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Regulation of the plant plasma membrane H^+-ATPase by its C-terminal domain: what do we know for sure?

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ABSTRACT

The plant plasma membrane H^+-ATPase is kept at a low activity level by its C-terminal domain, the inhibitory function of which is thought to be mediated by two regions (region I and II) interacting with cytoplasmic domains essential for the catalytic cycle. The activity of the enzyme is well known to be regulated by 14-3-3 proteins, the association of which requires phosphorylation of the penultimate H^+-ATPase residue, but can be abolished by phosphorylation of residues close-by. The current knowledge about H^+-ATPase regulation is briefly summed up here, combined with data that query some of the above statements. Expression of various C-terminal deletion constructs of PMA2, a H^+-ATPase isoform from *Nicotiana plumbaginifolia*, in yeast indicates that three regions, which do not correspond to regions I or II, contribute to autoinhibition. Their individual and combined action can be abolished by (mimicking) phosphorylation of three threonine residues located within or close to these regions. With respect to the wild-type PMA2, mimicking phosphorylation of two of these residues increases enzyme activity. However, constitutive activation of wild-type PMA2 requires 14-3-3 association. Altogether, the data suggest that regulation of the plant H^+-ATPase occurs in progressive steps, mediated by several protein kinases and phosphatases, thus allowing gradual as well as fine-tuned adjustment of its activity. Moreover, mating-based split ubiquitin assays indicate a complex interplay between the C-terminal domain and the rest of the enzyme. Notably, their tight contact does not seem to be the cause of the inactive state of the enzyme.

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Introduction

The plasma membrane H^+-ATPases of plants and fungi are primary active pumps that couple ATP hydrolysis to proton transport out of the cell. The established pH and potential gradients across the plasma membrane provide the driving force for secondary transport of metabolites. In addition to its fundamental role in nutrient uptake, the plant enzyme plays a major role in processes essential for normal growth, such as stomatal movement, cell elongation, pH homeostasis, salt tolerance (reviewed in (Palmgren, 2001; Duby and Boutry, 2009)) and also pathogen attack (Liu et al., 2009). Several of these require balanced regulation, suggesting that multiple regulatory pathways may converge in order to fine-tune H^+-ATPase activity. P-type H^+-ATPases are characterized by C-terminal domains serving as autoinhibitors (Baekgaard et al., 2005). The mechanism of pump activation, however, seems to differ significantly between plants and fungi, since their respective autoinhibitory domains do not share any sequence homology and, moreover, are of different length. With regard to the latter, the plant domain contains twice as many amino acids (ca. 100) as compared to the fungal domain, suggesting a more complex regulation of the plant enzyme.

On the basis of mutagenesis experiments, as well as systematic alanine scanning, two regions (regions I and II, Fig. 1) have been identified which seem to be important for the autoinhibitory function of the C-terminal domain of the plant enzyme, more precisely the *Arabidopsis thaliana* isoform AHA2 (Baunsgaard et al., 1996; Axelsen et al., 1999) and PMA2 from *Nicotiana plumbaginifolia* (Morsomme et al., 1996, 1998). Since mutations within these regions abolish the inhibitory effect of the C-terminus, they have been assumed to interact intramolecularly with the rest of the pump, which, however, is still to be verified.

Moreover, the constraint exerted by the autoinhibitor is released upon binding of 14-3-3 dimers (Oecking et al., 1997; Piotrowski et al., 1998; Fuglsang et al., 1999; Svennelid et al., 1999; Maudoux et al., 2000; Jaspert and Oecking, 2002), which are well known as discrete phosphoserine/threonine-binding modules that operate by enforcing conformational changes, among others (MacKintosh, 2004). Interaction with 14-3-3s *(i)* requires phosphorylation of the penultimate H^+-ATPase residue (Fuglsang et al., 1999; Svennelid et al., 1999; Maudoux et al., 2000), a highly

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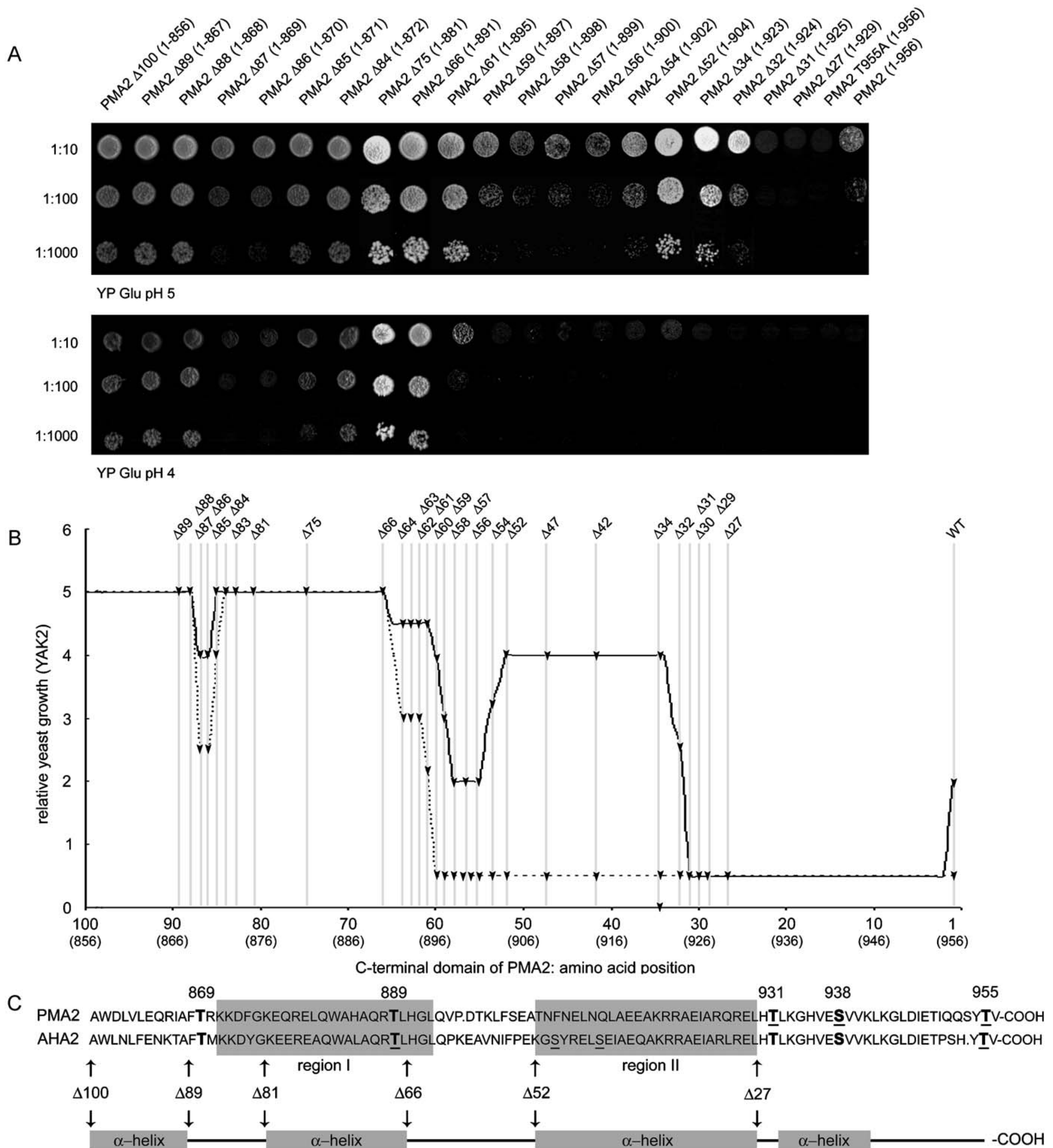

Fig. 1. (A) Growth of YAK2 transformed with wild-type and C-terminal deletion mutants of PMA2. Each strain was spotted at serial 10-fold dilution onto solid rich medium (pH 5.0 and pH 4.0, respectively), containing glucose as a sole carbon source. (B) Relative growth of YAK2 expressing the indicated PMA2 versions. The yeast growth was classified between 0 and 5, with category 0 indicating no and category 5 indicating strong growth. (C) Amino acid sequence of the C-terminal regulatory domains of PMA2 and AHA2. Region I and II are marked by grey background. Phosphorylated residues described in the literature are underlined and their homologs are shown in bold. The bars correspond to helices, that are either resolved by a crystal structure (downstream of Δ52; Ottmann et al., 2007) or predicted (secondary structure prediction according to PROF).

conserved threonine, *(ii)* involves approximately 50 C-terminal H^+-ATPase residues (Jelich-Ottmann et al., 2001; Fuglsang et al., 2003) (see Fig. 7) – altogether constituting the part that is absent in fungal pumps, and *(iii)* results in formation of a H^+-ATPase hexamer (Kanczewska et al., 2005; Ottmann et al., 2007). The crystal structure of 14-3-3, in complex with the entire binding motif, clearly shows that the C-terminal 27 residues are directly bound within the typical amphipathic groove of a 14-3-3 monomer, while the remaining amino acids, which correspond to region II (Fig. 1), protrude from the center of the 14-3-3 dimer (Ottmann et al., 2007) (see Fig. 7).

Notably, following the identification of 14-3-3s as 'master regulators' of the H^+-ATPase, several active enzyme versions carrying mutations within regions I or II have been shown to be strongly phosphorylated and to bind 14-3-3 more efficiently than the wild type (Maudoux et al., 2000; Jahn et al., 2002). Such mutations might therefore allow better accessibility to the C-terminal end for phosphorylation and subsequent 14-3-3 association.

Nevertheless, recent approaches have impressively demonstrated that phosphorylation does not only promote but also interferes with 14-3-3 binding to the H^+-ATPase, hence shifting the pump into its low activity state. In this context, phosphorylation of either a threonine (Duby et al., 2009) or a serine residue (Fuglsang et al., 2007; Duby et al., 2009) upstream of, but close to, the residue functioning as an 'attachment signal' for 14-3-3s (see Fig. 7), in return prevents and/or abolishes 14-3-3 binding to the H^+-ATPase. Such effects have already been predicted on the basis of the crystal structure (Ottmann et al., 2007). Altogether, this suggests the involvement of several kinases mediating either activation or inhibition of the H^+-ATPase, but does it indicate phosphorylation-dependent 14-3-3 association/dissociation to be the only mechanism involved in H^+-ATPase regulation?

By analyzing sucrose-induced phosphorylation changes in plasma membrane proteins of *Arabidopsis*, Niittylä et al. (2007) identified a new phosphorylation site in region I and, thus, outside the 14-3-3 binding motif of AHA2 that was claimed to produce an activated pump without the need for additional partners such as 14-3-3s (Niittylä et al., 2007). However, the critical experiment, in which the mutation of T881 is combined with the exchange of the penultimate AHA2 residue, has not yet been performed. Thus, our knowledge about 14-3-3-independent regulation of the H^+-ATPase is marginal. The same applies to the proposed interactions of regions I and II with the membrane-embedded 'rest' of the enzyme, especially the large loop and the attenuator. The recently obtained crystal structure of AHA2 has led to important insights into the mechanism of proton translocation (Pedersen et al., 2007), but was devoid of the C-terminal domain and hence, up to now, we do not have any structural clues about its inhibitory function.

In order to re-evaluate the inhibitory action of the PMA2 C-terminus in the absence of 14-3-3s, we decided to investigate a multitude of C-terminal deletion mutants after heterologous expression in the *Saccharomyces cerevisiae* strain YAK2.

Materials and methods

Growth media

S. cerevisiae cells were grown at 30 °C either in rich or minimal medium, the former containing 1% (w/v) yeast extract, 2% (w/v) peptone, 0.002% (w/v) adenine and 2% (w/v) glucose (YAK2, THY.AP4, THY.AP5) or galactose (YAK2) as carbon source. The medium for YAK2 was supplemented with 20 mM KH_2PO_4 (YAK2) to allow pH adjustment (KOH). Minimal medium consists of 0.67% (w/v) yeast nitrogen base and 2% (w/v) glucose (YAK2, THY.AP4, THY.AP5) or galactose (YAK2), supplemented with all amino acids and nucleotides required for growth.

Yeast strains and plasmids

The YAK2 strain of *S. cerevisiae* and the construction of the plasmid 2μp(PMA1)pma2 have been described in detail (de Kerchove d'Exaerde et al., 1995). In brief, the multicopy plasmid 2μp(PMA1)pma2 harbors the *N. plumbaginifolia* H^+-ATPase PMA2 cDNA under the control of the constitutive yeast PMA1 promoter. The yeast YAK2 carries the yeast H^+-ATPase *pma1* gene under the control of the galactose-dependent GAL1 promoter on a centromeric plasmid and is deleted of its endogenous H^+-ATPase genes.

cDNA fragments encoding the C-terminal domain of PMA2 and characterized by different length and/or carrying a specific mutation were exchanged for the respective wild-type DNA fragment in 2 μp(PMA1)pma2. The transformation of YAK2 was conducted as described (Elble, 1992). Transgenic YAK2 was used to test the ability of wild-type or mutant PMA2 to functionally replace the yeast H^+-ATPase on medium containing glucose as sole carbon source.

THY.AP4/THY.AP5 strains of *S. cerevisiae* and the plasmids pMetYCgate, pNXgate32-3HA have been described in detail (Grefen et al., 2007). The vector pMetYCgate is used to create C-terminal Cub fusion proteins and the vector pNXgate32-3HA is used to create N-terminal Nub fusion proteins. Following mating of the two transgenic yeast strains, interaction between Cub and Nub fusion proteins can be visualized by growth on selective medium.

Preparation of yeast plasma membrane

YAK2 cells were first grown in 100 ml of minimal medium containing 2 % (w/v) galactose until their OD_{600} reached 0.8. After sedimentation and washing (three times with cold water), the cells were used to inoculate 300 ml rich medium containing 2 % (w/v) glucose (OD_{600}: 0.2). When the OD_{600} reached 0.8, the cells were collected by centrifugation and washed (three times with cold water).

Membrane preparation followed the procedure described by Svennelid et al. (1999). Plasma membranes were collected by centrifugation, resuspended in 20% (w/v) glycerol, 10 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol, and 1 mM $MgSO_4$, frozen in liquid nitrogen and stored at -80 °C.

Gel electrophoresis and immunoblotting

Denaturing gel electrophoresis was performed according to (Laemmli, 1970). Protein blotting onto nitrocellulose was done electrophoretically overnight (4 °C, 64 mA) or within 2 h at room temperature (200 mA) as described by (Towbin et al., 1979). Immunodetection of PMA2 followed standard procedures using an antibody raised against an N-terminal peptide of AHA2.

Purification of recombinant $(His)_6$-14-3c

For expression of the *N. tabacum* 14-3-3 isoform T14-3c as a 6-His-tagged protein in *Escherichia coli*, the corresponding cDNA (Piotrowski et al., 1998) was amplified by PCR and cloned into the expression vector pQE-30 (Qiagen, Chatsworth, CA). The construct was expressed in *E. coli* M15, and the native protein was purified from 100 ml of culture by using Ni-NTA agarose, according to the manufacturer's protocol.

14-3-3 protein overlay

Yeast plasma membrane proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose. Non-specific sites were blocked by incubation for 1 h at room temperature with 2% (w/v) milk powder in 50 mM Tris-HCl, pH 7.8, 150 mM NaCl, and 1 mM $MgCl_2$ (Tris-buffered saline). Subsequently, the membrane was incubated overnight at 4 °C with purified $(His)_6$-tagged T14-3c isoform of *N. tabacum*, diluted to 20 μg/ml in 20 mM MOPS, pH 6.5 (KOH), 20% (w/v) glycerol,

5 mM $MgSO_4$, 2 mM dithiothreitol. After washing with Tris-buffered saline, immunodetection of $(His)_6$-tagged 14-3-3 was performed by applying the anti-RGS$(His)_6$ antibody (Qiagen; monoclonal antibody raised against the RGS$(His)_6$ epitope encoded by the pQE vector) in combination with goat anti-mouse immunoglobulin conjugated to horseradish peroxidase and its respective substrates.

Protein interaction assays using the mating-based split ubiquitin system

The protein–protein interaction experiments were done as described (Grefen et al., 2007). The membrane integral part of PMA2 (aa 1-855) and different C-terminal domains (aa 856-856) were amplified by PCR using PMA2-specific primers with added B1- and B2-linker sequences. In vivo cloning was performed by using the yeast strain THY.AP4 for Cub- and THY.AP5 for Nub-constructs, respectively. Positive clones were selected by yeast-colony-PCR and sequencing of the PCR product. Following mating of the two transgenic yeast strains, protein–protein interactions were detected by growth on selective medium. The expression of the recombinant Nub fusion proteins could not be detected by immunoblotting because the C-terminal B2-linker and the following HA-tag were removed, since modification of the C-terminus of PMA2 has been shown to negatively influence its interaction capacity (Maudoux et al., 2000; Würtele et al., 2003).

Results and discussion

Inhibitory regions within the C-terminal domain of PMA2

In order to analyze the capability of the cytoplasmic C-terminal domain to inhibit PMA2, a multitude of C-terminal deletion mutants was investigated after heterologous expression in the *S. cerevisiae* strain YAK2, the survival of which on galactose medium is made possible by the presence of the yeast H^+-ATPase gene under the control of a galactose-dependent promoter. In contrast, PMA2 is constitutively expressed, and hence, the plant H^+-ATPase can be analyzed for its ability to functionally replace the endogenous H^+-ATPase on glucose medium. The wild-type plant PMA2 is able to sustain yeast growth provided that the external pH is kept at or above pH 5.0 (de Kerchove d'Exaerde et al., 1995; Dambly and Boutry, 2001), due to the fact that at least part of the protein is activated upon association of yeast 14-3-3s (Piotrowski et al., 1998; Maudoux et al., 2000). As a control, mutation of the penultimate residue, T955A, which abolished phosphorylation and 14-3-3 binding, prevented PMA2 activation and yeast growth (see Fig. 4).

The results obtained by analyzing the C-terminal deletion constructs of PMA2, all of which are unable to bind 14-3-3, were completely unexpected and clearly indicate that our understanding of how this protein is regulated by its C-terminal domain is still far from complete. In brief, PMA2, truncated by the entire C-terminal domain (Δ100, Fig. 1), is – as expected – fully active since it allows yeast growth even at a pH of 4. This activity status is maintained until the first helix (Δ89, 88, secondary structure prediction according to PROF) is established. Addition of the first linker (Δ87, 86, 85) significantly reduces the activity at pH 4, and this inhibition is released upon attachment of region I (Δ66) which is also predicted to form an α-helix (Fig. 1). Further extension by the second linker (Δ58, 57, 56, 54) brings the activity down again, leading to a complete loss of yeast growth capacity at pH 4. Regarding pH 5, the observed inactivation is partly reversed by adding region II (Δ42, 34, 32). Finally, if the C-terminal 30 amino acid residues are reached, inhibition of the enzyme seems to be complete (Fig. 1).

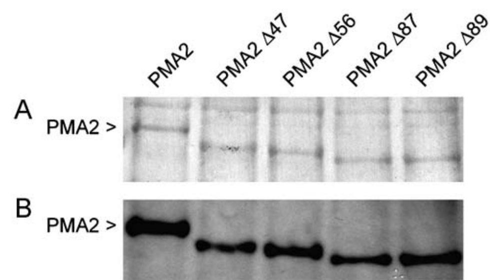

Fig. 2. Analysis of plasma membrane proteins of yeast cells expressing wild-type or mutant PMA2. Plasma membrane proteins (10 μg) were electrophoretically separated (8% SDS-PAGE), transferred to nitrocellulose membranes and stained with Coomassie (A) or processed for immunodetection of PMA2 (B).

Analysis of the amount of truncated PMA2 in yeast plasma membranes, with emphasis on those deletions which significantly reduce enzyme activity as compared with more active deletions close-by (Δ87 versus Δ89; Δ56 versus Δ47), indicated that there is no correlation between the growth phenotype of transgenic yeast and the expression level of the plant enzyme (Fig. 2). According to these results neither region I nor region II seem to function directly as autoinhibitory regions. Instead, the regions linking the predicted helices within the C-terminal domain (regions A and B in Fig. 7) as well as the C-terminal 30 amino acids (region C in Fig. 7) seem to act in an inhibitory manner. The fact that the inhibitory function of regions A and B can be (partly) reversed by the addition of (parts of) regions I and II indicates that the latter impact on the conformation of the C-terminal domain. Altogether, this suggests that inhibition of the H^+-ATPase by its C-terminal domain might occur in a progressive and complex manner, with region C exerting the most negative influence on enzyme activity.

Effect of putative phosphorylation sites

As already mentioned, a network of kinases seems to be involved in the regulation of the H^+-ATPase, most of which, however, impact negatively or positively on 14-3-3 association (see Introduction; Fig. 7). Interestingly, two putative PMA2 phosphorylation sites (T869, T889) are localized within or close to the inhibitory regions A and B (see Fig. 7). Both residues are well conserved within the H^+-ATPase family, and the amino acid corresponding to T869 of the rice H^+-pump OSA2 was shown to be phosphorylated by a Ca^{2+}-dependent protein kinase associated with the plasma membrane (Ookura et al., 2005). Whether this phosphorylation occurs in vivo is still unknown and its detection might be complicated by the multitude of trypsin cleavage sites nearby (Fig. 1). As depicted by Duby and Boutry (2009), mass spectrometry analyses revealed in vivo phosphorylation of PMA2 T889, and phosphorylation of the corresponding AHA2 residue (T881) was proposed to override activation mediated by 14-3-3 binding (see Introduction; Niittylä et al., 2007). We, therefore, mimicked phosphorylation by exchanging these threonine residues for aspartic acid and analyzed the growth capacity of yeast expressing PMA2 characterized by a C-terminal domain encompassing region A (Δ87) or region A/B (Δ56). While the substitution T869D completely restores the activity of PMA2Δ87, full activation of PMA2Δ56 requires the exchange of both T869 and T889 (Fig. 3), suggesting that the phosphorylation of these residues impacts on the constraint exerted by regions A and B, probably by conferring conformational changes, respectively. Does this

indicate that these posttranslational modifications bypass the inhibitory effect of region C and, thus, act independently of 14-3-3 in the full-length PMA2? Mimicking phosphorylation of either T869 or both threonine residues in the wild-type PMA2 results in an activity which is comparable to that of PMA2 deleted of its entire C-terminal domain (Δ100) (Fig. 4; compare with Fig. 1A). This shows the effect of T869D to be more distinct on full-length PMA2 than on PMA2Δ56, which might be related to region C. We therefore performed a 14-3-3 overlay assay with plasma membrane proteins prepared from transgenic yeast. As shown in Fig. 5, in vitro 14-3-3 binding to PMA2 T869D is significantly increased as compared to the wild type, indicating that phosphorylation of T869 does not only bypass the negative effect of region A but also impacts on region C, making it more accessible for phosphorylation and 14-3-3 association. However, since the T869D mutation in PMA2 T955A, which is unable to bind 14-3-3s, nevertheless partly activates the enzyme (Fig. 4) the effect of phosphorylation of this residue seems to be a combination of conformational changes affecting both region A and region C. The activity of PMA2 T955A is even higher on condition that not only phosphorylation of T869 but also that of T889 is mimicked (Fig. 4; PMA2 T889/869D T955A), again arguing for a combinatorial effect, this time also encompassing region B.

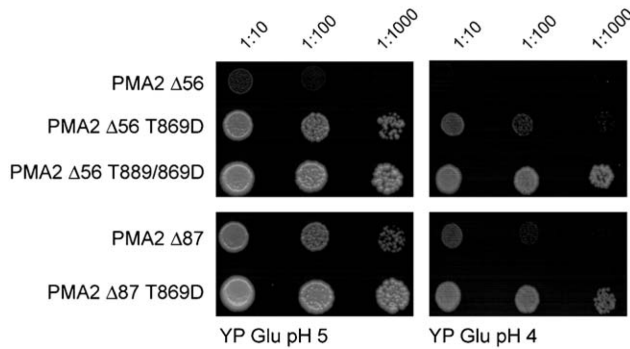

Fig. 3. Growth of YAK2 transformed with mutant PMA2. Each strain was spotted at serial 10-fold dilution onto solid rich medium (pH 5.0 and pH 4.0, respectively), containing glucose as a sole carbon source.

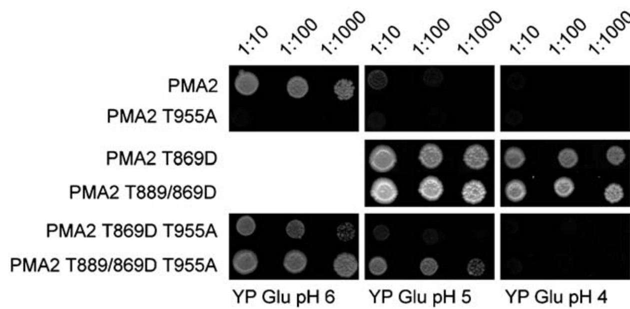

Fig. 4. Growth of YAK2 transformed with wild-type (PMA2) and mutant PMA2. Each strain was spotted at serial 10-fold dilution onto solid rich medium (pH 6.0, pH 5.0 and pH 4.0, respectively), containing glucose as a sole carbon source.

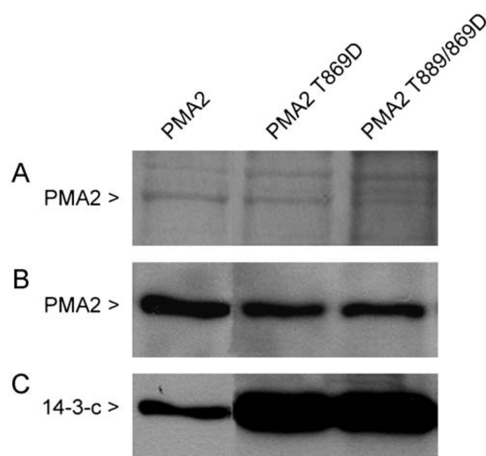

Fig. 5. 14-3-3 protein overlay analysis of plasma membrane proteins of yeast cells expressing wild-type and mutant PMA2. Plasma membrane proteins were electrophoretically separated (8% SDS-PAGE), transferred to nitrocellulose membranes and stained with Coomassie (A) or processed for immunodetection of PMA2 (B) or 14-3-3 protein overlay assay (C).

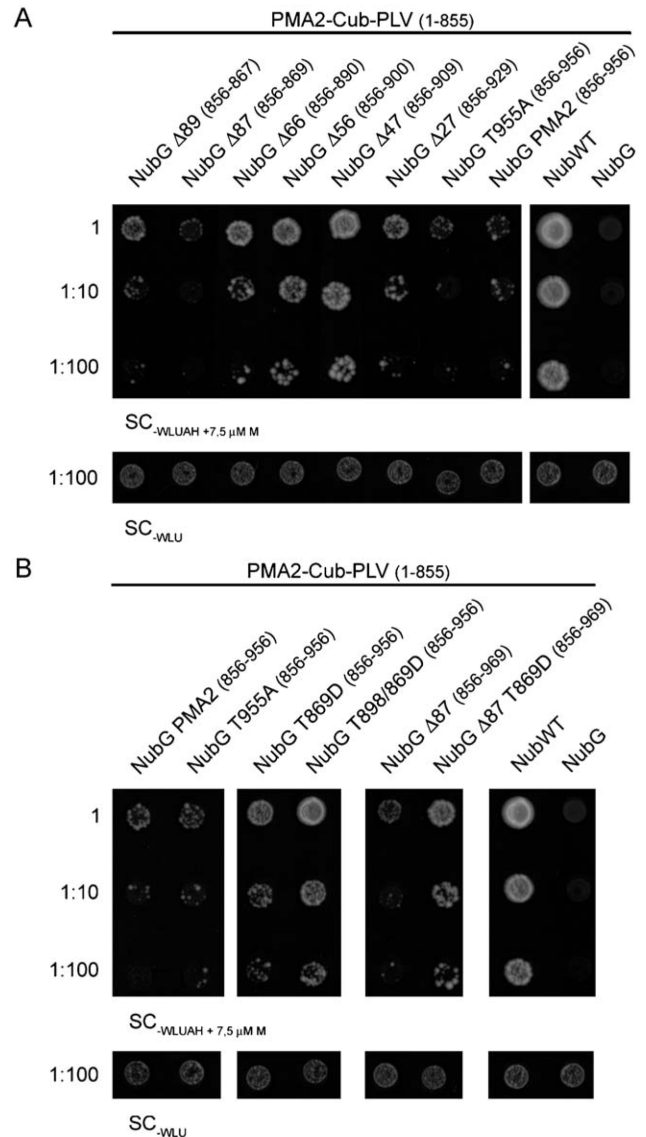

Fig. 6. Analysis of intramolecular interactions of PMA2 C-terminal domains of different lengths (A) and/or bearing mutations of threonine residues (B) with the membrane-embedded rest of the protein by means of mating-based split ubiquitin system assays. The membrane integral part (1–855) and the C-terminal regulatory domains (856 - xxx) of PMA2 were expressed as X-Cub-PLV and NubG-X fusion protein, respectively. Protein–protein interaction causes cleavage of the PLV transcription factor from Cub. PLV activates the reporter genes *ADE2* as well as *HIS3*, expression of which is visualized by growth on selective medium. Diploid cells expressing PMA2-Cub-PLV - NubWT (empty vector) or PMA2-Cub-PLV - NubG (empty vector) were used as positive and negative control, respectively. In each case, the cells were spotted at serial 10-fold dilution onto solid selective medium ($SC_{\text{-WLUAH+0.75 μM M}}$). Growth on $SC_{\text{-WLU}}$ is shown as control.

Notably, even this double exchange does not fully activate PMA2 T955A (Fig. 4), indicating that, although phosphorylation of these residues partly activates the H^+-pump, the major effect is finally mediated by 14-3-3 binding to the inhibitory region C.

Intramolecular interactions of the PMA2 C-terminal domain

Regions I and II have been proposed to interact with the 'rest' of the H^+-ATPase, thereby preventing the action of the cytoplasmic domains known to be essential to sustain the catalytic cycle (Morsomme and Boutry, 2000; Palmgren, 2001; Baekgaard et al., 2005; Duby and Boutry, 2009). In its activated state, however, the C-terminal regulatory domain is thought to adopt a more open structure, swinging away from the important catalytic domains of the enzyme. The fact that the deletion of the entire C-terminal domain leads to a constitutively activated ATPase in yeast (Palmgren and Christensen, 1993) (Δ100, Δ89; Fig. 1), supports this view. However, the activity of PMA2, encompassing most of region I (Δ66), is comparable to PMA2Δ100 while that of PMA2Δ87 is significantly reduced (Fig. 1), raising the question of the respective molecular basis. We therefore analyzed intramolecular interactions by using the mating-based split ubiquitin system (Obrdlik et al., 2004; Grefen et al., 2007, 2009). Adequate yeast strains were transformed, either with various C-terminal deletion constructs of PMA2 (fused to NubG) or the rest of the protein ($PMA2_{1-855}$ fused to Cub-PLV), and subsequently allowed to mate. Interactions were visualized via growth of diploid yeast cells on selection medium containing 0.075 mM methionine (Fig. 6). While both PMA2Δ89 and PMA2Δ66 interacted weakly with $PMA2_{1-855}$, the more inactive PMA2Δ87 did not. Introducing the T869D mutation, interestingly, does not only result in constitutive activity of PMA2Δ87 (Fig. 3) but also in a significant intramolecular interaction with the rest of the enzyme, resembling that of PMA2Δ89/Δ66 (Fig. 6). This indicates that an interaction of this particular region of the C-terminal domain with $PMA2_{1-855}$ positively affects enzyme activity (see Fig. 7). Increasing the length of the C-terminal regulatory domain further increases its capacity to bind to $PMA2_{1-855}$, with PMA2Δ47 showing the major interaction (Fig. 6). Taking the low activity state of PMA2Δ56 into consideration, this might suggest that interaction of region B with the catalytic domains of PMA2 inhibits the H^+-pump while additional binding of further amino acid residues (Δ47) reduces this negative effect (see Fig. 7). However, introducing the T869/889D double mutation into PMA2Δ56 does not significantly influence its interaction capability (data not shown) although the enzyme becomes constitutively active (Fig. 3). This might be due to the combination of an increased interaction of region A and a decreased interaction of region B and reveals the limitations of the experimental system which can only visualize the interaction capacity of the particular C-terminal domain as a whole. Interestingly, however, gradually prolonging the PMA2 C-terminus to the full length (Δ27, WT, T955A) and, thus, effectively shifting the enzyme into its low-activity state (Fig. 1), dramatically decreases its interaction with $PMA2_{1-855}$ (Fig. 6), arguing against the proposed intramolecular interactions of the H^+-ATPase in its inhibited state. This is consistent with the fact that mimicking phosphorylation of either T869 or both threonine residues, which activates PMA2 (Fig. 4), also increases the interaction of the entire C-terminal domain with the rest of the H^+-pump (Fig. 6). Altogether, our data indicate a complex interaction network of individual regions within the C-terminal domain with the membrane-embedded rest of PMA2, either positively or negatively affecting enzyme activity. These interactions can be modulated by phosphorylation of particular residues (T889, T869), shifting the pump to a higher activity level. Moreover, a complete dissociation of the C-terminal domain from the rest of the protein does not seem to be essential for enzyme activation. In contrast, a detectable interaction between the catalytic and the regulatory domains of PMA2 appears to support enzyme activity.

Summary and conclusions

Our data suggest that regions I and II do not inhibit PMA2 in a direct manner but rather impact on the overall conformation of the C-terminal domain. In contrast, we identified three regions that seem to function directly as autoinhibitors (referred to as inhibitory regions A, B and C; the latter being part of the 14-3-3 binding site; see Fig. 7) and that, upon sequential addition, efficiently lock the enzyme in an inactive state. The inhibitory function of these regions can (partly) be reversed by (mimicking) phosphorylation of three specific threonine residues (region A:

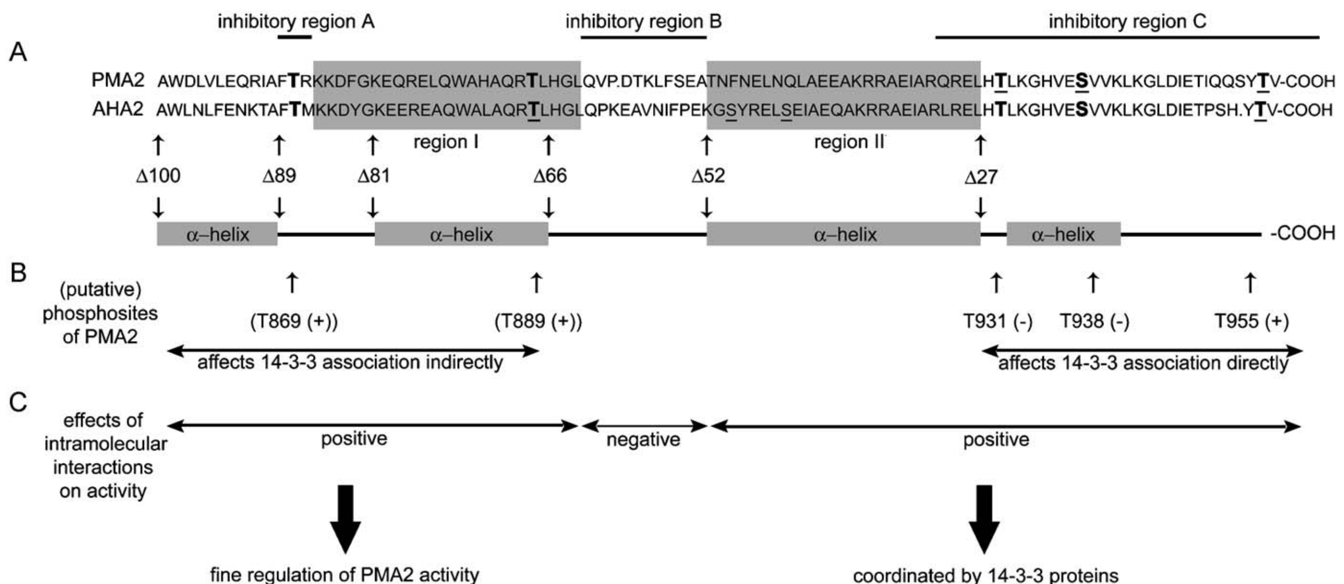

Fig. 7. Schematic overview of the PMA2 C-terminal structure and function of different subdomains in regulation of enzyme activity.

T869, region B: T889, region C: T955, already well known to mediate 14-3-3 binding). The analysis of the corresponding mutants in full-length PMA2 indicates that phosphorylation of most if not all residues within the C-terminal domain finally impacts on 14-3-3 binding, which is required to constitutively activate the H^+-ATPase. Altogether, enzyme regulation might occur in progressive steps, mediated by distinct protein kinases and phosphatases, thus allowing a gradual increase or decrease of activity. The identification of the respective kinases and phosphatases will be a major challenge in the future.

Moreover, in contrast to the prevailing belief, our data indicate that interaction of the C-terminal domain as a whole with the membrane-embedded rest of the H^+-ATPase does not necessarily decrease its activity. A deeper understanding of this interplay will require a high-resolution structure of the entire H^+-ATPase.

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