A High-Affinity Ca^{2+} Pump, ECA1, from the Endoplasmic Reticulum Is Inhibited by Cyclopiazonic Acid but Not by Thapsigargin[1]

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To identify and characterize individual Ca^{2+} pumps, we have expressed an Arabidopsis *ECA1* gene encoding an <u>e</u>ndoplasmic reticulum-type <u>C</u>a^{2+}-<u>A</u>TPase homolog in the yeast (*Saccharomyces cerevisiae*) mutant K616. The mutant (*pmc1pmr1cnb1*) lacks a Golgi and a vacuolar membrane Ca^{2+} pump and grows very poorly on Ca^{2+}-depleted medium. Membranes isolated from the mutant showed high H^+/Ca^{2+}-antiport but no Ca^{2+}-pump activity. Expression of ECA1 in endomembranes increased mutant growth by 10- to 20-fold in Ca^{2+}-depleted medium. $^{45}Ca^{2+}$ pumping into vesicles from *ECA1* transformants was detected after the H^+/Ca^{2+}-antiport activity was eliminated with bafilomycin A_1 and gramicidin D. The pump had a high affinity for Ca^{2+} ($K_m = 30$ nM) and displayed two affinities for ATP (K_m of 20 and 235 μM). Cyclopiazonic acid, a specific blocker of animal sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase, inhibited Ca^{2+} transport (50% inhibition dose = 3 nmol/mg protein), but thapsigargin (3 μM) did not. Transport was insensitive to calmodulin. These results suggest that this endoplasmic reticulum-type Ca^{2+}-ATPase could support cell growth in plants as in yeast by maintaining submicromolar levels of cytosolic Ca^{2+} and replenishing Ca^{2+} in endomembrane compartments. This study demonstrates that the yeast K616 mutant provides a powerful expression system to study the structure/function relationships of Ca^{2+} pumps from eukaryotes.

The role of Ca^{2+} in signaling and development is well recognized in eukaryotes (Bush, 1995; Clapham, 1995); however, the regulation of cytosolic and organellar Ca^{2+} in plants is still poorly understood. In plant cells the cytosol usually contains 30 to 600 nM free Ca^{2+} (Reed et al., 1992), whereas the cell wall and intracellular stores, such as the ER and vacuole, contain 1,000- to 10,000-fold higher concentrations. The Ca^{2+} gradients across the PM and the intracellular membranes are energized by primary Ca^{2+}-ATPases and by H^+-coupled Ca^{2+} antiport (Hirschi et al., 1996). A variety of signals induces transient increases in cytosolic Ca^{2+} because of the opening of specific Ca^{2+} channels (Bush, 1995). Recent studies with Ca^{2+}-indicator dyes demonstrated that the perception of a nodulation signal results in Ca^{2+} waves or oscillations in root-hair cells of alfalfa (Ehrhardt et al., 1996). In frog oocytes inositol 1,4,5-trisphosphate induced repetitive Ca^{2+} waves; however, the frequency of the waves increased in cells overexpressing a SER Ca^{2+} pump (Camacho and Lechleiter, 1993). Because the SERCA pumps cytosolic Ca^{2+} into endomembrane compartments, intracellular Ca^{2+} pumps could be an important factor in controlling Ca^{2+} oscillations in plant and in animal cells.

Ca^{2+} is also essential for tip growth of pollen tubes. A recent study showed that the tip-focused intracellular $[Ca^{2+}]$ gradient oscillates with the same period as growth (Holdaway-Clarke et al., 1997). High levels of Ca^{2+} in intracellular compartments serve a variety of essential functions. This divalent cation is a cofactor for specific enzymes (Bush et al., 1989) and can bind to several chaperones in the ER (Bergeron et al., 1994). Thus, endoluminal Ca^{2+} supplied by Ca^{2+} pumps on the ER, Golgi, and secretory vesicles could affect processing and sorting and determine the ultimate fate of membrane and secreted proteins (Rudolph et al., 1989). Furthermore, nuclear cisternal Ca^{2+} controls nuclear pore permeability and thus regulates transport across the nuclear envelope (Perez-Terzic et al., 1997).

Although high-affinity, Ca^{2+}-pumping ATPases have been identified in a variety of membranes, including the PM, ER, and tonoplast, the characterization of individual pumps separate from other related pumps has been difficult in many plant studies. Extensive biochemical studies demonstrated that plants have two major types of Ca^{2+} pumps with distinct properties. The PM-type Ca^{2+}-ATPase is stimulated by calmodulin (Bonza et al., 1998), whereas the ER type is not (Bush, 1995; Hwang et al., 1997). However, unlike the PM-bound Ca^{2+} pumps from animals, the PM-type Ca^{2+}-ATPase is localized to several membranes in plants (Askerlund, 1997; Hwang et al., 1997), suggesting a family of calmodulin-stimulated pumps. Several genes encoding Ca^{2+}-ATPase homologs from plants have been isolated. Based on similarities of the deduced amino acid sequence with animal Ca^{2+} pumps, LCA1 from tomato

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Abbreviations: BTP, 1,3-bis[tris(hydroxymethyl)methylamino]-propane; PM, plasma membrane; SC-URA, synthetic complete medium minus uracil; SER, sarcoplasmic/ER; SERCA, SER Ca^{2+} ATPase.

(Wimmers et al., 1992) and a gene from rice (Chen et al., 1997) encoded ER-type Ca^{2+} pumps. PEA1 from Arabidopsis (Huang et al., 1993) and BCA1 from cauliflower (Malmstrom et al., 1997) encoded a PM-type Ca^{2+}-ATPase homolog. Except for BCA1, the biochemical activities of these gene products have not been demonstrated.

To study individual Ca^{2+} pumps, we recently identified two distinct Ca^{2+}-ATPases by functional expression of two plant genes in a yeast triple mutant. One cDNA (*ECA1*) encoded an ER Ca^{2+}-ATPase homolog from Arabidopsis (Liang et al., 1997), and another gene (*ACA2*, accession no. AF025842) encoded a PM-type Ca^{2+}-ATPase with a unique N-terminal regulatory domain (Harper et al., 1998). *ECA1* encoded a 116-kD polypeptide that was more homologous to animal SERCA than to PM-type Ca^{2+} pumps. When *ECA1* was expressed in a yeast triple mutant defective in both a Golgi and a vacuolar Ca^{2+} pump (*pmr1pmc1cnb1*) (Cunningham and Fink, 1994), growth of this mutant in Ca^{2+}-depleted medium was restored. Furthermore, ECA1 could be phosphorylated in a Ca^{2+}-dependent manner in vitro similarly to phosphoenzyme intermediates formed in the reaction cycle of Ca^{2+}-ATPases. Therefore, *ECA1* encoded a Ca^{2+}-dependent ATPase (Liang et al., 1997); however, we were unable to demonstrate Ca^{2+}-pumping activity because of high activity from the Ca^{2+} antiporter (Vcx1).

Here we provide the first biochemical characterization, to our knowledge, of a plant Ca^+ pump functionally expressed in yeast. We show that *ECA1* encodes a high-affinity plant Ca^{2+} pump that is blocked by cyclopiazonic acid. ECA1 shares many similarities with animal SER-type Ca^{2+}-ATPases; however, it is unique in its insensitivity to thapsigargin. We also demonstrate that a yeast triple mutant provides a powerful expression system with which to study individual Ca^{2+} pumps from heterologous systems.

MATERIALS AND METHODS

Yeast Strain, Plasmid, and Growth Medium

Yeast (*Saccharomyces cerevisiae*) strain K616 (*MATa pmr1::HIS3 pmc1::TRP1 cnb1::LEU2, ura3*; Cunningham and Fink, 1994) is often referred to as the triple mutant. The full-length cDNA of the *ECA1* gene (accession no. U96455) from Arabidopsis was constructed into the yeast expression vector p426Gal1 under the control of the Gal-inducible promoter (Liang et al., 1997). The mutant K616 was transformed with this construct or the empty vector using the lithium acetate method (Chen et al., 1992). Transformants were selected on SC-URA. The growth medium consisted of 6.7 g/L yeast nitrogen base without amino acids, 2 g/L drop-out mixture without uracil, and 2% Gal (Rose et al., 1990).

Yeast Growth

To measure the growth of mutant K616 strains transformed with either *ECA1* or with vector alone, cells at the late-log phase were harvested by centrifugation and suspended in 10 mL of SC-URA that contained 1 mM Ca^{2+}.

The cell suspension was used to inoculate 20 mL of SC-URA (pH 6.2) to an initial A_{600} of 0.01. Either 10 mM Ca^{2+} or varying concentrations of EGTA was added to the medium to control free-Ca^{2+} levels. Growth at 30°C was monitored by the change in A_{600} of 0.8-mL samples for 2 d.

Isolation of Membrane Vesicles

Vesicles were isolated after cell disruption using the glass-bead method (Liang et al., 1997) with some modification. Transformants were inoculated into 25 mL of SC-URA and incubated overnight at 30°C. The seed culture was diluted into 250 to 300 mL of SC-URA/Gal and incubated until the A_{600} reached 1.5 to 2.0. Cells were pelleted at 4000g for 5 min, washed with 10 mL of distilled water, and pelleted. To isolate vesicles for transport studies, 2 mM $MgCl_2$ was included in all of the solutions to facilitate separation of the ER from the vacuolar vesicles (see below). The cell pellet was suspended in 10 mL of glass-bead buffer and pelleted. The glass-bead buffer consisted of 10% Suc, 25 mM Hepes-BTP, pH 7.5, 2 mM $MgCl_2$, 2 mM DTT, and 1 mM EGTA.

Typically, 3 to 4 mL of cells was resuspended in 1 volume of glass-bead buffer plus 1 mM PMSF, 10 mM benzamidine, 5 μg/mL pepstatin, 5 μg/mL leupeptin, and 0.5% BSA, and split into two Corning tubes (50 mL). An equal volume of glass beads (Sigma) was added and the mixture was vortex mixed four times for 30 s each. The lysate was centrifuged at 5,000g for 5 min and the supernatant was saved. The pellet was suspended in 1 volume of glass-bead buffer plus protease inhibitors, vortex mixed, and centrifuged as described above. Then, 2 to 3 mL of the pooled supernatant was layered onto a step gradient containing 6 mL each of 25% and 45% Suc in 20 mM Hepes-BTP (pH 7.0), 1 mM DTT, 2 mM $MgSO_4$, 0.2 mM PMSF, and 5 mM benzamidine, and centrifuged (model SW 28 centrifuge, Beckman) at 108,000g for 2 h. Membranes at the 26%/45% Suc interface were collected and diluted 6- to 8-fold in a suspension solution containing 25 mM Hepes-BTP (pH 7.0), 1 mM DTT, 2 mM $MgSO_4$, and protease inhibitors. After the sample was centrifuged at 108,000g for 50 min, the pellet was suspended in the same solution and stored at −80°C. The protein concentration was determined with the Bio-Rad reagent.

To determine the distribution of ECA1 in yeast membranes, microsomes were isolated in the presence or absence of Mg^{2+}. About 0.5 mL of cells from 50 mL of overnight culture was suspended in 1 volume of glass-bead buffer with either 2 mM $MgSO_4$ or 2 mM EDTA. The glass-bead buffer included 0.5 mM PMSF, 2 mM benzamidine, 5 μg/mL pepstatin, 5 μg/mL leupeptin, and 0.5% BSA. Cells were disrupted with the buffer as described above. The lysate was centrifuged at 5,000g for 5 min and the supernatant was saved. The pellet was suspended in glass-bead buffer, vortexed, and centrifuged as described above. The supernatants were pooled and pelleted at 108,000g for 50 min. The microsomal pellet was resuspended in 0.8 mL of the above solution without BSA and layered onto a step gradient with 1.2 mL each of 12%, 15%, 18%, 21%, 24%, 27%, 30%, 33%, 36%, 39%, 42%, and 45% Suc. The Suc

solutions contained 25 mM Hepes-BTP, pH 7.0, 1 mM DTT, 0.1 mM PMSF, and 2 mM benzamidine with either 2 mM MgSO$_4$ or 2 mM EDTA. After the sample was centrifuged at 110,000g for 16 h, 0.75-mL fractions were collected and stored at $-80°$C.

^{45}Ca^{2+} Uptake

Ca^{2+} uptake into membrane vesicles was measured by the filtration method. Typically, transport was initiated with 3 mM ATP in a reaction mixture (250 μL) containing 250 mM Suc, 25 mM Hepes/BTP (pH 7.0), 10 mM KCl, 0.4 mM NaN$_3$, 3 mM MgSO$_4$, 100 μM EGTA, and 10 μM ^{45}CaCl (3000 Ci/mmol, NEN-Dupont) so the final specific activity was 1 to 2 μCi/2.5 nmol Ca^{2+} per reaction. Under these conditions, the calculated free-Ca^{2+} concentration is about 0.1 μM (Bers et al., 1994). For measuring ΔpH-independent Ca^{2+}-pumping activity, 0.5 μM bafilomycin A1 and 5 μM gramicidin D were routinely included. After incubation at 22°C, 220 μL was run through a filter (0.45-μm pore size, Millipore) moistened with a rinse solution containing 250 mM Suc, 2.5 mM Hepes-BTP (pH 7.0), and 0.2 mM CaCl$_2$. The filter was washed with 5 mL of a cold rinse solution. The ^{45}Ca^{2+} radioactivity associated with the filter was determined by liquid-scintillation counting. Active transport was determined as the difference between activity in the presence and absence of ATP, and is expressed as nanomoles of Ca^{2+} per milligram of protein. All inhibitors were preincubated with membranes at 22°C for 15 min before the reaction was started. To determine the K_m for Ca^{2+}, the reaction mixture contained 500 μM EGTA and various amounts of Ca^{2+} to give the desired range of the free-Ca^{2+} concentration (10 nM to 2 or 3 μM).

Electrophoresis, Immunostaining, and Calmodulin Overlay

These procedures were described previously (Hwang et al., 1997; Liang et al., 1997) and are briefly described in the figure legends. After SDS-PAGE, proteins were transferred to Immobilon-P membranes (Millipore) in 25 mM Tris, pH 8.3, 192 mM Gly, and 15% methanol. To determine the calmodulin binding, the blot was blocked for 1 h with 1% BSA in TBS/CaMg (50 mM Tris, pH 7.5, 0.2 M NaCl, and 50 mM MgCl$_2$, with or without 0.5 mM CaCl$_2$). Then the blot was incubated with 100 ng/mL biotinylated calmodulin in TBS/CaMg for 2 h at 22°C and washed twice in TBS/CaMg plus 0.05% Tween 20. After the sample was incubated with streptavidin conjugated to alkaline phosphatase, color was developed with 5-bromo-4-chloro-3-indoyl phosphate and nitroblue tetrazolium (Sigma). To detect nonspecific binding, electroblotted proteins were incubated with biotinylated calmodulin in the presence of 1 mM EGTA. Bovine calcineurin (Sigma) was used as a positive control.

Chemicals

Erythrosin B, cyclopiazonic acid, and gramicidin D were obtained from Sigma, and thapsigargin was purchased from LC Service Co. (Woburn, MA). Bafilomycin A1 was a gift from Dr. Karlheinz Altendorf (University of Osnabrueck, Germany). All other chemicals were of a reagent grade.

RESULTS

An Arabidopsis Ca^{2+}-ATPase ECA1 Increased Yeast Mutant K616 Growth on Ca^{2+}-Depleted Medium

The yeast mutant K616 defective in both the Golgi (Pmr1) and the vacuolar Ca^{2+} (Pmc1) pumps was unable to grow on SC-URA plates containing 10 mM EGTA; therefore, we tested the growth rates of mutants expressing an Arabidopsis Ca^{2+}-ATPase, ECA1, as a function of Ca^{2+} concentration. Although the control K616 mutant transformed with vector alone had a doubling time of 5 h in medium containing 1 to 10 mM Ca^{2+}, growth was severely retarded as the free-Ca^{2+} concentration was lowered to 0.3 μM by 20 mM EGTA (Fig. 1A). In contrast, mutants transformed with the *ECA1* gene had a doubling time of about 5 h when external Ca^{2+} was <1 μM (Fig. 1B). The initial free-Ca^{2+} concentration was estimated with the Max-Chelator program (Bers et al., 1994) based on the amount of EGTA added to the medium, which contained 1 mM Ca^{2+}. At 31 h after inoculation, the relative density of *ECA1*-transformed mutants was 10- to 20-fold higher than that of the control mutants grown in medium containing approximately 0.3 μM Ca^{2+}. The differential growth rate was consistently observed even when the medium pH was buffered to 6.0 (not shown). Therefore, expression of an Arabidopsis Ca^{2+}-ATPase enhanced yeast mutant growth in submicromolar levels of Ca^{2+} to a rate nearly comparable to that of wild-type strains (not shown).

To determine the subcellular location of ECA1 in yeast, microsomes were isolated from *ECA1*-transformed mutants and fractionated by a Suc-density gradient. The fractions were analyzed by SDS-PAGE, blotted, and immunostained with a polyclonal antibody against the carboxyl terminus of ECA1. In the presence of EDTA, ECA1 was broadly distributed with an apparent peak at approximately 32% Suc. When Mg^{2+} was present during cell isolation and in gradient fractionation, ECa1p was concentrated at 38% to 40% Suc (Fig. 2). As EDTA chelates Mg^{2+} and dissociates ribosomes from the ER, the shift in ECA1 suggested that this polypeptide was associated with the ER. This interpretation agrees with other studies in which yeast ER was found at 40% Suc (Antebi et al., 1992). However, overexpression of this protein could result in localization to other endomembranes, such as the Golgi. To investigate the transport properties of ECA1, vesicles were routinely isolated from the interface of a 26%/45% Suc step gradient containing Mg^{2+}.

The Control K616 Mutant Has High H$^+$/Ca^{2+}-Antiport Activity

We first tested for background Ca^{2+} transport in vesicles isolated from the control mutant K616 transformed with vector alone. At a free-Ca^{2+} concentration of approximately 0.1 μM, ATP increased ^{45}Ca^{2+} associated with ves-
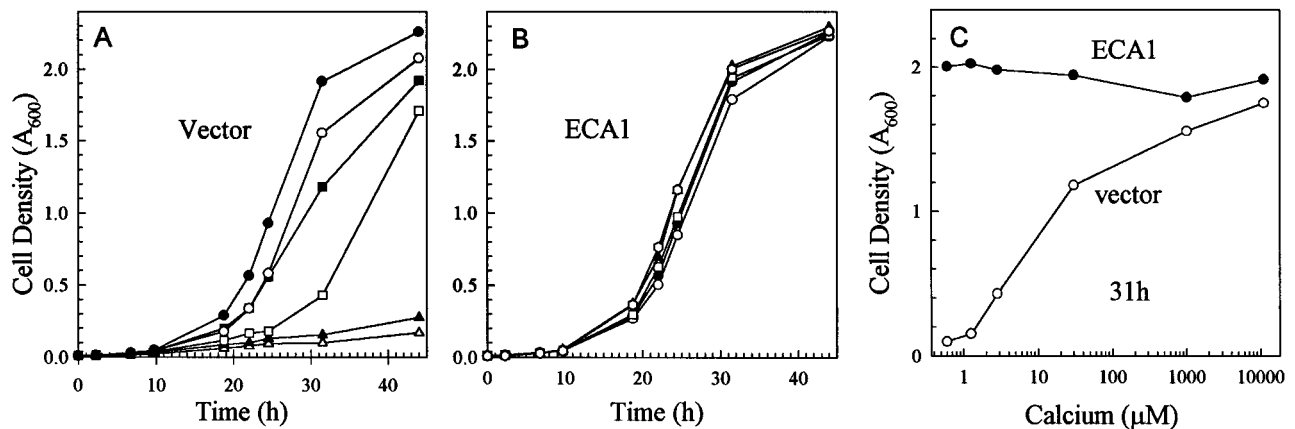
Figure 1. ECA1 enhanced growth of yeast mutant K616 in Ca^{2+}-depleted medium. A and B, Time course of yeast growth. Mutants transformed with vector (A) or ECA1 (B) were suspended in SC-URA (pH 6.2) to a final A_{600} of 0.01 and incubated at 30°C. The medium was either used directly (○) or supplemented with 10 mM $CaCl_2$ (●) or 2 (■), 5 (□), 10 (▲), or 20 (△) mM EGTA. The turbidity of the culture was measured at 600 nm over time. C, Effect of free-Ca^{2+} concentration on growth of mutant K616 harboring vector control (○) or ECA1 (●). A_{600} was determined after 31 h of growth.

icles. The Ca^{2+} was released by the ionophore A23187, indicating that it had accumulated against a concentration gradient. Transport was resistant to sodium vanadate, a P-type cation-pumping ATPase inhibitor (Nechay, 1984), but was decreased by gramicidin D, bafilomycin A_1, or both (Fig. 3). Since bafilomycin specifically inhibits proton pumping by the vacuolar H^+-ATPase and gramicidin dissipates proton gradients, the results demonstrated that more than 95% of Ca^{2+} transport was ΔpH dependent (Fig. 3). Thus, nearly all of the Ca^{2+} uptake was driven by the H^+/Ca^{2+}-antiport activity of the Vcx1 (Cunningham and Fink, 1996). Either gramicidin or bafilomycin alone inhibited 80% to 90% of the ΔpH-dependent Ca^{2+} uptake (data not shown); however, both compounds were needed to achieve maximum inhibition of antiport activity.

The ΔpH-dependent Ca^{2+} transport had an apparent affinity for Ca^{2+} of 350 ± 50 nM (n = 5; Fig. 4), which is lower than the published K_m for Ca^{2+} of 10 to 100 μM reported for wild-type yeast (Ohsumi and Anraku, 1983; Dunn et al., 1994). A small component (<10%) of the vesicle-associated Ca^{2+} appeared to be vanadate sensitive when free $[Ca^{2+}]$ was >0.1 μM; however, this component was mainly due to ATP-dependent binding because the

Ca^{2+} was not released by the ionophore A23187 (not shown).

ECA1-Catalyzed Ca^{2+}-Pump Activity Is ΔpH Independent

To determine whether ECA1 encoded a functional Ca^{2+} pump, membrane vesicles were isolated from ECA1-transformed mutants and assayed for ^{45}Ca uptake. In contrast to the findings shown in Figure 3, sodium vanadate consistently inhibited ATP-driven Ca^{2+} uptake partially (Fig. 5A). Furthermore, a component (50%) of the Ca^{2+} accumulated was resistant consistently to a combination of 5 μM gramicidin and 0.5 μM bafilomycin A_1 (Fig. 5). Thus, the transport component that was ΔpH independent and vanadate sensitive resembled activity from a Ca^{2+} pump.

Ca^{2+} uptake driven by the antiport, but not by the pump, was enhanced 4-fold and 3-fold by 10 mM potassium oxalate and 10 mM potassium phosphate, respectively (Table I). Oxalate stimulation of Ca^{2+} uptake is thought to be caused by formation of Ca^{2+} oxalate precipitate inside the vesicles, thus decreasing the magnitude of the Ca^{2+} chemical gradient. Formation of Ca^{2+} phosphate precipitate in vesicles will result in a similar stimulatory effect of Ca^{2+} uptake. The differential stimulation of anions suggested that an oxalate carrier colocalized to the same vesicle membrane as the antiporter and that the pump, in spite of overexpression, resided on another membrane that was not permeable to oxalate or phosphate.

Although the initial rate of Ca^{2+} uptake by the pump (4 nmol mg^{-1} min^{-1}) was 3- to 4-fold higher than that of the antiporter (1 nmol mg^{-1} min^{-1}), net uptake by antiport activity was 3- to 4-fold higher than that by the pump. This difference could be due to one or more of the following: (a) differential driving force, (b) differential transport rate, or (c) differential Ca^{2+} leakage from the compartments via channels. These results further support the idea that the pump and antiport reside in separate compartments.
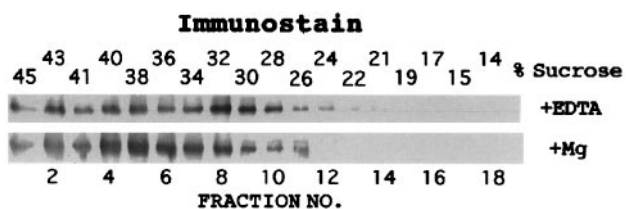

Figure 2. ECA1 was associated with ER vesicles from yeast. Microsomes were isolated from the ECA1-transformed mutant and fractionated with a Suc gradient containing 2 mM EDTA (top) or 2 mM $MgCl_2$ (bottom). Proteins (1–5 μg/lane) were separated by 7% SDS-PAGE, blotted, and immunostained with polyclonal antibodies against ECA1 (1:10,000).
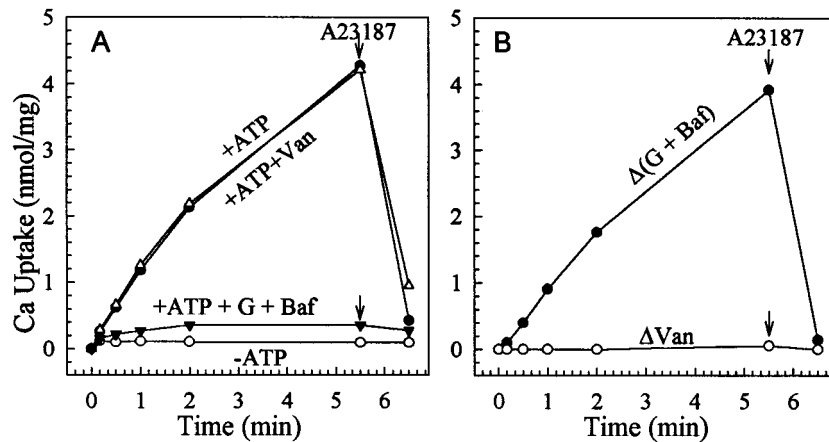
Figure 3. Active ^{45}Ca^{2+} uptake into vesicles of control mutant is ΔpH dependent. Microsomal vesicles were isolated from the vector-transformed K616 strain. Uptake was assayed in a mixture containing 0.1 μM free Ca^{2+}. A, Total uptake. Activity was assayed in the absence (−ATP, ○) or presence (+ATP) of 3 mM ATP with or without inhibitors (●). When added, the concentrations of sodium vanadate (Van, △) or gramicidin D and bafilomycin A$_1$ (G + Baf ▼) were 200 or 5 and 0.5 μM, respectively. B, ΔpH-dependent (ΔpH, ●) and vanadate-sensitive (ΔVan, ○) Ca^{2+} transport. ΔpH or Δ(G + Baf) refers to the difference between total activity (+ATP) and that measured with bafilomycin and gramicidin (+ATP + G + Baf). ΔVan refers to the difference between total activity (+ATP) and that measured in the presence of vanadate (+ATP + Van). The arrows indicate addition of 1 μM ionophore A23187.

Although pump activity could be determined as either ΔpH-independent or vanadate-inhibitable Ca^{2+} activity, net uptake of the vanadate-sensitive component declined over time (Fig. 5B). It is possible that the high-capacity Vcx1 competed with the pump for free Ca^{2+} in the absence of bafilomycin and thus depleted the free-Ca^{2+} concentration in the medium. Therefore, subsequent *ECA1*-pump activity was measured as ΔpH-independent transport in the presence of gramicidin and bafilomycin A$_1$.

High Affinity for Ca^{2+} and ATP

The initial rate of pumping was measured as bafilomycin- and gramicidin-resistant Ca^{2+} uptake at 40 s when uptake was nearly linear with incubation time (Fig. 5A). Free-Ca^{2+} concentration was controlled by varying the total Ca^{2+} added in the presence of 0.5 mM EGTA to give a final free-ion concentration ranging from 10 to 2000 nM (not shown). Surprisingly, the average K_m for Ca^{2+}

determined from five independent experiments was 30 ± 10 nM (Fig. 6). Thus, this ER-type Ca^{2+}-ATPase has a high affinity for Ca^{2+} and reached a maximum velocity when Ca^{2+} was approximately 0.1 μM.

The initial rate of Ca^{2+} transport showed a biphasic dependence on ATP (Fig. 7), which is analogous to animal Ca^{2+} pumps. All assays were conducted at 40 s, when the ATP concentration had not changed significantly. These data are consistent with a model of two ATP-binding sites with K_m values of 20 and 235 μM, respectively. GTP also energized Ca^{2+} pumping; however, we did not detect evidence for biphasic kinetics. The affinity of the pump for GTP was low, and the estimated K_m for GTP was greater than 1.5 mM.

Inhibitors of the ECA1 Pump

The initial rate of ECA1-catalyzed Ca^{2+} uptake was inhibited 50% by 1.5 μM sodium orthovanadate (Fig. 8A), a
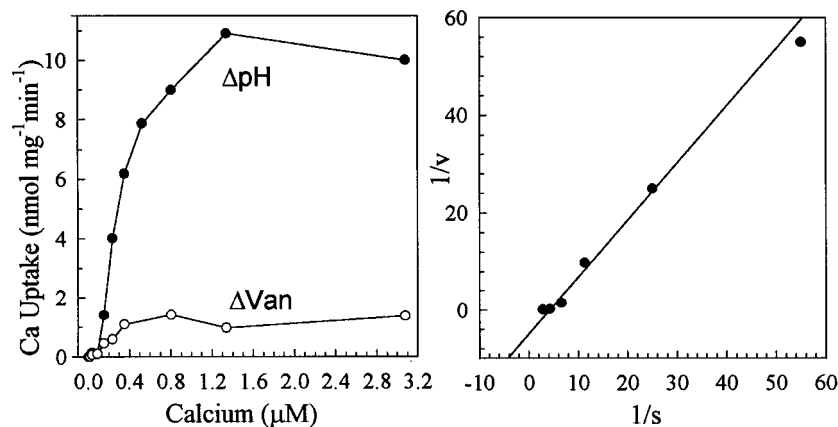

Figure 4. Ca^{2+} affinity of ΔpH-dependent Ca^{2+} transport. Vesicles were isolated from mutant K616 transformed with vector. Left, Uptake was determined at 40 s after 3 mM ATP was added to a reaction medium containing 10 nM to 3 μM free Ca^{2+}. ΔpH-dependent Ca^{2+} transport (ΔpH, ●) was determined as activity that was inhibited by 5 μM gramicidin and 0.5 μM bafilomycin. Vanadate-sensitive Ca^{2+} transport refers to activity inhibited by 200 μM sodium vanadate (ΔVan, ○). Right, Lineweaver-Burk plot. ΔpH-dependent uptake has a K_m for Ca^{2+} of 350 ± 50 nM (n = 5).

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Figure 5. Vanadate-sensitive and ΔpH-independent Ca^{2+}-pumping activity. Vesicles were isolated from the *ECA1*-transformed mutant K616. ^{45}Ca uptake was assayed as described in Figure 3. A, Total uptake. Uptake was determined without (−ATP, ○) or with ATP in the absence (+ATP, ●) or presence of 200 μM sodium vanadate (+Van, △) or 5 μM gramicidin and 0.5 μM bafilomycin (G + Baf, ■). B, ΔpH-independent (●) and vanadate-sensitive (ΔVan, ○) uptake were similar initially. The initial rate was approximately 4 nmol mg^{-1} protein min^{-1}. The arrows indicate the addition of 1 μM ionophore A23187.
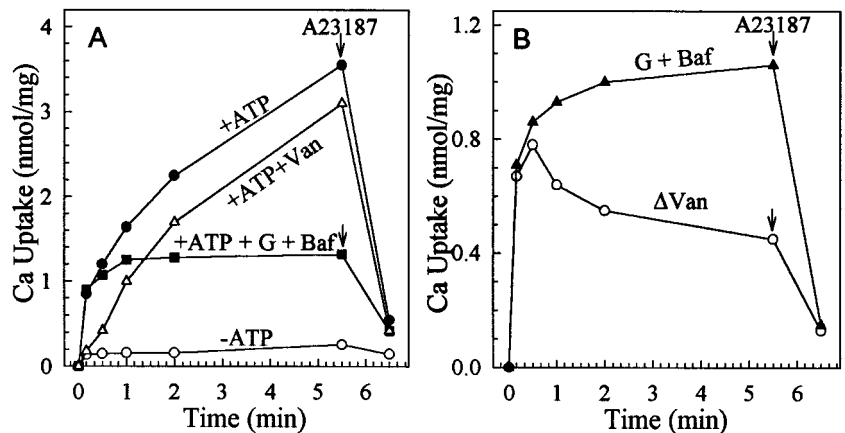
diagnostic inhibitor of P-type ATPases. Erythrosin B at about 0.5 μM inhibited Ca^{2+}-pump activity by 50% (Fig. 8B). Erythrosin B is a halogenated derivative of fluorescein that could modify a Lys residue close to the ATP-binding site, causing inhibition of ATP binding (Mignaco et al., 1996).

Cyclopiazonic acid, an indole tetramic acid, has been reported to be a specific inhibitor of SER-type Ca^{2+}-ATPase (Seidler et al., 1989). Pump activity from ECA1 was blocked 50% by 3 nmol cyclopiazonic acid mg^{-1} protein (Fig. 8C). This value is comparable to the concentration required to block SERCA. However, thapsigargin, a potent and specific inhibitor of SER-type Ca^{2+} pumps, had no effect on Ca^{2+} transport at concentrations up to 3 μM (or 66 nmol mg^{-1} protein; Fig. 8D).

No Effect by Calmodulin

Because calmodulin-stimulated Ca^{2+} transport has been detected in the ER fraction from carrot cells (Hsieh et al., 1991; Hwang et al., 1997), we tested the effect of calmodulin on pump activity. Net Ca^{2+} uptake at 5 min was 0.65 nmol mg^{-1} protein with or without 1 μM calmodulin, suggesting that calmodulin did not stimulate this pump. To test whether ECA1 bound calmodulin, membranes were isolated and the proteins were separated by SDS-PAGE, blot-

ted, and probed with biotinylated calmodulin (Fig. 9). As a positive control, we showed that calmodulin bound the 60-kD calcineurin A subunit in a Ca^{2+}-dependent manner (Fig. 9, lanes 6 and 9). ECA1 was detected by immunostaining only in *ECA1*-transformed, but not in control, mutants (Fig. 9, lanes 1 and 2). However, calmodulin failed to bind any protein at approximately 116 kD from either membrane preparation. Thus, the ER-type Ca^{2+} pump encoded by *ECA1* was not directly regulated by calmodulin.

DISCUSSION

A Functional Plant Ca^{2+} Pump Expressed in Yeast

Here we provide the first biochemical characterization, to our knowledge, of a functional Ca^{2+} pump encoded by a plant gene, *ECA1*. The pump properties were determined after the gene was expressed in a yeast mutant strain, K616, that lacked both the Golgi (Pmr1) and the vacuolar (Pmc1) Ca^{2+} pumps (Cunningham and Fink, 1994; Catty et al., 1997). The advantages of the triple mutant are: (a) the

Table I. *Potassium oxalate and potassium phosphate stimulated Ca^{2+}/H^+-antiport but not Ca^{2+}-pump activity*

Net ATP-dependent $^{45}Ca^{2+}$ uptake at 6 min was determined with 0.1 μM Ca in the reaction mixture as described in Figure 5. Uptake was measured in the absence (Total) or presence (Pump) of 5 μM gramicidin and 0.5 μM bafilomycin. Antiport activity was determined as the difference between "total" and "pump" activity. Potassium oxalate or potassium phosphate was added to a final concentration of 10 mM. Data represent the averages of two experiments.

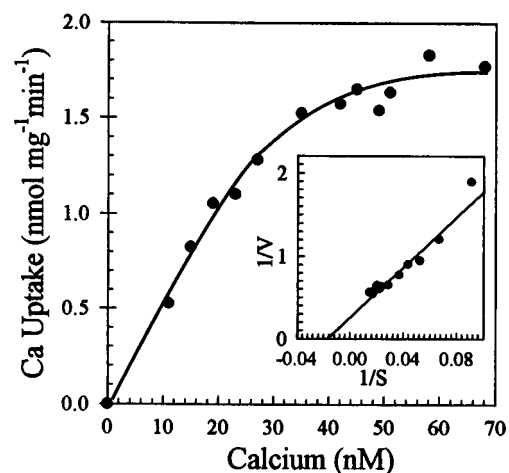| Potassium Salt	Net Ca²⁺ Uptake		
	Total	Pump	Antiport
	nmol/mg (-fold)		
None	1.92 (1.00)	0.98 (1.00)	0.94 (1.00)
+Potassium oxalate	5.00 (2.63)	1.04 (1.06)	3.96 (4.21)
+Potassium phosphate	3.95 (2.06)	0.90 (0.92)	3.05 (3.25)

Figure 6. ECA1 catalyzed high-affinity Ca^{2+}-pumping activity. Ca^{2+} transport into vesicles isolated from the *ECA1*-transformed mutant K616 was performed as described in Figure 4, except that the free-Ca^{2+} concentration was buffered from 10 to 70 nM. Lineweaver-Burk plot (inset) showed an apparent K_m for Ca^{2+} of 30 ± 10 nM (*n* = 5).
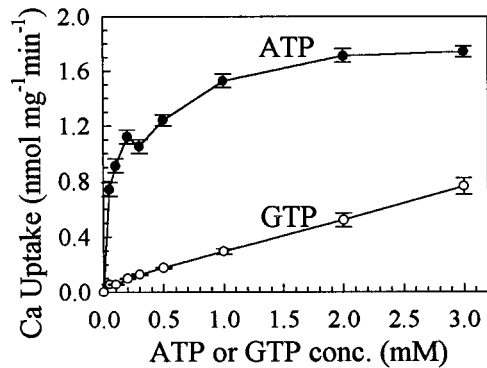
Figure 7. High affinity for ATP. Vesicles were isolated from *ECA1*-transformed mutants for ATP- or GTP-dependent ^{45}Ca transport. Reaction mixtures contained 0.1 μM free Ca^{2+}, 5 μM gramicidin, 0.5 μM bafilomycin, and 25 μM to 3 mM ATP (●) or GTP (○). Uptake was measured at 40 s. Apparent ATP K_m values were 20 and 235 μM. The results are the averages of three experiments. conc., Concentration.

growth rate of the mutant on Ca^{2+}-depleted medium provides an in vivo assay for functional identification of active Ca^{2+} pumps, (b) the control mutant is devoid of any background Ca^{2+}-pump activity (Fig. 3) or Ca^{2+}-dependent phosphoproteins (Liang et al., 1997), and (c) any heterologous gene encoding a Ca^{2+} pump could be overexpressed with a strong inducible promoter. Thus, the triple mutant provides an extremely valuable expression system for determining the nature and structure/function relationships of individual Ca^{2+} pumps from other eukaryotes.

Despite these advantages, Ca^{2+}-pump activity is frequently masked by high H$^+$/Ca^{2+}-antiport activity in the triple mutant. Cunningham and Fink (1994, 1996) found that the double mutant *pmr1pmc1* was not viable unless calcineurin function was disrupted. Evidence suggested that calcineurin acted as a negative regulator of *VCX1* in wild-type strains. Loss of calcineurin function resulted in a triple mutant K616 (*pmr1pmc1cnb1*) that could tolerate high

levels of Ca^{2+}, apparently by activating the H$^+$/Ca^{2+} antiport. Three strategies were used to separate pump activity from the vacuolar H$^+$/Ca^{2+}-antiport activity. First, vesicles enriched in ECA1 were collected from a 26%/45% Suc interface to minimize vacuoles at 22% Suc (Antebi and Fink, 1992; Sorin et al., 1997). Second, pump activity was assayed after inhibition of H$^+$/Ca^{2+}-antiport activity with proton ionophore, gramicidin, and a specific vacuolar H$^+$-ATPase inhibitor, bafilomycin. Third, the free-Ca^{2+} concentration was reduced to 0.1 μM to reduce antiport activity and to minimize Ca^{2+} binding to membranes.

A High-Affinity Ca^{2+} Pump

The most striking feature of the ECA1 pump is its high affinity for Ca^{2+}. The pump showed a K_m for Ca^{2+} of about 30 nM (Fig. 6) relative to the K_m of 350 nM of the H$^+$/Ca^{2+} antiport. Transport was dependent on both Mg^{2+} (data not shown) and ATP (Fig. 5), and 1 to 3 mM was required for maximum pump activity (Fig. 7). It is interesting that the ECA1 pump displayed two affinities for ATP, 20 and 235 μM. In animal Ca^{2+} pumps the high-affinity site of about 2 μM is the hydrolytic site, whereas the low-affinity site ranging from 100 to 300 μM accelerates the reaction (Schatzmann, 1989). It is not clear whether the two sites are separate or whether one site changes in affinity in the reaction cycle.

Inhibition by Cyclopiazonic Acid but Not by Thapsigargin

Although sodium vanadate inhibited Ca^{2+} transport with a 50% inhibition of approximately 1.5 μM (Fig. 8A), it had little or no effect on the initial rate of phosphorylation (F. Liang, unpublished data). These results indicate that vanadate does not block the early steps in the reaction cycle. The reaction cycle of animal SERCA can move in the direction of ATP synthesis. In this mode, vanadate inhibits the Pi-dependent formation of E$_2$P by competing for the
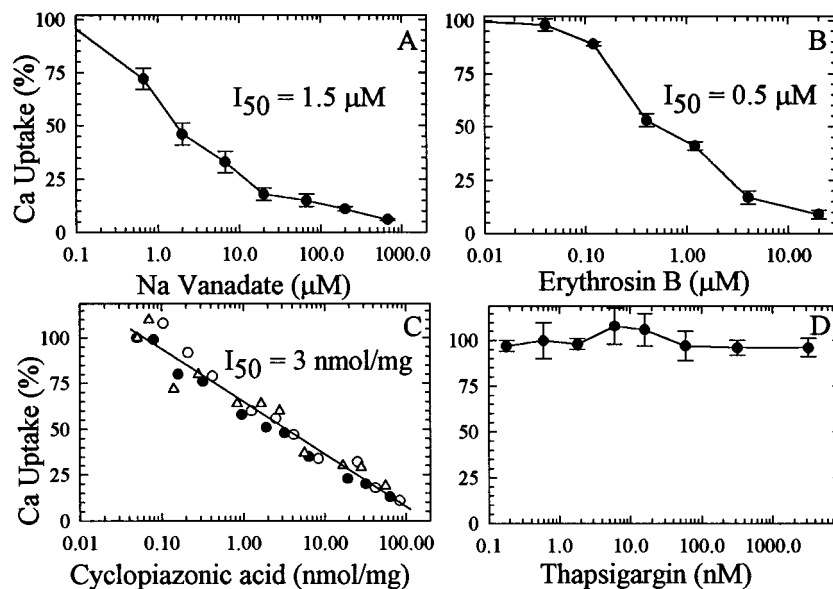

Figure 8. Inhibition of the *ECA1* pump by vanadate and cyclopiazonic acid but not by thapsigargin. Vesicles were preincubated with each inhibitor or DMSO for 15 min at room temperature in the absence of ATP. To start the reaction, ATP was added to a mixture containing 0.1 μM free Ca^{2+} and various concentrations of sodium vanadate (A), erythrosin B (B), cyclopiazonic acid (C), or thapsigargin (D). The transport was stopped at 40 s. Control activity (100%) was 1.8 nmol Ca^{2+} mg^{-1} protein min^{-1}. Data are the averages of three experiments. I$_{50}$, Inhibitor concentration for 50% displacement.
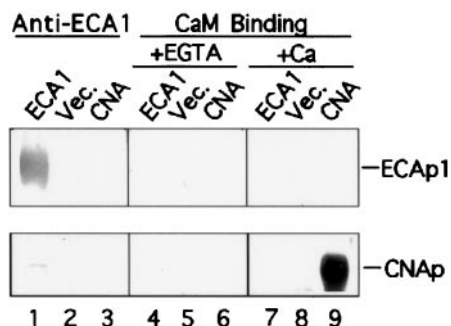
Figure 9. ECA1 did not bind calmodulin. Membranes were isolated from cells transformed with either vector alone (Vec.) or *ECA1*. Vesicle protein (5 μg) from the 25%/45% Suc interface was analyzed by SDS-PAGE, blotted onto an Immobilon-P membrane, and probed with an antibody against ECA1 (lanes 1–3). Another blot was probed with biotinylated calmodulin in the absence (+1 mM EGTA, lanes 4–6) or presence of (+Ca^{2+}, lanes 7–9) 0.5 mM Ca^{2+}. The bovine calcineurin A subunit (CNA; 0.2 μg protein/lane) is a calmodulin-binding protein.

phosphate-binding site (Schatzmann, 1989). Thus, vanadate inhibits Ca^{2+} transport, which depends on completion of many reaction cycles.

However, the ECA1 pump is insensitive to thapsigargin (Fig. 8D), a specific inhibitor of SERCA (Sagara and Inesi, 1991). It binds to SERCA with a one-to-one stoichiometry and a subnanomolar affinity on the third transmembrane segment (Norregaard et al., 1994). During turnover thapsigargin slowly inhibits activity by binding to the Ca^{2+}-deprived intermediate at each cycle, leading to total inactivation. Thus, in a 1-mL reaction mixture containing 1 μg of sarcoplasmic reticulum protein, 4 pmol of thapsigargin (4 nM) will completely inhibit 4 pmol of the SERCA pump (Sagara and Inesi, 1991). We have estimated the relative amount of the Arabidopsis ER Ca^{2+}-ATPase expressed in yeast membranes. The steady-state level of phosphoenzyme formed at 300 nM ATP (when rate was near maximum) was 120 to 150 pmol mg^{-1} membrane protein (F. Liang, unpublished data). With a molecular mass of 116 kD, the plant Ca^{2+} pump represents at least 1.4% to 1.7% of the membrane protein. Thus, the plant Ca^{2+} pump is 15- to 20-fold overexpressed in yeast membranes relative to native Ca^{2+}-ATPases, which make up approximately 0.01% of plant membranes (Chen et al., 1993). However, 3 μM thapsigargin, which corresponds to 66 nmol mg^{-1} membrane protein, did not inhibit *ECA1* pumping. Thus, this isoform of plant ER-type Ca^{2+} pump does not bind to thapsigargin, although the third transmembrane segment of ECA1 shared 67% identity with the thapsigargin-interaction site of SERCA. The difference between plant and animal ER-type Ca^{2+} pumps might be because thapsigargin is a plant-derived sesquiterpene lactone.

It is interesting that the plant ECA1 pump is inhibited by another specific SERCA inhibitor, cyclopiazonic acid, although the mode of action is still unclear. Cyclopiazonic acid inhibited sarcoplasmic reticulum Ca^{2+}-ATPase stoichiometrically, but the indole tetramic acid at 1000 nmol mg^{-1} had no effect on the PM Ca^{2+}-ATPase or other ion-pumping ATPases (Seidler et al., 1989). Binding of cyclo-

piazonic acid decreases the enzyme affinity for ATP 10-fold, and recent studies suggested that cyclopiazonic acid and ATP do not compete for the same binding site (Plenge-Tellechea et al., 1997). Both phosphoenzyme formation and Ca^{2+} transport of the plant ECA1 pump are inhibited with a 50% inhibition of approximately 3 nmol mg^{-1} (Fig. 8C). Thus, binding of cyclopiazonic acid could reduce the affinity of ECA1 for ATP and consequently decrease the initial rate of phosphoenzyme formation (Liang et al., 1997) and Ca^{2+} transport (Fig. 8C).

Function of ECA1 in Arabidopsis

It is interesting that the localization of ECA1 on the ER and perhaps the Golgi of Arabidopsis plants (Liang et al., 1997) is similar to its expression on the ER and on endomembrane compartments of yeast. Furthermore, ECA1 is localized on membranes distinct from the vacuolar H^+/ Ca^{2+} antiport in yeast (Table I), suggesting that a potential ER retrieval signal, KXKXX, at the carboxyl terminus of the plant pump is recognized in yeast. If so, the function of the plant Ca^{2+} pump in yeast may reflect in part its native role in plants. ECA1 could restore *pmr1* mutant growth on Ca^{2+}-depleted medium (Liang et al., 1997), indicating that it resembled the function of the native yeast Golgi Ca^{2+} pump (Sorin et al., 1997). However, ECA1 could pump other divalent cations, including Mn^{2+}, because expression of *ECA1* restored the growth of a yeast *pmr1* mutant on Mn^{2+}-containing medium (Liang et al., 1997). It is interesting that plants treated with cyclopiazonic acid showed aggregation of ER membranes (Busch and Sievers, 1993). Furthermore, gravitropic responses are inhibited in cress roots treated with cyclopiazonic acid (Sievers and Busch, 1992). After overexpression of a rice ER-type Ca^{2+}-ATPase, the GA_3 requirement for α-amylase induction and secretion in aleurone protoplasts was bypassed (Chen et al., 1997). These results support the idea that: (a) luminal Ca^{2+} supplied by ER Ca^{2+}-pumping ATPase could play a role in protein processing and vesicle trafficking of the ER and Golgi (Beckers and Balch, 1989), and (b) ER Ca^{2+} pumps modulate cytoplasmic Ca^{2+} waves induced by various stimuli. Thus, like yeast cells, the normal growth and development of plant cells are dependent on active high-affinity Ca^{2+} pumps on the ER and Golgi.

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