Autoinhibition of a Calmodulin-dependent Calcium Pump Involves a Structure in the Stalk That Connects the Transmembrane Domain to the ATPase Catalytic Domain*

The regulation of Ca\(^{2+}\)-pumps is important for controlling [Ca\(^{2+}\)]\(_i\) in the cytosol and organelles of all eukaryotes. Here, we report a genetic strategy to identify residues that function in autoinhibition of a novel calmodulin-activated Ca\(^{2+}\)-pump with an N-terminal regulatory domain isoform ACA2 from Arabidopsis. Mutant pumps with constitutive activity were identified by Arabidopsis thaliana domain (isoform ACA2 from type ATPases. The stalk is of general importance in regulating diverse P-type ATPases. Fifteen mutations were found that disrupted a segment of the N-terminal autoinhibitor located between Lys\(^{23}\) and Arg\(^{54}\). Three mutations (E167K, D219N, and E341K) were found associated with the stalk that connects the ATPase catalytic domain (head) and with the transmembrane domain. Enzyme assays indicated that the stalk mutations resulted in calmodulin-independent activity, with V\(_{\text{max}}\)K\(_{\text{mATP}}\) and K\(_{\text{mCa}^{2+}}\) similar to that of a pump in which the N-terminal autoinhibitor had been deleted. A highly conservative substitution at Asp\(^{219}\) (D219E) still produced a deregulated pump, indicating that the autoinhibitory structure in the stalk is highly sensitive to perturbation. In plasma membrane H\(^+\)-ATPases from yeast and plants, similarly positioned mutations resulted in hyperactive pumps. Together, these results suggest that a structural feature of the stalk is of general importance in regulating diverse P-type ATPases.

The regulation of ion transport across membranes is an essential feature of all living cells. In many cases ions are transported against their concentration gradient (i.e. “uphill”) via energy-dependent pumps or cotransporters. The ion pumps belonging to the P-type ATPase superfamily all share a similar enzymatic mechanism with an aspartyl-phosphate intermediate (1). Members include proton pumps, which function to energize the plasma membrane of fungi and plants with an electrochemical potential, and Na\(^+/K\)-ATPases, which provide an analogous function for animal cell plasma membranes. Eukaryotic cells also use P-type ATPases to transport Ca\(^{2+}\), heavy metals, and lipids across different membranes. Many P-type ATPases have been shown to be regulated by autoinhibitors or interacting proteins, with their activation involving a “release of inhibition” (2–5). However, there are no examples in which the mechanisms of inhibition and activation are understood at a structural level (4, 6–9).

The regulation of Ca\(^{2+}\)-pumps is important because their activity can alter the magnitude, duration, and frequency of a Ca\(^{2+}\) signal (10, 11). Here, we investigate the autoinhibition of ACA2, a calmodulin-activated pump that belongs to the type IIB family of Ca\(^{2+}\)-pumps (1, 12). Type IIB pumps include the plasma membrane-localized Ca\(^{2+}\)-ATPase (PMCA), first characterized in animals, and a sub-group of unique Ca\(^{2+}\)-pumps recently identified in plants, including ACA2, which was cloned from a model plant system, Arabidopsis. There are two features that distinguish these plant pumps from an animal PMCA. First, their regulatory domains are located at the N- instead of C-terminal end of the pump. Second, some plant isoforms have been found in non-plasma membrane locations, such as ACA2 (endoplasmic reticulum) (13), and BCA1 (tonoplast) (14). Despite these differences, biochemical and genetic studies on ACA2 and BCA1 suggest mechanisms of autoinhibition and calmodulin activation that are analogous to a PMCA (12, 15–17).

For a PMCA, autoinhibition was proposed to occur through an interaction of a C-terminal autoinhibitor sequence with the first and second cytoplasmic loops (18, 19). Together, these two loops form the ATPase catalytic domain (head), which is connected via a stalk structure to the transmembrane domain through which Ca\(^{2+}\) ions are translocated. A synthetic peptide, C28W, corresponding to the autoinhibitory domain of the human plasma membrane Ca\(^{2+}\)-ATPase (hPMCA4), was found to inhibit hPMCA4 with an IC\(_{50}\) of 20 \(\mu\)M. This peptide was found to interact with residues Ile\(^{206}\)-Val\(^{271}\) and Cys\(^{537}\)-Thr\(^{544}\) located in the first and second cytoplasmic loops, respectively, as revealed by cross-linking studies. This cross-linking experiment has led to

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| Amino acid mutation | Primer | Sequence$^a$ |
|---------------------|--------|--------------|
| D218A               | 207    | TGTGTGGTGTACGCCTAGTCAAGCCTACGCACTCTTGTGAGTTC |
| D219K               | 208    | ACACGTCAGTGACGCAGCCAGCCTTGTGAGTTC |
| D219E               | 209    | ACACGTCAGTGACGCAGCCAGCCTTGTGAGTTC |
| Y220A               | 210    | ACACGTCAGTGACGCAGCCAGCCTTGTGAGTTC |
| R221A               | 211    | ACACGTCAGTGACGCAGCCAGCCTTGTGAGTTC |
| Q222A               | 212    | ACACGTCAGTGACGCAGCCAGCCTTGTGAGTTC |
| R253A               |        | GGAATGCTGAGCCAGCCTACGCACTCTTGTGAGTTC |
| R25A                |        | CTGTGAGAAGGTCCTATCCTGAGTGTGTT |
| K32A                |        | TGTGGCGTGTACGCAGCCTAACGTCGG |
| K35A                |        | GGTGAAGACCCCTACGCCTGGTCTGGTTC |
| R36A                |        | AAGAACCCTAAAACGCAGCCAGGTCGGT |
| R37A                |        | GANCCCTAACGCTGTTCTTTATCAACCC |
| R39A                |        | AACATCCTGTCCTTACGTCAGGTTACCTT |
| K46A                |        | CCTATCCTTTCGCAGCATAGAAGCCTG |
| R47A                |        | GCTAATCCTTTGAAAGCCTATGAGGAGCCTG |
| K54A                |        | GCCGCCGCCAGTGGTCCAGACAAACAGAG |

$^a$ Primers are written 5' to 3', bold letters indicate bases that have been mutated in the ACA2 cDNA, and underlined bases indicate where a silent SpeI restriction site was introduced.

a model in which autoinhibition of a type IIB pump involves intramolecular binding between the autoinhibitor and the ATPase catalytic domain.

Here, we present a genetic selection to identify mutations that disrupt autoinhibition in a representative member of the plant subfamily of type IIB pumps, ACA2. Our genetic approach was made possible by the discovery that only a deregulated version of ACA2 complemented a yeast host K616 (12) grown on calcium-deficient media. K616 lacks a functional Golgi Ca$^{2+}$-ATPase (PMR1), a vacuolar Ca$^{2+}$-ATPase (PMC1), and calcineurin B (20). Complementation appears to require a constitutively active Ca$^{2+}$-pump in the endoplasmic reticulum/Golgi in order to scavenge trace Ca$^{2+}$ for proper functioning of the secretory pathway. Of seven mutants obtained through random mutagenesis, three had mutations predicted to alter the stalk that connects the ATPase to the transmembrane domains. Our results suggest that the stalk may provide a structure that functions in autoinhibition by interacting directly or indirectly with the N-terminal autoinhibitor of ACA2. This regulatory structure may also be important for other P-type ATPases because similarly positioned mutations were also found associated with hyper-activation of proton pumps from plants and yeast (21–23).

**EXPERIMENTAL PROCEDURES**

**Yeast Strain, Transformations, and Growth Media**—The Saccharomyces cerevisiae strain K616 (MATa pmr1::HIS3 pmcl::TRP1 cnu::Leu2, ura3) was used to express ACA2 mutant enzymes (20). For transformations, yeast strain K616 was grown in synthetic drop-out media containing 2% dextrose, 6.7% YNB, 0.67% complete supplemented medium minus histidine (CSM-HIS, Bio 101), 10 mM CaCl$_2$ (C$_{10}$), and 0.5 g/liter MES, pH 6.0. 

A lithium acetate/polyethylene glycol method of yeast transformation was utilized (24), and transformants were selected for their ability to grow in the absence of uracil on plates containing 2% dextrose, 6.7% YNB, 0.67% complete supplemented medium minus URA (Ura$^-$), C$_{10}$, 0.5 g/liter MES, pH 6.0, and 2% agar. For complementation studies, Ura$^-$ colonies were streaked onto plates lacking uracil and containing 10 mM EGTA (E$_{50}$), pH 6.0.

**Random Mutagenesis**—Yeast harboring a wild-type ACA2 cDNA were mutagenized by resuspending a cell pellet in water plus 1% ethyl methane sulphonate (EMS) (v/v) and incubating for 30 min. Cells were washed in 5% sodium thiosulfate and plated on selection media consisting of YNB-U$^-$ E$_{10}$. Plasmids were isolated from the yeast colonies that grew on E$_{10}$, then transformed Escherichia coli (25), and were subsequently sequenced at The Scripps Biochemistry Core Facility using an automated sequencer (Prism 373XL, ABI, Foster City, CA).

**Site-directed Mutagenesis**—Portions of ACA2 were amplified by a two-step PCR process to obtain the desired mutations in a cloneable fragment. First, pYX-ACA2-1 (wild-type ACA2 under the control of a constitutive promoter from triose-phosphate isomerase in a vector containing a Ura$^+$ marker (12)) was used as a template in a PCR reaction using TaKaRa Taq polymerase per the manufacturer's instructions, with primers 207–213 and primer 153 (5'-GACCCAGTGACGTCTCTCCT-3') to obtain the desired point mutation. Table I shows the sequence of the primers used in these reactions. The reaction mix was then cleared of unused dNTPs and salts using the PCR cleanup kit (Qiagen). The entire “cleaned” mix was used in a subsequent PCR step using the products of the first PCR reaction as a mega-primer and primer 153 (5'-GTCCATCGTGGTCTCCG-3') as an upstream primer to amplify an easily subclonable fragment of ACA2 (i.e. from NcoI to HindIII). This reaction was cleaned as before, and the amplified fragment was digested with NcoI and HindIII and subcloned into the pYX-ACA2-1. Potential site-directed ACA2 mutant plasmids were digested with SpeI, and those plasmids that were cut with SpeI were sequenced to ensure that only the desired mutation was present and that the remaining cloned portion was free of PCR-generated errors.

To express the PCR-generated mutations Y220A, R221A, and Q222A in the ACA2 N-terminal truncated background, the NcoI/HindIII fragments of the cDNAs encoding the Y220A, R221A, and Q222A mutant pumps (pYX ACA2-32, -33, -34, respectively) were subcloned into the NcoI/HindIII site of the plasmid containing the N-terminal truncated ACA2 (pYX-ACA2-2, as described previously (12)). The resulting plasmids were expressed in K616, and transformants were streaked onto E10 media for complementation studies.

A directed alanine-scanning mutagenesis of the basic residues in the N terminus (Lys$^{23}$–Arg$^{25}$) was conducted according to the manufacturer's protocol (QuickChange site-directed mutagenesis kit, Stratagene) using primers listed in Table I.

To verify the absence of unintended mutations, all regions of clones derived from PCR modifications were sequenced by automated sequencing procedures at Core Facilities located at The Scripps Research Institute or the Center for Agricultural Biotechnology (University of Maryland).

**Membrane Fractionation**—Yeast membranes were isolated and fractionated as described previously (12). Briefly, yeast was grown in 400 mL of YNB U$^-$ C$_{10}$ media to an approximate A$_{600}$ = 0.8. Cells were pelleted, washed with 20 mL of water, and resuspended in 7.5 mL of homogenization buffer (100 mM Tris, pH 7.5, 20% glycerol, 20 mM EDTA, 1 mM dithiothreitol), 40 g/mL pepstatin, 20 g/mL chymostatin, and 0.4 mM phenylmethylsulfonyl fluoride; protease inhibitors were added fresh). 23 g of glass beads (0.5-mm diameter) were added to the resuspended cells and vortexed for 6 × 1 min, alternating vortexing and incubation on ice. Glass beads and large cell debris were pelleted, and the supernatant was diluted 4-fold in GTED (20% glycerol, 50 mM Tris, pH 7.5, 10 mM EDTA, 1 mM dithiothreitol), and spun at 25,000 rpm (141,000 × g) in a SW28 rotor (Beckman) for 2 h. Membrane pellets were homogenized in GTED + protease inhibitors (50 g/mL pepstatin, 25 g/mL chymostatin, 90 g/mL phenylmethylsulfonyl fluoride) in a glass homogenizer, layered onto a 20–60% continuous sucrose gradient (20–60% sucrose, 50 mM Tris, pH 7.5, 1 mM EDTA, 1 mM dithiothreitol) and spun for 19 h at 30,000 rpm (150,000 × g) in a SW41T rotor (Beckman). Fractions (1 ml) were taken from the top of the gradients, frozen in liquid nitrogen, and stored at −70 °C. The peak fraction of
AACA2p (typically, fraction 5), as indicated by Western blots, was used for ATPase assays.

**Western Blotting and Protein Quantification**—Protein concentration of the individual sucrose fractions was determined by the method of Bradford (26). For SDS-polyacrylamide gel electrophoresis, protein samples were mixed with 0.5% SDS, 0.1% bromophenol blue and incubated for 10 min at 37 °C. Samples were then electrophoresed through an 8% polyacrylamide gel (37:5:1, acrylamide:bisacrylamide, Fisher) and transferred to nitrocellulose using a Bio-Rad transfer apparatus. The transfer buffer consisted of 25 mM Tris, pH 8.3, 192 mM glycine, 20% (v/v) methanol, and 0.12% SDS. After overnight transfer at 150 mA, blots were blocked in blocking buffer (10 mM Tris, pH 7.6, 137 mM NaCl, 0.5% (v/v) Tween 20 (TBS-T), with 5% (w/v) nonfat dry milk) for at least 1 h at room temperature with shaking. The blots were then incubated for 1 h with the primary antibody 1371 raised against the AACA2 protein (12) diluted 1:2000 in blocking buffer. The blots were then washed 4 × 10 min in TBS-T and detection was made using ECL (Amersham Pharmacia Biotech). Following second antibody incubation, the blots were washed 4 times for 10 min in TBS-T, and detection was made using ECL (Amersham Pharmacia Biotech).

**ATPase Assays**—ATPase assays were performed using a modified method of Lanzetta et al. (27). Sucrose fractions enriched in endomembranes (typically fraction 5 from the top, approximately 30% (v/v) sucrose (28)) were diluted to 0.125 μg/μl in GTED, and 10 μl of a diluted fraction was added to the reaction mix to start the reaction. The reaction mix consisted of 90 μl of 20 mM MOPS, pH 7.0, 5 mM MgSO4, 50 mM KNO3 (which inhibits V-ATPase), 1 mM NaMoO4 (inhibits phosphatase), 1 mM EGTA, 1 mM NaN3 (inhibits mitochondrial ATPase), ± 1 mM CaCl2 and ± 100 mM calmodulin. For each assay, a plus vanadate (90 μM sodium orthovanadate) reaction was included and used to determine the vanadate-sensitive ATPase activity. A minus CaCl2 reaction was also included to determine Ca2+-dependent, vanadate-sensitive ATPase activity. Ca2+-stimulated, vanadate-sensitive ATPase activity was determined in the presence and absence of calmodulin. Each reaction was incubated at 22 °C for 40 min and then stopped by the addition of 800 μl of stop solution (8.5 mM (NH4)6Mo7O24·4H2O (99.98%, Aldrich), 0.88 mM malachite green, 1 mM HCl, and 0.04% tertigol (tertigol was added fresh)). After a 8–12-min incubation at room temperature, 100 μl of quenching solution (34% (w/v) trisodium citrate) was added to stabilize the color development. Quenched reactions were incubated for 1 h at 22 °C before measuring the Amax. The amount of phosphate produced in each reaction was determined by comparing the Amax to a standard curve of known phosphate concentrations. A Western blot analysis comparing the amount of mutant enzyme to a dilution series of wild-type enzyme was used to correct for the amount of enzyme added to each ATPase reaction. Therefore, ATPase activity is expressed as the nmol Pi produced/min/unit of enzyme.

**Measurements**—The ATP concentration was varied between 0.3 and 3 mM ATP. For each ATP concentration, an ATP, and a vanadate reaction was included, and the Ca2+-stimulated, vanadate-sensitive activity was determined by subtracting vanadate-sensitive activity in the absence of the Ca2+ from that in the presence of Ca2+. For all KmaxATP measurements, the reaction mix included 2 mM phosphoenolpyruvate and 50 μM/ml pyruvate kinase as an ATP regeneration system. The KmaxATP measurements, [Ca2+]i, measured using fluo-3, pentapotassium salt, and the calcium calibration concentrated buffer kit per the manufacturer’s instructions (Molecular Probes). In brief, a 1 μM fluo-3 stock was made in distilled water, aliquoted, and stored in the dark at −20 °C. A μM fluo-3 was used to construct a standard curve of known [Ca2+]i by exciting the sample at 425 nm and reading the emission at 520 nm in a spectrophotometer. The emission at 520 nm of 425 nm-excited samples containing the ATPase reaction mix and various [Ca2+]i were determined, and the [Ca2+]i value was calculated using the standard curve.

**RESULTS**

**Mutagenesis Reveals Two Regions of the Pump Involved in Autoinhibition**—To select for mutations that deregulate AACA2 activity, yeast K616 expressing a wild-type ACA2 were mutagenized with EMS and plated on Ca2+-deficient media. From more than 10,000 colonies that survived an initial selection (E10-resistant), an estimated 2,000 (20%) harbored a deregulated ACA2 mutant. Most of the initial survivors failed to grow after continued passage on E10 media. Of those that survived multiple selections, more than 50% were found to have mutations linked to a plasmid encoding an ACA2 pump, as shown by the isolation of the plasmid and its reintroduction into a K616 host. A sequence analysis of 81 independent ACA2 mutants revealed only seven different point mutations (Fig. 1, Table II), four of which occurred in the putative autoinhibitor domain (R36H, R37W, R39P, R47P), whereas the remaining three occurred in the stalk region (E167K, D219N, and E341K).

The D219N mutation was found in 80% of the sequenced clones (Table II). This mutation was always observed as a nucleotide change from G to A in the context of dACT AGC (G)>AAT TAC CGC. Because this mutation occurred with such high frequency, we set up multiple screens to look selectively for mutations downstream of this Asp219. For example, from a pool of approximately 2000 potential mutants, plasmid DNA was isolated, and DNA fragments containing potential mutations in the C-terminal portion of the pump downstream of Asp219 were subcloned into a wild-type ACA2 background. This subcloned DNA was transformed back into yeast, and the yeast were subjected to selection on E10 media. Of thousands of transformed yeast, only one survived selection and was subsequently shown to harbor a mutant pump with an E341K mutation. Three independent searches with this strategy confirmed that mutations that are located downstream of Asp219 and confer deregulation were generated at a very low frequency by EMS mutagenesis. Because the N-terminal domain was previously shown to contain an autoinhibitor, it was not surprising to find four mutations in this region produced by random EMS mutagenesis. To obtain more mutations in this region, we selectively mutagenized the N-terminal autoinhibitory region through mutagenic PCR and site-directed Ala scanning mutagenesis (Table II). Mutagenic PCR yielded 3 additional point mutations that conferred E10 resistance, N33D, T41P, and A42D. The directed alanine substitutions of all 9 basic residues between Lys23 and Arg54 revealed that all but 2 (K35A and K46A) directed alanine substitutions of all 9 basic residues between Lys23 and Arg54 revealed that all but 2 (K35A and K46A) resulted in approximately equal growth of K616 on Ca2+-deficient media. From those that survived multiple selections, more than 50% were found to have mutations linked to a plasmid encoding an ACA2 pump, as shown by the isolation of the plasmid and its reintroduction into a K616 host. A sequence analysis of 81 independent ACA2 mutants revealed only seven different point mutations (Fig. 1, Table II), four of which occurred in the putative autoinhibitor domain (R36H, R37W, R39P, R47P), whereas the remaining three occurred in the stalk region (E167K, D219N, and E341K).

Three Stalk Mutations Result in Constitutively Active Pumps—All three stalk mutants (E167K, D219N, and E341K) resulted in approximately equal growth of K616 on Ca2+-deficient media (Fig. 2A). Their expression levels in K616 were slightly lower than a wild-type ACA2 control (Fig. 2B), indicating that complementation was not an artifact of a pump simply being over-expressed. To determine whether the mutant pumps were deregulated, membranes were partially purified from yeast and their Ca2+-stimulated, vanadate-sensitive ATPase activities determined in the presence and absence of calmodulin (Fig. 2B). All three stalk mutants showed a high constitutive level of ATPase activity that was insensitive to calmodulin stimulation. Their specific activities, when normalized per unit of wild-type pump, were comparable with the deregulated pump created by an N-terminal truncation of the autoinhibitor (ACA2–2N) (12, 16). The KmaxATP, KmCa2+, and catalytic efficiencies (Vmax/Km) of these mutant enzymes were measured and compared with truncated (∆N) and wild-type pump controls (Table III). In all cases, the stalk mutants, like the truncated enzyme, showed kinetic parameters most similar to the fully activated state of a wild-type enzyme. Interestingly, each of the stalk mutants

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**Fig. 1.** Diagram showing the predicted topology of ACA2 and the mutations investigated in this study. Each circle represents a single amino acid residue. Mutations resulting in deregulated pumps are shown as **black** circles, and those that retain a wild type-like activity are shown as **black boxes**. The autoinhibitor is shown as a **shaded box**. The **bold P** indicates the conserved Asp residue that forms the phosphoenzyme intermediate. The **open boxes** in the cytosolic domains represent sequences homologous to the two domains in hPMCA4 that interact with the autoinhibitor. Predicted \(\alpha\)-helical structures are shown as groups of **angled rows** of three residues. In the text, the ATPase catalytic domain refers to the first two cytosolic loops. **ER**, endoplasmic reticulum.

showed a different catalytic efficiency with respect to ATP, with D219N being the highest at “287,” whereas E341K was more than two-fold lower at “120”; the mutant E167K at “226” was most similar to the truncated deregulated enzyme. In contrast, the catalytic efficiency with respect to Ca\(^{2+}\) was similar for all three stalk mutants, but at a level 30% lower than the truncated pump. Thus, all three stalk mutations resulted in deregulated pumps but were nevertheless distinguished from each other by different kinetic parameters.

**Mutation D219E Results in a Deregulated Pump**—The remainder of this study focused on mutations in the immediate vicinity of the Asp\(^{219}\) position. This position was considered of the highest priority for further investigation because alignments of different P-type ATPases indicated that this position was the most highly conserved among all pumps transporting Ca\(^{2+}\), H\(^+\), or Na\(^+\)/K\(^+\) (28). To determine whether the D219N mutation resulted in a deregulated pump because of the loss of the Asp or the gain of an Asn, site directed mutagenesis was performed to replace Asp\(^{219}\) with mutations D219A, D219E, and D219K. These mutant pumps all provided complementation when expressed in K616 (data not shown). In vitro ATPase assays confirmed that each mutant pump showed calmodulin-independent ATPase activity comparable with the activity of the calmodulin-stimulated wild-type enzyme (Fig. 3). Since even a conservative substitution of D219E resulted in a deregulated pump, autoinhibition of ACA2 appears to have a strict requirement for an Asp at the Asp\(^{219}\) position.

**Autoinhibition Is Not Disrupted by Three Ala Substitutions Downstream of Asp\(^{219}\)**—To determine whether the stalk region immediately downstream of Asp\(^{219}\) is also involved in autoinhibition, the three neighboring residues were independently substituted (Y220A, R221A, Q222A). All three mutations failed to generate pumps conferring E\(_{10}\) resistance in yeast host K616 (data not shown). A control showed that the same mutations introduced into a truncated pump (\(\Delta N\)) still allowed these pumps to function (i.e. provide E\(_{10}\) resistance), indicating that the mutations did not merely create “dead pumps”. A normal ATPase activity was subsequently confirmed by in vitro assays on each full-length mutant (Fig. 3). Thus, the Asp\(^{219}\) position alone at the beginning of stalk segment 2 represents a highly sensitive position in a regulatory structure.

**DISCUSSION**

**Selection of Deregulated Ca\(^{2+}\)-ATPase Mutants in Yeast**—We have used a molecular genetic approach to examine the structure and function of ACA2, a representative member of a unique subfamily of calmodulin-dependent Ca\(^{2+}\)-pumps with an N-terminal autoinhibitor. The results presented here demonstrate that complementation of the yeast strain K616, deficient in two endogenous Ca\(^{2+}\)-pumps, can be used to select for random mutations that constitutively activate ACA2. Using this strategy, we identified three residues in the stalk domain that play a critical role in autoinhibition. The power of this genetic approach is illustrated by the fact that despite an extensive literature on the structure and function of Ca\(^{2+}\)-pumps, there was no a priori reason to suspect the identified stalk residues as part of a regulatory structure.

Prior studies on Ca\(^{2+}\)-pumps (mainly animal SERCA) have analyzed hundreds of rationally designed mutations (28, 29). However, a severe limitation of these studies has always been the laborious requirement that each mutant be individually expressed and biochemically characterized, which was further complicated by the fact that suitable expression systems all contained endogenous Ca\(^{2+}\)-pump activities. Here, we demonstrate the efficacy of (i) a genetic strategy to select for a deregulated pump from millions of potential mutants by functional expression in yeast strain K616, and (ii) a rapid biochemical assay to determine a mutant pump’s activity in yeast membranes lacking endogenous Ca\(^{2+}\)-ATPase activity.

Our genetic strategy appears to be applicable to analyzing...
other divergent members of the type IIB Ca\textsuperscript{2+}-pump family. Although complementation of K616 appears to require Ca\textsuperscript{2+} transport into the endoplasmic reticulum/Golgi system, it is notable that complementation has been reported for mutant (deregulated) pumps related to ACA2 that normally target to other locations, including the plasma membrane or tonoplast in plant cells (5, 30). It is likely that these non-endoplasmic reticulum pumps nevertheless accumulate to sufficient levels in the yeast secretory pathway to provide the necessary activity in this non-native location. Whether a deregulated animal PMCA will function in this system has not yet been reported.

Functional complementation has also been reported for other endomembrane calcium pumps, including ECA1 from Arabidopsis (31), SERCA1a from rabbit (32), and SMA2 from Schistosoma mansoni (33). However, these pumps differ from the type IIB pumps described above in that they (i) do not have calmodulin-regulated autoinhibitors and (ii) provide complementation in yeast when expressed as wild-type enzymes. As delineated by mutations, the N-terminal autoinhibitor of ACA2 encompasses nearly all of a peptide sequence corresponding to Val\textsuperscript{200}-Leu\textsuperscript{204}, which was previously shown to function as a peptide inhibitor of ACA2 (16). This peptide inhibited Ca\textsuperscript{2+} trans-

formed here were limited.

As delineated by mutations, the N-terminal autoinhibitor encompasses nearly all of a peptide sequence corresponding to Val\textsuperscript{200}-Leu\textsuperscript{204}, which was previously shown to function as a peptide inhibitor of ACA2 (16). This peptide inhibited Ca\textsuperscript{2+} transport by a wild-type ACA2 and a constitutively active, N-terminally truncated ACA2 (\textDelta N). In addition, it inhibited the activity of ECA1, a type IIA pump that is more closely related to the SERCA pumps in animals. Similarly, an inhibitory peptide (C28W) derived from the analogous C-terminal autoinhibitor of a human PMCA (hPMCA4) was also shown to inhibit a SERCA pump. Together, these results suggest that both N- and C-terminal autoinhibitors of type IIB pumps function by a similar mechanism, presumably by interacting with regions of the pump that are structurally conserved in distinctly related type IIA pumps (e.g. SERCA, ECA1).

It is unclear how any of the mutations found here actually disrupt autoinhibition. We suspect that some substitutions pro-

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|}
\hline
Mutation & K616 growth on \textit{E}_{10} & Biochemistry & Mutagenesis \\
\hline
Wild type\textsuperscript{d} & No & CaM-dependent (A,T) & None \\
\Delta 2–80\textsuperscript{e} & Yes & Deregulated (A, T) & Directed \\
R221A & Yes\textast & ND & Directed \\
R225A & Yes & ND & Directed \\
R232A & Yes & ND & Directed \\
R352A & No & ND & Directed \\
N33D & Yes & ND & Directed \\
R360A & Yes & ND & Directed \\
R36H & Yes & ND & EMS (2) \\
R37A & Yes & ND & Directed \\
R37W & Yes & ND & EMS (4) \\
R39A & Yes & ND & Directed \\
R39P & Yes & ND & EMS (1) \\
T41P & Yes & ND & M-PCR \\
A42D & Yes & ND & M-PCR \\
K46A & No & ND & Directed \\
R47A & Yes & ND & Directed \\
R47P & Yes & EMS (1) & Directed \\
R54A & Yes\textast & ND & Directed \\
E167K & Yes & Deregulated (A) & EMS (6) \\
E341K & Yes & Deregulated (A) & EMS (2) \\
D219N & Yes & Deregulated (A) & EMS (65) \\
D219A & Yes & ND & Directed \\
D219E & Yes & Deregulated (A) & Directed \\
D219K & Yes & Deregulated (A) & Directed \\
Y220A & No & CaM-dependent (A) & Directed \\
\textDelta \text{N} + Y220A & Yes & ND & Directed \\
R221A & No & CaM-dependent (A) & Directed \\
\textDelta \text{N} + R221A & Yes & ND & Directed \\
Q222A & No & CaM-dependent (A) & Directed \\
\textDelta \text{N} + Q222A & Yes & ND & Directed \\
\hline
\end{tabular}
\caption{ACA2 mutations investigated in this study}
\end{table}
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A comparison of the $K_{\text{m,ATP}}$, $K_{\text{m,calmodulin}}$, and the catalytic efficiencies of wild-type ACA2, N-terminal truncated ACA2 (ΔN), and the three stalk domain mutants

| Enzyme     | Calmodulin |
|------------|------------|
|            | $K_{\text{m,ATP}}^a$ | $K_{\text{m,calmodulin}}^a$ | Catalytic efficiency$^b$ |
| Wild-type  | + 0.77 ± 0.04 (n = 2) | 1.0 ± 0.2 (n = 2) | 167 ATP | 128 Ca$^{2+}$ |
| Wild-type  | 0.52 ± 0.03 (n = 2) | ND$^c$ | 60 ATP | ND$^c$ |
| ΔN         | 0.79 ± 0.16 (n = 3) | 0.43 ± 0.14 (n = 4) | 219 ATP | 403 Ca$^{2+}$ |
| ACA2-D219N | 0.34 ± 0.17 (n = 5) | 0.35 ± 0.06 (n = 4) | 257 ATP | 279 Ca$^{2+}$ |
| ACA2-E167K | 0.55 ± 0.03 (n = 4) | 0.48 ± 0.11 (n = 3) | 226 ATP | 273 Ca$^{2+}$ |
| ACA2-E441R | 0.77 ± 0.25 (n = 5) | 0.31 ± 0.06 (n = 4) | 120 ATP | 298 Ca$^{2+}$ |

$^a$ Values represent the mean of at least two independent $K_{\text{m}}$ measurements ± the respective standard deviations. $n$ = number of independent $K_{\text{m}}$ measurements.

$^b$ Catalytic efficiency ($V_{\text{max}}/K_{\text{m}}$) was determined for each enzyme using the means of $V_{\text{max}}$ and $K_{\text{m}}$.

$^c$ ND, could not be accurately determined by ATPase assay used here.

Nevertheless, the overall kinetic parameters of these stalk mutants are most similar to the fully activated state, as opposed to the basal state, of a wild-type pump.

Site-specific mutagenesis of Asp$^{219}$ and neighboring residues revealed three additional insights. First, the three residues downstream of Asp$^{219}$ can be replaced by alanines without a detectable disruption of autoinhibition or loss of calmodulin-stimulated activity, as shown by complementation studies and enzyme assays (Fig. 3). Second, the loss of Asp at the Asp$^{219}$ position, as opposed to gain of an Asn, is responsible for deregulation, is indicated by the deregulated activity of the mutant containing a D219A substitution (Fig. 3). Third, the Asp itself is uniquely required at this position and cannot be replaced by a highly conservative substitution of Gln (Fig. 3); this is significant because Asp and Gln differ by only a single carbon in the length of their acidic side chains.

To our knowledge, this is the first study to show a regulatory role for a residue corresponding to position Asp$^{219}$ in any Ca$^{2+}$-pump. Of the three acidic residues identified here as stalk mutations, the Asp$^{219}$ position appears to be the most highly conserved in P-type ATPases, with a corresponding acidic residue present in PMCA and SERCA, as well as proton pumps and Na$^+$/K$^+$-ATPases (28). Although a mutation of a PMCA at this corresponding position has not been reported, the analogous position has been mutated in a rabbit fast twitches SERCA pump without a detectable effect (E109A) (34). Likewise, positions corresponding to the other two ACA2 stalk mutations were also mutated in a SERCA pump (E55Q, E243A, E244A, and D245A) without a detectable effect (34). However, these SERCA pump studies did not evaluate whether the mutations might disrupt an inhibitory interaction by phospholamban and therefore did not address the question of whether the stalk domain of a SERCA might be involved in regulation.

Similarities to Other P-type ATPases—There has been speculation that P-type ATPases may utilize similar mechanisms of inhibition regardless of whether the inhibition is accomplished by an autoinhibitor or a separate interacting protein (5, 35). Nevertheless, there are many potential ways in which a pump could be inhibited, and there are no clear structural paradigms for how any P-type ATPase is regulated.

The PMCA’s of animals and the plasma membrane proton pumps of plants and fungi represent the two best studied examples of autoinhibition of a P-type ATPase (2, 4, 21–23). Although the autoinhibitors of these pumps are all located at the C-terminal end, domain swapping in a PMCA indicated that its autoinhibitor was still partially active in an N-terminal location, suggesting that the mechanism of autoinhibition was very tolerant of structural rearrangements (36). Thus, the unique location of an N-terminal autoinhibitor in members of a subfamily of ACA2-like pumps does not preclude the possibility that both N- and C-terminal located autoinhibitors function by
a similar mechanism.

In genetic studies similar to those conducted here for ACA2, proton pumps from a tobacco plant (NpPMA2 (21, 22)) and yeast (ScPMA1 (23)) were also found to become hyperactivated as a result of mutations located in both the autoinhibitory domain and core regions of the pump. The relative positions of mutations found in the core regions of NpPMA2 and ScPMA1 are shown in Fig. 4 in comparison with the stalk mutations found in ACA2. Six of the 20 proton pump mutations were found in stalk segments 1, 2, and 3, in the same general vicinity as the three mutations found in ACA2. Thus, at least some proton and calcium pumps appear to have a structure involved in autoinhibition that can be perturbed by mutations located in the stalk.

The stalk mutations in the tobacco proton pump were identified by a genetic screen for pumps that could complement a deletion of the yeast plasma membrane proton pump when cells were grown under low pH growth conditions (21, 22). These mutant pumps were all shown subsequently to have increased specific activities, which correlated with a structural change causing the C-terminal autoinhibitory domain to be more sensitive to trypsin digestion. This structural change is consistent with the hypothesis that the mutations resulted in the displacement of the C-terminal autoinhibitor from its site of inhibition.

The mutations in the yeast proton pump PMA1 were identified by a genetic screen for second site mutations that reversed the inhibitory effect of a primary mutation in the C-terminal autoinhibitor (23). As with the tobacco pump, a small number of mutations were located in the stalk. The two mutations found nearest to a position corresponding to Asp$^{219}$ in the ACA2 calcium pump were A165V and VD170IN, predicted to be two and six residues downstream from an acidic residue that aligns with Asp$^{219}$ (28). The effect on enzyme activity of these second site mutations was examined in the absence of the primary mutation. In membranes harboring these mutant pumps, proton ATPase activity showed a $V_{\text{max}}$ approximately 5–10-fold higher than membranes containing wild-type enzyme. This apparent increase in activity supports the hypothesis that growth of yeast harboring the mutant pumps was because of a dominant mutation giving rise to a deregulated hyperactive pump.

During the reaction cycle of a P-type ATPase, the stalk domain plays a critical role in the transduction of conformational changes in the ATPase catalytic domain into conformational changes in the transmembrane helices that translocate ions through the channel domain (18). It is likely that the stalk domain also contributes to the structure of a pore through which ions enter the channel domain. Thus, there are multiple features of the stalk that could provide potential points of regulation. Which of these features is regulated by the N-terminal autoinhibitor in ACA2 is not known.

We offer two models for how the stalk may be involved in the autoinhibition of ACA2 (Fig. 5). In both models the N-terminal autoinhibitor “clamps” the enzyme in a non-active conformation that reduces activity by (i) blocking substrate binding sites (i.e. ATP or Ca$^{2+}$) or by (ii) preventing the relay of a conformational change between the ATPase catalytic domain and the channel domain. The primary distinguishing feature of the two models is the hypothesis that the autoinhibitor interacts directly with the ATPase catalytic domain in Model I, as suggested by cross-linking studies with the animal PMCA, compared with Model II in which the interaction is only with the stalk, at a position corresponding to the Asp$^{219}$ mutation identified here in ACA2. If Model I is correct (“loop interaction”), the mechanism by which the ACA2 stalk mutations deregulate the pump could be explained by a long distance conformational change that disrupts the docking of the autoinhibitor with the ATPase catalytic domain. If Model II is correct (“stalk interaction”), the mechanism of deregulation could be explained by a local change in conformation that directly disrupts an autoinhibitor binding site located in the stalk. Interestingly, all three stalk mutations identified here disrupted acidic residues, whereas eight of the mutations in the N-terminal autoinhibitor neutralized basic residues, consistent with the potential importance of an electrostatic interaction between these two regions.

In conclusion, the ability to select for active Ca$^{2+}$-pumps in the yeast host K616 provides a powerful approach in which random mutagenesis can be used to dissect the mechanism of autoinhibition and calmodulin activation of type-IIB Ca$^{2+}$-pumps. The discovery here of stalk mutations that deregulate ACA2 provides the first evidence for a regulatory structure associated with this region of a Ca$^{2+}$-pump. This observation, together with the findings of analogous mutations in proton pumps, provides genetic evidence supporting a similar structural basis for autoinhibition among two very distantly related P-type ATPases.
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