2B} Ca^{2+}-ATPases are predicted to have a large hydrophobic region in their R-domain as would be expected for domains interacting with transmembrane helices. Second, the regulatory mechanism by which pump autoinhibition is to be released is also problematic. In both plasma membrane H^{+}-ATPases and P_{2B} Ca^{2+}-ATPases, the constraint is relieved following binding of a small protein, 14-3-3 protein or CaM, to the R-domain of the pump. Since the 14-3-3 binding site of H^{+} pumps is situated at the extreme end of the C-terminus and the CaMBD of Ca^{2+} pumps is overlapping with the autoinhibitory domain (Fig. 1), both sites would probably have to be embedded in the membrane if the autoinhibitory domains are to bind to transmembrane helices. This would make it difficult for the activating proteins to approach and bind to the R-domains. In contrast, activation of SERCA is mediated by phosphorylation of PLN at a serine or threonine residue that resides in the cytoplasmic part of the bound protein (Simmerman and Jones, 1998) making it is easy for a protein kinase to approach. Consequently, although it cannot be ruled out that the transmembrane helices of plasma membrane H^{+}- and Ca^{2+}-ATPases bind the R-domains, further studies are needed to investigate this model.

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