Inhibition of the Formation of the Spf1p Phosphoenzyme by Ca\textsuperscript{2+}*

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P5-ATPases are important for processes associated with the endosomal-lysosomal system of eukaryotic cells. In humans, the loss of function of P5-ATPases causes neurodegeneration. In the yeast Saccharomyces cerevisiae, deletion of P5-ATPase Spf1p gives rise to endoplasmic reticulum stress. The reaction cycle of P5-ATPases is poorly characterized. Here, we showed that the formation of the Spf1p catalytic phosphoenzyme was fast in a reaction medium containing ATP, Mg\textsuperscript{2+}, and EGTA. Low concentrations of Ca\textsuperscript{2+} in the phosphorylation medium decreased the rate of phosphorylation and the maximal level of phosphoenzyme. Neither Mn\textsuperscript{2+} nor Mg\textsuperscript{2+} had an inhibitory effect on the formation of the phosphoenzyme similar to that of Ca\textsuperscript{2+}. The $K_m$ for ATP in the phosphorylation reaction was $\sim 1$ $\mu$M and did not significantly change in the presence of Ca\textsuperscript{2+}. Half-maximal phosphorylation was attained at $8 \mu$M Mg\textsuperscript{2+}, but higher concentrations partially protected from Ca\textsuperscript{2+} inhibition. In conditions similar to those used for phosphorylation, Ca\textsuperscript{2+} had a small effect accelerating dephosphorylation and minimally affected ATPase activity, suggesting that the formation of the phosphoenzyme was not the limiting step of the ATP hydrolytic cycle.

P5-ATPases are a large group of enzymes that couple the hydrolysis of ATP with the active transport of ions (14, 15). During the transport cycle, they transiently form a phosphoenzyme (EP) that plays a key role in the active transport mechanism. P-ATPases comprise a membrane domain (M) and a soluble portion with nucleotide binding (N), phosphorylation (P), and actuator (A) domains. These domains are involved in a kinase-phosphatase reaction cycle through two major conformations, E\textsubscript{1}-E\textsubscript{2}, and the transient formation of a catalytic EP. The binding of the transported ion to the $E_i$ form prompts the assembly of the phosphorylation site between the ATP-bound N domain and the P domain, whereas the A domain directs the occlusion of the bound ion. When the phosphorylation reaction occurs, it initially generates the high energy $E_i$-$P$ intermediate and releases ADP. $E_i$-$P$ then changes to $E_P$, and the A domain associates with the N-P complex and dephosphorylates the P domain. The binding of a counter transported ion is associated with the dephosphorylation of $E_P$. Finally, the cycle recommences with the transition of $E_P$ back to $E_i$.

At present, the biochemical characterization of P5-ATPases is limited, and the putative transported ion has not yet been identified (16). The best characterized P5-ATPase is Spf1p. Spf1p is capable of hydrolyzing ATP and forming the catalytic EP in a relatively simple reaction medium containing no added metal ions except Mg\textsuperscript{2+}, a cofactor of all P-ATPases (7, 17, 18). This result suggests either that the Spf1p transported ion is already present in the reaction medium, for example H\textsuperscript{+} ions, or that Spf1p is unique in that it can spontaneously adopt an $E_i$ conformation ready for phosphorylation by ATP. Furthermore, a substantial amount of the EP formed by Spf1p is of the $E_i$-$P$ type, as indicated by its fast decomposition in the presence of ADP (17, 18).

Earlier studies based on the phenotypes generated by Spf1p deletion led to the suggestion that Spf1p may be a Ca\textsuperscript{2+} transporter (7, 19, 20). However, direct biochemical measurements to confirm this assumption are still lacking (21). It has been recently reported that Ca\textsuperscript{2+} ions stimulate the decay of the EP of HvP5A1, a homolog of Spf1p from barley (17). The aim of the present study was to examine in more detail the kinetics of the

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3 The abbreviations used are: ER, endoplasmic reticulum; Spf1, sensitivity to killer toxin; C\textsubscript{12}E\textsubscript{10}, polyoxyethylene 10-lauryl ether; EP, phosphorylated enzyme.
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formation and decomposition of the Spf1p EP and the influence of Ca\textsuperscript{2+}.

Experimental Procedures

Chemicals—Polyoxyethylene-10-laurylether (C\textsubscript{12}E\textsubscript{10}), 1-α-phosphatidylcholine type XVI-E Sigma from fresh egg yolk, ATP (disodium salt, vanadium-free), SDS, yeast synthetic detergent medium supplement without leucine, yeast nitrogen base without amino acids, dextrose, enzymes and cofactors were obtained from Sigma. Trypsitone and yeast extract were from Difco. PerkinElmer Life Sciences provided the [γ\textsuperscript{32}P]ATP. Salts and reagents were of analytical reagent grade.

Yeast Strain and Growth Media—S. cerevisiae strain DBY 2062 (MATα his4-619 leu2-3,112) (18) was used for expression. Yeast cells were transformed with the pMP625 vector containing a Leu\textsuperscript{+} marker and the PMAI promoter and the cDNA coding for either Spf1p or the fusion protein GFP-Spf1p. The experiments reported here were done using GFP-Spf1p, which has the same ATPase activity and maximal phosphorylation level as Spf1p (18) and allows an easy quantitation of its expression by fluorescence microscopy. The growing medium contained 6.7% yeast-nitrogen base without amino acids, 0.67% dextrose, and 20% glycerol, 130 mM KCl, 1 mM MgCl\textsubscript{2}, 20 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, homogenized in a glass homogenizer, and solubilized at 4 °C for 15 min by adding 2 g of C\textsubscript{12}E\textsubscript{10}/g of total membrane protein. 10 mM imidazole was added to the supernatant, and then it was loaded onto a 2-mL nickel-nitriilotriacetic acid-agarose column (Qiagen) and separated by acidic SDS-PAGE. Slices of the gel containing the Spf1p phosphoenzyme were cut, and the radioactive label was diluted by adding 500 μM of cold ATP.

ATPase Activity—The ATPase activity was estimated at 28 °C from the release of [\textsuperscript{32}P] from [γ\textsuperscript{32}P]ATP (24) in a final volume of 0.25 ml of “ATPase medium” containing, 50 mM Tris-HCl (pH 7.2), 0.5 mM EGTA, 5 mM N\textsubscript{3}N\textsubscript{a}, 2 mM MgCl\textsubscript{2}, 30 μM ATP, and 1 μg of GFP-Spf1 in 50 μl of elution buffer. The GFP-Spf1 protein was supplemented with 0.85 μg of C\textsubscript{12}E\textsubscript{10} and 4.3 μg of phosphatidylcholine, the suspension was mixed and preincubated for at least 5 min on ice before being added to the reaction medium. The reaction was initiated by the addition of ATP and terminated by acid denaturation.

Data Analysis—Except where indicated, the data points represent the average values of two or three independent determinations performed with different purified protein preparations. Best fitting values of the parameters and their S.E. were obtained by fitting the equations indicated in the legends of the figures to the experimental data using the SigmaPlot 10 scientific data analysis and graphing software (Systat Software Inc., CA) for Windows.

Results

Phosphorylation of Spf1p by ATP—Purified Spf1p was preincubated in a medium containing 0.5 mM EGTA and 2 mM Mg\textsuperscript{2+} and phosphorylated by the addition of 0.5 μM [γ\textsuperscript{32}P]ATP at 4 °C. The results in Fig. 1 show that, in this condition, the reaction was fast and reached a maximal amount of EP of ~1 nmol/mg of protein at ~30 s. The value of the apparent phosphorylation rate constant (k\textsubscript{p}), obtained by fitting a monoequivalent rate expression to maximum function, was 0.14 s\textsuperscript{−1}. When the phosphorylation was initiated by adding ATP and CaCl\textsubscript{2} to give 100 μM Ca\textsuperscript{2+} in the phosphorylation medium, the levels of EP were significantly lower and increased slowly with time (k\textsubscript{p} = 0.02 s\textsuperscript{−1}) up to a maximal level of 0.75 nmol/mg of protein. At short times of phosphorylation the level of EP was ~8 times higher in the absence than in the presence of Ca\textsuperscript{2+}. The initial rate of phosphorylation (v\textsubscript{0}) is a function of the amount of E\textsubscript{i} and the apparent constant of the reaction (k\textsubscript{p}).

\[ v_0 = k_p[E_i] \]  
(Eq. 1)

The effect of Ca\textsuperscript{2+} decreasing the level of EP was readily observed when Ca\textsuperscript{2+} was added together with ATP, suggesting that it did not involve a change in the amount of E\textsubscript{i}. As shown in Fig. 1C, preincubation of the enzyme with Ca\textsuperscript{2+} before the beginning of phosphorylation resulted in a minimal decrease of the phosphorylation rate compared with that attained when Ca\textsuperscript{2+} was only present during phosphorylation. These results suggest that Ca\textsuperscript{2+} directly decreased the apparent rate constant of phosphorylation, as indicated in Equation 1.
Further information on the effect of Ca\(^{2+}\) was obtained by comparing its effects with those of vanadate (Fig. 2). Vanadate, a well known inhibitor of P-ATPases, binds to the nonphosphorylatable E2 conformation, displacing the equilibrium between E2 and E3 toward the former. In contrast with the effect of Ca\(^{2+}\), the formation of EP was significantly inhibited only when vanadate was in contact with the enzyme before phosphorylation. Moreover, when the enzyme was preincubated with vanadate, its apparent affinity as an inhibitor of phosphorylation was similar in the absence and in the presence of Ca\(^{2+}\). These results indicate that Ca\(^{2+}\) did not affect the E2-E3 equilibrium.

**Dependence of the Rate of Phosphorylation on the Concentration of Ca\(^{2+}\)—**The level of EP at 5s of phosphorylation was determined in medium containing increasing concentrations of Ca\(^{2+}\). As shown in Fig. 3A, the yield of EP decreased rapidly with a K\(_i\) of ~0.2 mM Ca\(^{2+}\) and then seemed to remain constant at concentrations higher than 100 mM Ca\(^{2+}\).

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**FIGURE 1. Time course of EP formation.** A, acidic gel electrophoresis of phosphorylated GFP-Spf1p showing the radioactivity (top panel) or the Coomassie Blue staining (bottom panel). 1.5 μg of GFP-Spf1p was suspended at 4 °C in a medium containing 2 mM Mg\(^{2+}\) and 0.5 mM EGTA. The reaction was started by adding 0.5 μM ATP plus 0.5 mM EGTA or 0.5 μM ATP plus enough CaCl\(_2\) to give a final concentration of 100 μM Ca\(^{2+}\). B, EP levels quantified as described under “Experimental Procedures.” The data points are the averages from three experiments. Error bars show the standard deviation. C, 0.5 mM EGTA, 100 μM Ca\(^{2+}\). The data were fitted by an exponential equation with the following parameters, in the absence of Ca\(^{2+}\) EP\(_{max}\) = 1.00 ± 0.03 nmol/mg, and k\(_p\) = 0.14 ± 0.01 s\(^{-1}\), and in the presence of Ca\(^{2+}\) EP\(_{max}\) = 0.75 ± 0.04 nmol/mg, and k\(_p\) = 0.020 ± 0.002 s\(^{-1}\). The phosphorylation was done in conditions similar to B except that either the enzyme was suspended in a reaction medium with 0.5 mM EGTA, and Ca\(^{2+}\) was added together with ATP (circles), or the enzyme was preincubated in a reaction medium with Ca\(^{2+}\) for 5 min at 4 °C before starting the phosphorylation (triangles). The data points are the averages from two experiments. Error bars show the standard deviation.

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**FIGURE 2. Comparison of the effects of Ca\(^{2+}\) and vanadate on the EP formation.** A, 1.5 μg of GFP-Spf1p was suspended at 4 °C in a medium containing 2 mM Mg\(^{2+}\) and 0.5 mM EGTA, and the phosphorylation was started by adding 0.5 μM ATP (EGTA); 0.5 μM ATP plus 100 μM vanadate (VAN); 0.5 μM ATP plus 100 μM Ca\(^{2+}\) (VAN+Ca\(^{2+}\)) or 0.5 μM ATP, 200 μM vanadate, and 100 μM Ca\(^{2+}\) (VAN+Ca\(^{2+}\)). The bar (VAN+pre) shows the level of EP formed in conditions similar to (VAN) except that the enzyme was preincubated for 5 min at 4 °C with 200 μM vanadate before starting the phosphorylation. The reaction time was 5 s. The values are the average from two experiments. Error bars show the standard deviation. B, GFP-Spf1p was suspended at 4 °C in a medium containing 2 mM Mg\(^{2+}\) and 0.5 mM EGTA and the indicated concentration of vanadate. The phosphorylation was started by adding 0.5 μM ATP (filled circles) or 0.5 μM ATP plus 100 μM Ca\(^{2+}\) (empty circles). The value of EP in each condition in the absence of vanadate was taken as 100%. The data points are the averages from three experiments, and the error bars show the standard deviation. The lines represent the best fit to the data given by the hyperbolic equation EP = EP\(_0\) + EP\(_{max}\)([vanadate]/K\(_i\) + [vanadate]), with the following parameters, in the absence of Ca\(^{2+}\), EP\(_0\) = 15 ± 7%, K\(_i\) = 267 ± 75 μM and EP\(_{max}\) = 81 ± 7% and in 100 μM Ca\(^{2+}\), EP\(_0\) = 10 ± 13%, K\(_i\) = 274 ± 144 μM, and EP\(_{max}\) = 82 ± 14%.

Fig. 3B shows the effect of increasing concentrations of Mn\(^{2+}\) on the level of EP. Somewhat lower levels of EP were observed as Mn\(^{2+}\) concentration increased from 0 to 1 mM. However, the effect of Mn\(^{2+}\) on EP was weaker than that of Ca\(^{2+}\).

**Apparent Affinity for ATP—**One possible explanation of the inhibitory effect of Ca\(^{2+}\) on the rate constant of phosphorylation could be a decrease in the affinity for ATP. To test this hypothesis, the level of EP was measured at increasing concentrations of ATP (Fig. 4). In the presence of 0.5 mM EGTA and 2 mM Mg\(^{2+}\), the level of EP at 5 s of phosphorylation increased rapidly with the concentration of ATP in the range of 0–30 μM, following a hyperbolic curve with K\(_m\) = 1 μM. The addition of ATP plus CaCl\(_2\) to give a final Ca\(^{2+}\) concentration of 100 μM lowered the levels of EP obtained at all the concentrations of ATP tested. The estimated K\(_m\) for ATP in the presence of Ca\(^{2+}\) was 0.9 μM. Thus, Ca\(^{2+}\) did not significantly change the apparent affinity for ATP at the high affinity site.

**Apparent Affinity for Mg\(^{2+}\)—**Mg\(^{2+}\) is a common cofactor of all P-ATPases. To test the effect of Mg\(^{2+}\) on the phosphorylation of Spf1p, we measured the level of EP at increasing concentrations of Mg\(^{2+}\). In the presence of 0.5 mM EGTA, the EP at 5 s of phosphorylation increased with the concentration of Mg\(^{2+}\), reaching a maximal level at ~100 μM (Fig. 5). When Ca\(^{2+}\) was
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![Diagram A](image_url)

**FIGURE 3. Dependence of the EP formation with the concentration of Ca\(^{2+}\) and Mn\(^{2+}\).** A, 1.5 \(\mu\)g of GFP-Spf1p was suspended at 4 °C in a medium containing 2 mM Mg\(^{2+}\), 0.5 mM EGTA, and increasing concentrations of CaCl\(_2\) to give the indicated concentrations of Ca\(^{2+}\) in the final reaction medium. The reaction was started by adding 30 \(\mu\)M ATP and terminated after 5 s. The data points shown are measurements from three independent experiments. The line represents the best fit to the data given by the hyperbolic equation \(EP = EP_0 + EP_{\infty} \cdot [Ca]/(K_c + [Ca])\), with the following parameters \(EP_0 = 0.14 \pm 0.01\) nmol/mg, \(K_c = 0.18 \pm 0.04\) \(\mu\)M, and \(EP_{\infty} = 0.74 \pm 0.06\) nmol/mg. B, GFP-Spf1p was suspended at 4 °C in a medium containing 2 mM Mg\(^{2+}\) and the indicated concentrations of Mn\(^{2+}\) and phosphorylated by the addition of 30 \(\mu\)M ATP.

![Diagram B](image_url)

**FIGURE 4. ATP dependence of EP formation.** 1.5 \(\mu\)g of GFP-Spf1p was suspended at 4 °C in a medium containing 0.5 mM EGTA and enough MgCl\(_2\) to give the indicated final Mg\(^{2+}\) concentrations in the phosphorylation medium. The phosphorylation was started by adding 30 \(\mu\)M ATP (filled circles), 30 \(\mu\)M ATP plus 0.2 \(\mu\)M Ca\(^{2+}\) (filled triangles), or 30 \(\mu\)M ATP plus 100 \(\mu\)M Ca\(^{2+}\) (empty circles). The reaction time was 5 s. The data points shown are measurements from five independent experiments. The lines represent the best fit to the data given by the Hill equation. The estimated values of \(K_m\) were 8, 280, and 1250 \(\mu\)M for no Ca\(^{2+}\), 0.2 \(\mu\)M Ca\(^{2+}\), and 100 \(\mu\)M Ca\(^{2+}\), respectively.

The Effect of Ca\(^{2+}\) on Dephosphorylation—Ca\(^{2+}\) has been shown to promote the dephosphorylation of HvP5A1, a barley homolog of Spf1p (17). Here, we examined the effects of Ca\(^{2+}\) on the decay of EP in conditions similar to those used for the phosphorylation reaction. To this end, Spf1p was phosphorylated in medium with EGTA and no added CaCl\(_2\), and the decay of EP was followed both in the absence of Ca\(^{2+}\) and after the addition of CaCl\(_2\) to give 100 \(\mu\)M Ca\(^{2+}\). The time courses of dephosphorylation were biphasic (Fig. 6). The addition of Ca\(^{2+}\) at the start of dephosphorylation increased ~2-fold the rate of the rapid component, whereas the slow component was minimally affected.

**ATPase Activity**—In previous studies, we did not detect a significant effect of Ca\(^{2+}\) on the ATPase activity of Spf1p (18). However, because here we found that Ca\(^{2+}\) changed the level and kinetics of EP, we reexamined its effects on ATPase by using a low concentration of ATP (30 \(\mu\)M) and short reaction times similar to those of the phosphorylation experiments. In these conditions, ATPase activity in the presence of 0.5 mM EGTA was slightly higher than that in the presence of 100 \(\mu\)M Ca\(^{2+}\) (Fig. 7).

Discussion

Here, we investigated the formation and decay of the catalytic phosphorylated intermediate of Spf1p in the presence and in the absence of Ca\(^{2+}\). In agreement with previous studies (7, 17, 18), we found that Spf1p readily accepted the \(\gamma\)-P from ATP, provided Mg\(^{2+}\) was present in the medium. The phosphorylation reaction attained maximal rate and maximal levels of EP in medium containing enough EGTA to reduce the concentration of Ca\(^{2+}\) to less than 0.1 \(\mu\)M. The estimated values of the rate constants for phosphorylation for Spf1p are in the range of those reported for other P-ATPases (25, 26). On the other hand, the maximal level of EP measured in different preparations of the purified protein allows estimating a stoichiometry of near 0.1 mol EP/mol of protein. Although this value is far from the theoretical stoichiometry of 1:1, it is close to the values reported for other P-ATPases like those of the P4 type (27). In addition, the amount of EP detected may be underestimated because of the inactivation of the protein during the purification process, the presence of a small amount of contaminant proteins in the purified preparation, and the decomposition of EP during the acidic gel electrophoresis. In any case, our results indicate that the absence of Ca\(^{2+}\) stabilizes Spf1p in its phosphorylated form.

Effects of Ca\(^{2+}\) on Phosphorylation—When the phosphorylation reaction took place in the presence of Ca\(^{2+}\), the apparent rate of phosphorylation and the maximum level of EP
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The phosphorylation rate increased with the concentrations of Mg\(^{2+}\) in the micromolar range. Interestingly, the amount of Mg\(^{2+}\) needed to activate phosphorylation increased in the presence of Ca\(^{2+}\), and high Mg\(^{2+}\) partially protected from Ca\(^{2+}\) inhibition.

For the mechanism of Ca\(^{2+}\) inhibition of EP formation, at least two possibilities could be considered. First, Ca\(^{2+}\) may compete with Mg\(^{2+}\) at the catalytic site of Spf1p, replacing the activating effect of Mg\(^{2+}\) with less efficiency. Such a competition occurs in other P-ATPases, with varying degree of catalytic efficiency (30, 31). Furthermore, if the inhibitory species were Ca\(^{2+}\) in complex with ATP, the affinity of Spf1p for Ca\(^{2+}\)-ATP should be extremely high, because it can be estimated that, in the conditions used for the phosphorylation reaction, more than 85% of ATP was bound to Mg\(^{2+}\). Alternatively, the inhibition of Spf1p phosphorylation by Ca\(^{2+}\) may involve a separate Ca\(^{2+}\) site on the protein. Modulatory Ca\(^{2+}\) sites have been identified in the nucleotide domain of other P-ATPases (32).

Actually, the nucleotide domain of P5-ATPases exhibits some unique amino acid motifs that may be relevant for the formation and stability of the Spf1p EP (17, 33).

Effects of Ca\(^{2+}\) on Dephosphorylation—Dephosphorylation involved both a fast and a slow component. We found that Ca\(^{2+}\) had a small effect accelerating the fast phase of dephosphorylation by \~2-fold. This type of biphasic dephosphorylation kinetics has already been described in other P-ATPases (34, 35) and may represent the fast decomposition of the preexistent E\(_2\)P formed from E\(_2\)P. If this were the case, our results would indicate that Ca\(^{2+}\) accelerates E\(_2\)P decay. On the other hand, because a substantial amount of the phosphorylated Spf1p is E\(_{\text{P}}\), the possibility that Ca\(^{2+}\) promotes the reaction of E\(_{\text{P}}\) with the ADP produced cannot be discarded. Further studies are needed to discriminate between these possibilities. Moreover, by using yeast membrane preparations, Sørensen et al. (17) showed that Ca\(^{2+}\) induces a spontaneous decay of the recombinant plant P5A-ATPase HvP5A1 EP. These authors showed that Ca\(^{2+}\) exerted this effect with relatively low affinity (K\(_i\) = \~250 \mu M) but was very effective in reducing EP, a fact that might have been helped by the ADP-producing hexokinase-glucose system used to deplete ATP and thus stop phosphorylation. In contrast, our present results showed that the most prominent effect of Ca\(^{2+}\) was directly inhibiting EP formation and that the effect of Ca\(^{2+}\) accelerating dephosphorylation was smaller. This is consistent with the lower level of EP detected at steady state in the presence of Ca\(^{2+}\). An interesting hypothesis that may explain the differences between our results and those reported previously is the modulation of the effect of Ca\(^{2+}\) by detergents and lipids. Indeed, the signaling lipids phosphatidic acid and phosphatidylinositol 3,5-biphosphate have been recently shown to increase the phosphorylation of the closely related P5B-ATPase ATP13A2 (36).

Effects of Ca\(^{2+}\) on the ATPase Activity of Spf1p—Spf1p ATPase activity, measured in conditions similar to those used for phosphorylation, was less affected by Ca\(^{2+}\) than expected on the basis on its effect on the formation of EP. This result suggests that the phosphorylation reaction is not limiting ATPase activity. This is in agreement with the fact that a sub-
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Stanscent fraction of the Spf1p EP is E\(_4\)P and does not transit to E\(_2\)P (17, 18). In our hands, the slow phase of dephosphorylation did not seem to change with Ca\(^{2+}\), which might be related to the lack of stimulation of ATPase activity. We believe that the effect of Ca\(^{2+}\) on Spf1p may depend on the temperature of the assay, the presence of other modulators, different lipid environments, and potential interacting partners that are unknown at present. This requires further investigation.

**Significance of the Effects of Ca\(^{2+}\) on the Function of Spf1p**—Because earlier studies indicated a connection between Spf1p and Ca\(^{2+}\) homeostasis (6, 7), it is tempting to speculate on the potential relevance of a Ca\(^{2+}\) modulation of the Spf1p function. Moreover, Ca\(^{2+}\) plays an important role in membrane trafficking, a process also affected by the function of P5-ATPases (3).

We have considered the possibility that the observed effects of Ca\(^{2+}\) are the consequence of its action as a transported counterion in the catalytic cycle of Spf1p. However, based on the results presented here and the comparison with the behavior of other P-ATPases, we believe that this option is unlikely.

Although functional reconstitution of Spf1p into liposomes has not yet been reported, it should be soon available for a direct testing of Spf1p transporting activity. Nevertheless, Ca\(^{2+}\) may modulate the functions of Spf1p even if it is not transported. Indeed, the catalytic subunit of P4-ATPase Drs2p interacts with its Cdc50p subunit preferentially when it is phosphorylated (27). Our results indicate that at the low concentrations of Ca\(^{2+}\) present in the cytosol at resting conditions, Spf1p would be stabilized in the phosphorylated form, and this might influence its interaction with other protein partners. In this line, the effects of Ca\(^{2+}\) on the formation of the catalytic EP of Spf1p may be part of a signaling pathway from the cytosol to the ER.

**Author Contributions**—G. R. C. and N. A. C. designed, performed, and analyzed the experiments. L. R. M. and N. S. performed the experiments and contributed to the preparation of the figures. H. P. A. designed the study, analyzed the experiments, and wrote the paper. All authors analyzed the results and approved the final version of the manuscript.

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