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Activation of H^+-ATPase by glucose in *Saccharomyces cerevisiae* involves a membrane serine protease

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ABSTRACT

Background: Glucose induces H^+-ATPase activation in *Saccharomyces cerevisiae*. Our previous study showed that (i) *S. cerevisiae* plasma membrane H^+-ATPase forms a complex with acetylated tubulin (AcTub), resulting in inhibition of the enzyme activity; (ii) exogenous glucose addition results in the dissociation of the complex and recovery of the enzyme activity.
Methods: We used classic biochemical and molecular biology tools in order to identify the key components in the mechanism that leads to H^+-ATPase activation after glucose treatment.
Results: We demonstrate that glucose-induced dissociation of the complex is due to pH-dependent activation of a protease that hydrolyzes membrane tubulin. Biochemical analysis identified a serine protease with a kDa of 35–40 and an isoelectric point between 8 and 9. Analysis of several knockout yeast strains led to the detection of Lpx1p as the serine protease responsible of tubulin proteolysis. When *lpx1Δ* cells were treated with glucose, tubulin was not degraded, the AcTub/H^+-ATPase complex did not undergo dissociation, and H^+-ATPase activation was significantly delayed.
Conclusion: Our findings indicate that the mechanism of H^+-ATPase activation by glucose involves a decrease in the cytosolic pH and consequent activation of a serine protease that hydrolyzes AcTub, accelerating the process of the AcTub/H^+-ATPase complex dissociation and the activation of the enzyme.
General significance: Our data sheds light into the mechanism by which acetylated tubulin dissociates from the yeast H^+-ATPase, identifying a degradative step that remained unknown. This finding also proposes an indirect way to pharmacologically regulate yeast H^+-ATPase activity and open the question about mechanistic similarities with other higher eukaryotes.

In the memory of Marina Rafaela Amaiden, "Rafa", who will remain forever in our hearts.

1. Introduction

In the yeast *Saccharomyces cerevisiae*, plasma membrane H^+-ATPase plays key roles in the regulation of intracellular pH (by countering the pH-reducing effect of environmental factors) and in the transport of nutrients into the cell (by generating the necessary extracellular H^+ gradient). The activation of the H^+-ATPase pump by glucose is a well-known process that was first described by Serrano [1]. The underlying mechanism involves changes in kinetic parameters (*e.g.*, increased V_{max} and decreased Km_{ATP}) and increased expression of the*pma1* gene at the transcriptional level. The decrease in intracellular pH induced by organic acids [2] was shown to induce H^+-ATPase activation *via* a Ca^{2+}-dependent mechanism [3–5]. Lecchi and co-workers[6] demonstrated that this

Abbreviations: AcTub, acetylated tubulin; Ig, immunoglobulin; mAb, monoclonal antibody; pH_e, extracellular pH; pH_i, intracellular pH; PMSF, phenyl methyl sulfonyl fluoride

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mechanism involves phosphorylation of the Ser 911 and Thr 912 residues of the ATPase and that such events are related to kinetic changes of the enzyme.

The production of H^+ because of glucose metabolism leads to a reduction in cytosolic pH. The pH reduction is counteracted (allowing the cell to reach pH homeostasis) by the activation of plasma membrane H^+-ATPase that pumps H^+ out of the cell and of vacuolar H^+-ATPase that pumps H^+ into the vacuole [7]. We demonstrated previously that acetylated tubulin (AcTub) interacts with plasma membrane H^+-ATPase in *S. cerevisiae*, resulting in the formation of an AcTub/H^+-ATPase complex in which the enzyme is inactive. Following glucose stimulation, the tubulin dissociates from the enzyme, which consequently becomes activated [8]. We have described similar interactions involving Na^+, K^+-ATPases and Ca^{2+}-ATPases in a variety of tissues and cell lines [9–15]. In each case, tubulin formed a complex with the ATPases resulting in the inhibition of the enzyme activity, and experimental treatment causing dissociation of the complex resulted in the recovery of enzyme activity. These previous studies did not address the mechanism whereby dissociation of the AcTub/ATPase complex caused activation of the enzyme. In the present study, we investigated the activation of H^+-ATPase in *S. cerevisiae* and found that a serine protease is involved in the dissociation of the complex and the consequent recovery of enzyme activity.

2. Materials and methods

2.1. Materials

ATP, glucose, 2-deoxy-D-glucose, mouse monoclonal antibody (mAb) DM1A specific to α-tubulin, mouse mAb6-11B-1 specific to AcTub, anti-mouse and anti-rabbit IgGs conjugated to peroxidase, fluorescein-conjugated anti-mouse IgG, rhodamine-conjugated anti-rabbit Ig, and several protease inhibitors (bestatin, TLCK, antipain, chymostatin, pepstatin, leupeptin, E64, PMSF, 1,10-phenanthroline) were from Sigma Chemical Co. (St. Louis, MO, USA). [γ-^{32}P]ATP was from Perkin-Elmer (Wellesley, MA, USA). Rabbit polyclonal antibody Pma1p specific to H^+-ATPase was provided by R. Serrano (Instituto de Biología Molecular y Celular de Plantas, Valencia, Spain). 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester (BCECF-AM) was from Molecular Probes/Invitrogen, Grand Island, NY, USA. The non-esterified form of this compound (BCECF) was from Fluka, St Louis, MO, USA.

2.2. Yeast strains and growth conditions

S. cerevisiae strain CECT 1891 was from Spanish Type Culture Collection, Univ. of Valencia, Valencia, Spain. Knockout yeast strains (in which the deleted gene was replaced by the kanamycin resistance gene *kanMX4*) were from EUROSCARF (Frankfurt, Germany). Cells were grown on synthetic medium YP [0.5% (w/v) yeast extract and 0.5% (w/v) peptone] containing 4% (w/v) glucose in a 200 rpm rotary incubator (model G24; New Brunswick Scientific, Enfield, NJ, USA) at 30 °C until the end of the exponential phase. Some cells (referred to as "glucose-starved cells") were harvested by centrifugation (1000 ×*g*, 10 min), suspended in Mes/Tris buffer (100 mM Mes/Tris, pH 6.5), and magnetically stirred for 60 min to eliminate glucose activation [1]. A protease inhibitor cocktail was composed of 8 mM benzamidine, 1 mM phenanthroline, 0.5 mM aprotinin, and 1 mM PMSF.

2.3. Cloning

The LPX1 gene was cloned using conventional molecular biology techniques. Forward primer: 5′AAAGGTACCATGGAACAGAACAGGTCC3′; reverse primer: 5′TTTTCTAGATTACAGTTTTTGTTTAGTCG3′; introduces a *Kpn1* and a*Xba1* site, respectively. Briefly, upon grade PCR reaction using yeast genomic DNA as template, the LPX1 gene was digested with *Kpn1* and *Xba1* and then inserted in the same sites of the previously digested yCPlac33 derived plasmid pJV29 (provided by Dr. J. Valdez-Taubas, CIQUIBIC, CONICET, Córdoba, Argentina). Transformed yeast cells were selected in adequate medium (0.2% yeast nitrogen base without amino acids [Difco], 0.6% ammonium sulfate, 55 mg/L adenine, 55 mg/L tyrosine, 2% D + -glucose, 2% agar–agar) plus the corresponding amino acid Drop Out solution. *Escherichia coli* strain DH5α was used for plasmid amplification in bacteria. The absence of undesired mutations was confirmed by DNA sequencing (DNA Sequencing and Genotyping Facility, Univ. of Chicago, IL, USA).

2.4. Yeast cell homogenization, detergent-solubilized yeast preparation, and plasma membrane fraction preparation

Yeast cells were suspended in Mes–Tris buffer supplemented (or not) with 1 mM PMSF, homogenized by vigorous shaking with glass beads, and centrifuged at 1000 ×*g* for 10 min to yield "supernatant A". In one procedure, 1000 g of supernatant A was added with Triton X-100 (final concentration 0.1%), agitated gently for 5 min, and centrifuged (70,000 ×*g*, 15 min, 4 °C). The resulting supernatant ("detergent-solubilized yeast preparation") was stored at −20 °C until use. In a separate procedure, a plasma membrane fraction was prepared as described by Villalba et al. [16]. Supernatant A (1000 g) was centrifuged at 70,000 ×*g* for 60 min to obtain a total membrane fraction. A total membrane fraction from 10 g yeast was suspended in 3 mL Mes/Tris buffer containing 1 mM PMSF and the suspension was applied to a discontinuous gradient from 5.0 mL of 60% (w/v) sucrose to 5.0 mL of 40% (w/v) sucrose in Tris–HCl buffer (10 mM Tris–HCl, pH 7.6, 1 mM EDTA, 1 mM dithiothreitol). The plasma membranes were centrifuged for 3 h at 100,000 ×*g*, collected from the 40/60% sucrose interface, diluted 10-fold with Mes–Tris/PMSF buffer, and centrifuged at 100,000 ×*g* for 1 h. The resulting pellet was resuspended in Mes–Tris/PMSF buffer and stored at −70 °C until use (maximum storage time = 3 months). When PMSF-free membranes were used, the stored membranes were prewashed by centrifugation.

2.5. Plasma membrane H^+-ATPase activity assay

H^+-ATPase activity was assayed by the [γ-^{32}P]ATP hydrolysis method of [17]. The incubation mixture (final volume 0.5 mL) consisted of Mes–Tris/PMSF buffer, 10 mM $MgCl_2$, 2 mM [γ-^{32}P]ATP (450 dpm/nmol) and 10 μg/mL plasma membrane protein. The reaction which proceeded for 20 min at 30 °C, was stopped by adding 50 μL of 66% (w/v) trichloroacetic acid per mL of incubation mixture, and the released [γ-^{32}P]Pi was quantified. The plasma membrane H^+-ATPase activity was calculated as the difference of ATP hydrolysis in the presence *vs.* absence of 100 μM sodium orthovanadate.

2.6. Proteolytic assay

Proteolytic activity based on tubulin degradation was determined by electrophoresis, immunoblotting, and densitometry of the tubulin band. A protein fraction (5–10 μg protein) was added to the reaction mixture (100 mM Mes–Tris, pH 6.5, containing 2 mM $MgCl_2$) in a final volume of 35 μL. The mixture was kept for 10 min at room temperature, and the reaction was initiated by the addition of 8 μg purified rat brain tubulin. Incubation was continued for 15 min at 30 °C, the reaction was stopped by heating (95 °C for 5 min), 30 μL Laemmli buffer was added, and tubulin degradation fragments were determined by SDS-PAGE and immunoblotting as described in the figure legends.

2.7. Size exclusion chromatography

A protein preparation (~0.8 mg/mL) solubilized in phosphate buffer (50 mM NaH_2PO_4, pH 6.8) was subjected to molecular exclusion

chromatography on an SE-FPLC system (ISCO Inc, Lincoln, NE, USA), using a BioSep-Sec®-S3000 column (300 × 7.8 mm; Phenomenex, Torrance, CA, USA) pre-equilibrated with phosphate buffer at a flow rate of 1.5 mL/min. Running conditions: volume sample, 500 μL; flow rate, 1 mL/min; fraction volume, 1 mL; room temperature. Molecular weight standards (Sigma) were separated under identical conditions. Proteolytic activity toward tubulin was assayed in the collected fractions.

2.8. Ion exchange chromatography

A Q-Sepharose column (1 × 7 cm, 0.25 meq/mL; Sigma) was equilibrated with Buffer A (10 mM Tris–HCl, pH 7.0). A fraction with proteolytic activity (10 mg/mL) was applied to the column at a flow rate of 30 mL/h. The column was washed with Buffer A until all unbound proteins were removed. The bound proteins were eluted using a discontinuous NaCl gradient (0–1.0 M NaCl) in Buffer A. Proteolytic activity toward tubulin was assayed in the collected fractions. The active fractions were pooled, dialyzed against Buffer B (10 mM Tris–HCl, pH 7.5), and concentrated in a Centrivap concentrator (Labconco, Kansas, MO, USA). The resulting protein solution was loaded onto a CM-cellulose column (1 × 8 cm, 0.7 meq/g; Bio-Rad Laboratories, Hercules, CA, USA) pre-equilibrated with Buffer B and separated at a flow rate of 12 mL/h. The bound proteins were eluted using a discontinuous NaCl gradient (0–1.0 M NaCl) in Buffer B. Proteolytic activity was assayed in each collected fraction. Excess salt was removed, and the proteins were used immediately or stored at −20 °C.

2.9. One-dimensional electrophoresis and immunoblotting

Proteins were separated by SDS-PAGE on 10% (w/v) polyacrylamide slab gels [18], transferred to nitrocellulose sheets, and reacted with mouse mAb6-11B-1 (dilution 1:1000) to determine AcTub content [19], with mouse mAbDM1A (dilution 1:1000) to determine total α-tubulin content, or with rabbit polyclonal antibody Pma1p (dilution 1:1000) to determine plasma membrane H^+-ATPase activity. Each sheet was reacted with the corresponding anti-IgG antibody conjugated with peroxidase and stained using the 4-chloro-1-naphthol method. Band intensities were quantified using the Scion Image program.

2.10. Two-dimensional electrophoresis

Proteins (25–50 μg) were dialyzed against water, vacuum concentrated in a rotary concentrator and solubilized in a solution of 60% glycerol and 4% ampholytes. Isoelectric focusing was performed in a 1.5 mm, 7.5% acrylamide and 1.8% pH 3–10 carrier ampholyte gel (Sigma). Isoelectric point markers (4.45–9.6; BioRad) were included. Electrophoresis was performed at 200 V for 2.5 h at 4 °C. When the first dimension was finished, a strip containing the isoelectric point markers and a strip with sample proteins were stained with Coomassie Brilliant Blue R-250. Another strip with sample proteins was incubated in equilibration buffer (75 mM Tris-HCl, pH 8.8, 30% glycerol, 2% SDS) for 30 min at room temperature. The equilibrated strip was applied onto a 10% acrylamide gel and proteins were separated in the second dimension.

2.11. Proteolytic activity assay on copolymerized tubulin-polyacrylamide gel

In gel proteolytic activity toward tubulin was assayed by the method of Heussen and Dowdle [20] with slight modifications. Purified mouse brain tubulin (1.5 mg/mL) was copolymerized with the SDS-polyacrylamide gel matrix at the time of casting, providing *in situ* substrates for the separated protease bands. Protease-containing samples were treated with Laemmli buffer (without 2-mercaptoethanol) without heating or other denaturing conditions. Electrophoresis was performed at 4 °C at a constant current of 8 mA for ~5 h. The gel was then gently agitated at room temperature for 1 h in 2.5% Triton X-100 in water to remove SDS. The gel slabs were transferred to a bath containing 0.1 M glycine-NaOH, pH 8.3, incubated at 37 °C for 3–5 h, fixed and stained by immersion for 1 h in a 0.1% solution of Amido Black in methanol: acetic acid: water (30:10:60) and destained with the same solvent solution.

2.12. Tubulin preparations

Rat brain tubulin preparations containing differing proportions of the acetylated isotype were isolated as described previously [10]. These preparations contained low and high AcTub proportions that differed by a factor of ~4.

2.13. Yeast tubulin preparations

High-quality yeast tubulin preparations were obtained by the method of Bellocq et al. [21] with slight modification. Cells grown in YPD medium were harvested, washed twice in water, resuspended in Mes–Tris buffer supplemented with 1 mM PMSF and lysed by shaking with glass beads. The homogenate was centrifuged at 100,000 ×*g* for 60 min, and the resulting supernatant was loaded on a 7 mL DEAE-Sephadex A-50 column pre-equilibrated with Buffer 1(100 mM Pipes/KOH, pH 6.9, 1 mM $MgCl_2$, 2 mM EGTA) supplemented with 0.2 M KCl. The column was washed with 10 volumes of Buffer 1, and tubulin was eluted by Buffer 1 supplemented with 0.5 M KCl. This material was loaded onto an immunoaffinity column prepared as described by Paturle et al. [22] by coupling mAbDM1A to cyanogen bromide-activated Sepharose4B at an antibody concentration of 5 mg/mL activated gel. The column, pre-equilibrated with Buffer 1, was loaded with tubulin-containing fractions from the preceding DEAE-Sephadex column and washed with 30 bed volumes of Buffer 1 containing 0.1 M KCl. Tubulin fractions (~8 mL) were eluted by raising the concentration of KCl in Buffer 1 to 0.8 M and immediately desalting in Bio-Gel P-6DG. The resulting sample was concentrated to ~1.0 mg/mL with a Centricon-30 microconcentrator (Amicon, Billerica, MA, USA). The concentrated tubulin was divided into aliquots, frozen in liquid nitrogen, and stored at −70 °C.

2.14. Extracellular pH measurements

Extracellular pH (pH_e) was measured fluorometrically using BCECF. Cells were grown on synthetic medium YP [0.5% (w/v) yeast extract and 0.5% (w/v) peptone] containing 4% (w/v) glucose at 30 °C until the end of the exponential phase and them cells were harvested by centrifugation (1000 ×*g*, 10 min), suspended in Mes/Tris buffer (100 mM Mes/Tris, pH 6.5), and magnetically stirred for 60 min to eliminate glucose activation. After starvation, cells were counted in a Neubauer chamber, resuspended to a final density of 5×10^7–1×10^8 cells/mL in physiological solution (0.9% NaCl) supplemented with 1 mM $MgCl_2$ and 2 μM BCECF (final volume1400 μL). The fluorescence ratios (excitation wavelength 500–440 nm; emission wavelength 530 nm) were recorded and translated into pH values based on the ratios obtained at various pH values [23]. See the calibration curve showed in Fig. 1A. The stimulus volume was never greater than 5% of the final volume of the mixture.

2.15. Protein concentrations

Protein concentrations were determined by the method of Bradford [24].

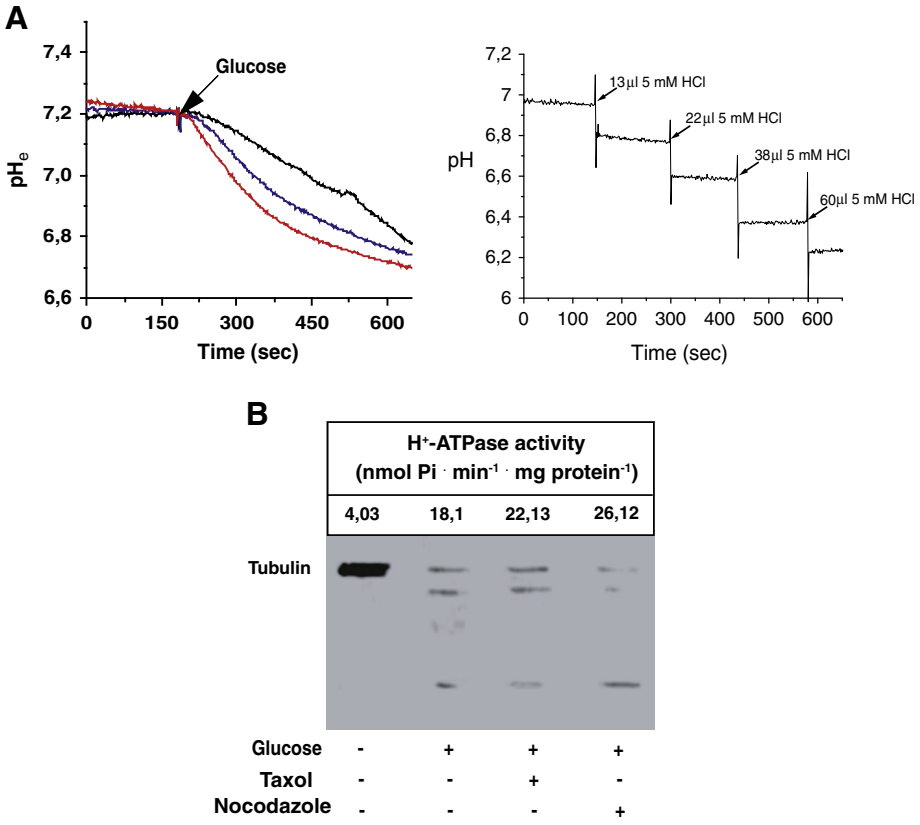


Fig. 1. Effects of glucose, taxol, and nocodazole on extracellular pH and on membrane tubulin integrity in *S. cerevisiae*. Starved yeast cells were incubated in the absence (black) or presence of 5 μM taxol (red) or 50 μM nocodazole (blue) for 30 min at 30 °C. The cells (5×10^7) were washed twice with 100 mM Mes–Tris, pH 6.8, and added with BCECF for the determination of pH_e (A, left) (see Materials and Methods). 10 mM glucose was added at 180 s (arrow). The calibration curve (A, right) was performed by adding an amount of 5 mM HCl capable of decrease 0.2 pH units and fluorescence was registered and transformed in pH units. (B) Tubulin proteolysis in plasma membrane (50 μg protein) was examined by immunoblotting using mAb6-11B-1. The membranes analyzed were obtained from cells processed as described in (A) immediately before or 420 s after glucose addition. Plasma membrane H^+-ATPase activity was assayed as described in Materials and Methods [17]. The curves in A, from fluorescence assays, are representative of two independent experiments.

3. Results

3.1. Effects of glucose on H^+-ATPase activation and membrane tubulin integrity

We found previously that glucose treatment of *S. cerevisiae* leads to H^+-ATPase activation through the dissociation of the AcTub/H^+-ATPase complex [8]. In the present study, we examined the effects of glucose treatment on extracellular pH as a measure of H^+-ATPase activity and tubulin membrane integrity. After glucose addition, yeast cells display a fast and short decrease in pH_i [25,26] that is quickly restored by H^+ removal from cytoplasm in hands of the vacuolar V-ATPase and Pma1p [27], leading to cytoplasm alkalinization, and vacuole and extracellular media acidification. By 7 min after the addition of glucose (Fig. 1A, "600 s" on abscissa), the pH of the extracellular medium decreased by 0.6 and 0.5 units due to Pma1p activation [1]. Glucose treatment also eliminated the co-localization of AcTub with H^+-ATPase [8].We attribute this loss of co-localization to the dissociation of the AcTub/H^+-ATPase complex. Interestingly, glucose treatment induced membrane tubulin degradation (Fig. 1B), suggesting that the dissociation of the AcTub/H^+-ATPase complex may be a consequence of tubulin degradation.

In our previous studies of other cell types, we found that microtubule stability affected the enzymatic activity of certain P-type ATPases (Na^+, K^+-ATPase, Ca^{2+}-ATPase) [12,13]. We therefore examined the effects of microtubule stability-modifying drugs (taxol, nocodazole) on H^+-ATPase activity and membrane tubulin content in the present study. Glucose-starved cells were pre-incubated with taxol or nocodazole and H^+-ATPase activity was then induced by glucose addition. The presence of taxol or nocodazole prior to glucose addition did not affect pH_e (Fig. 1A). The H^+-ATPase activity as determined by the [γ-^{32}P]ATP hydrolysis method and the membrane tubulin content were also not significantly affected by taxol or nocodazole treatment (data not shown). Glucose addition caused a more pronounced decrease in pH_e in cells that were pretreated with taxol or nocodazole than in cells that were not pretreated. For example, the pH_e decrease 2 min after glucose addition (300 s on the abscissa) was 0.2 and 0.3 units, respectively, for taxol- and nocodazole-pretreated cells but only 0.1 unit for non-pretreated cells (Fig. 1A), this enhanced ability to decrease pH_e in taxol and nocodazole pre-treated cells correlates with an increase in H^+-ATPase activity of 22,2% and 44%, respectively, when compared with non treated cells (Fig. 1B). Treatment of cells with these drugs did not affect glucose-induced tubulin degradation (Fig. 1B). These findings indicate that taxol and nocodazole efficiently facilitate the activation of H^+-ATPase by glucose in yeast.

Glucose addition caused the hydrolysis of tubulin in the membrane (Fig. 1B) and it is also known that glucose leads to a short term pH_i decrease [25,26]. To determine whether these effects were related, we incubated yeast plasma membranes at various pH levels and measured the subsequent H^+-ATPase activity and tubulin integrity. At pH 6.2 and 6.5, the H^+-ATPase activity was maximal and was correlated with the amount of hydrolyzed membrane tubulin (Fig. 2A). This finding is consistent with the idea that a decrease in physiological pH activates a protease that hydrolyzes membrane tubulin and consequently activates the proton pump. This idea is also consistent with the observations that membrane tubulin underwent partial proteolysis and H^+-ATPase activity increased 4.5-fold in glucose-treated cells (Fig. 2B). When cells were treated with a protease inhibitor cocktail prior to glucose addition, there

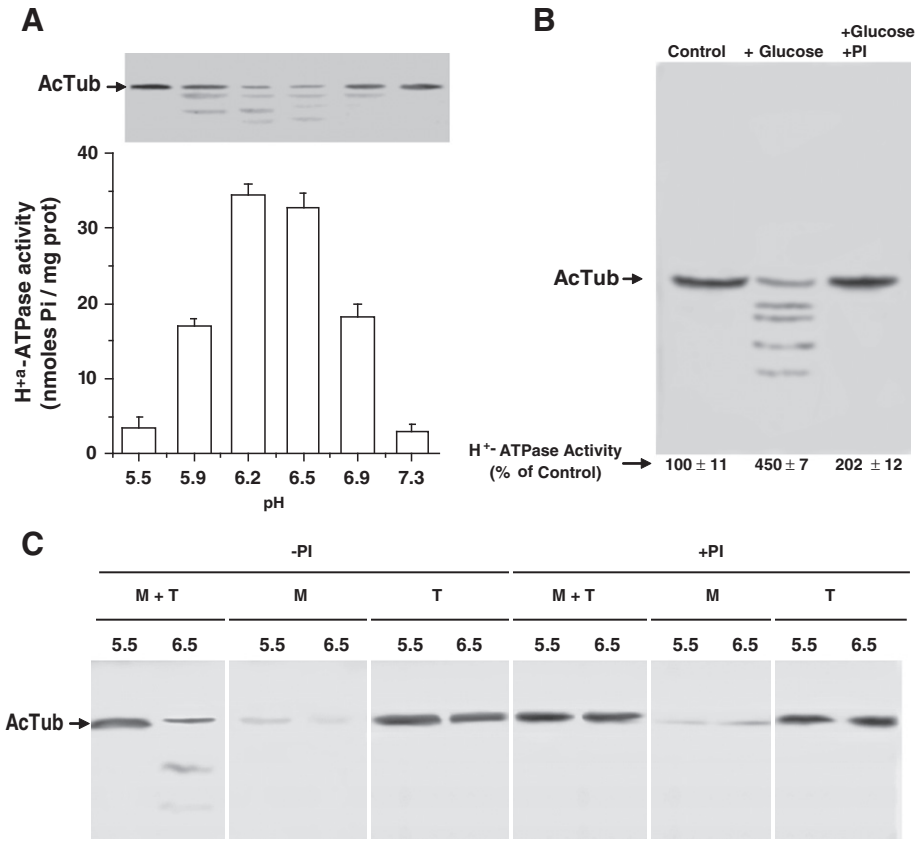


Fig. 2. *In vitro* and *in vivo* degradation of membrane tubulin. (A) Plasma membranes from starved yeast cells were incubated at various pH values in 100 mM Mes–Tris buffer for 30 min at 30 °C and washed twice with the same buffer. H^+-ATPase activity was measured as described in Material and Methods and membrane tubulin was analyzed by Western blotting using mAb6-11B-1. (B) Cells starved for 2 h were incubated with (+PI) or without (−PI) protease inhibitor cocktail for 30 min, added with glucose (final concentration 10 mM) and incubated for 10 min. Membranes were isolated, and H^+-ATPase activity and membrane tubulin were analyzed as in A. (C) Plasma membranes (1 mg protein) from starved cells were incubated with purified rat brain tubulin (200 mg protein) in the presence or absence of protease inhibitor cocktail at pH 5.5 or 6.5 and incubated for 30 min at 30 °C. Tubulin degradation was measured by immunoblotting. The self-degradation of plasma membrane endogenous tubulin and pure brain tubulin was controlled. M + T: yeast plasma membrane + tubulin; M: yeast plasma membrane + MES-Tris buffer; T: tubulin + MES-Tris buffer. ATPase activity values are the mean ± SD from three independent experiments. The AcTub bands and degradation fragments shown are from representative experiments.

was only a 2-fold increase in H^+-ATPase activity and no proteolysis was detected in membrane tubulin (Fig. 2B). These findings suggest that H^+-ATPase activation is due entirely or in part to a mechanism that involves membrane tubulin proteolysis. We demonstrated the presence of a protease in the yeast membrane by incubating a mixture of isolated membranes and purified mouse brain tubulin in the presence *vs.* absence of the protease inhibitor cocktail at pH 5.5 and 6.5. The tubulin was degraded at pH 6.5 in the absence but not in the presence of protease inhibitors (Fig. 2C). No tubulin proteolysis was observed at pH 5.5 regardless of the presence or absence of protease inhibitors. These findings indicate that the membrane protease activity in these cells degrades not only membrane tubulin but also soluble tubulin. The degradation of membrane tubulin is not obvious in Fig. 2C because a low amount of membrane was incubated to avoid masking the degradation of exogenous tubulin.

3.2. Biochemical characterization of a tubulin-hydrolyzing protease

We performed pull-down experiments to isolate and characterize the protease that hydrolyzes membrane-associated or soluble AcTub. Tubulin was used as an affinity ligand to precipitate all tubulin-interacting proteins from yeast cell homogenate. Purified mouse brain tubulin or yeast tubulin was covalently bound to Sepharose beads, and the resin was incubated with a yeast total homogenate in a detergent-containing buffer. Sepharose-tubulin beads with bound proteins were washed 3 times with Mes–Tris buffer to eliminate nonspecific bound proteins. The presence of a protease in the precipitate was determined by incubating the beads at 37 °C and analyzing the products by Western blot. Yeast tubulin bound to Sepharose was able to precipitate protease activity from the total homogenate (Fig. 3A). The Western blots appeared to show that the proteases retained by the tubulin-Sepharose resin hydrolyzed yeast tubulin more efficiently than mouse tubulin; ~80% of yeast tubulin but only ~5–10% of mouse tubulin was degraded in 80 min. This observation may be due to a higher affinity of the protease for yeast tubulin, either because of higher proteolytic activity or a greater amount of protease precipitated with Sepharose-tubulin.

The proteins retained by Sepharose-tubulin were eluted by a solution of 10 mM Tris–HCl, pH 7, containing 0.5 M NaCl and subjected to size exclusion chromatography. Tubulin degradation activity was measured in all of the eluted fractions. Fractions 10 to 14 displayed tubulin proteolytic activity (Fig. 3B). The quantification of hydrolyzed tubulin indicated that fraction 12 was the most active fraction (Fig. 3C). The protease had an estimated molecular mass of ~35–50 kDa based on a comparison with the chromatographic behavior of standard proteins (result not shown). To obtain a more accurate estimate of the molecular mass of the protease, the proteins in fraction 12 were subjected to semi-denaturing electrophoresis to detect proteolytic activity against tubulin *in situ*. Following protein separation, we observed tubulin degradation in the region corresponding to 35–40 kDa in the gel (Fig. 3D). No such degradation zone was observed in the control gel (which contained gelatin instead of tubulin), suggesting that the protease was substrate-specific for tubulin. Those fractions that showed proteolytic activity after size-exclusion chromatography were pooled and subjected to anion (Fig. 4A) and cation (Fig. 4B) exchange chromatography and tubulin degradation activity was monitored by Western blotting. In the anion column (equilibrated with buffer at pH 7), the protease

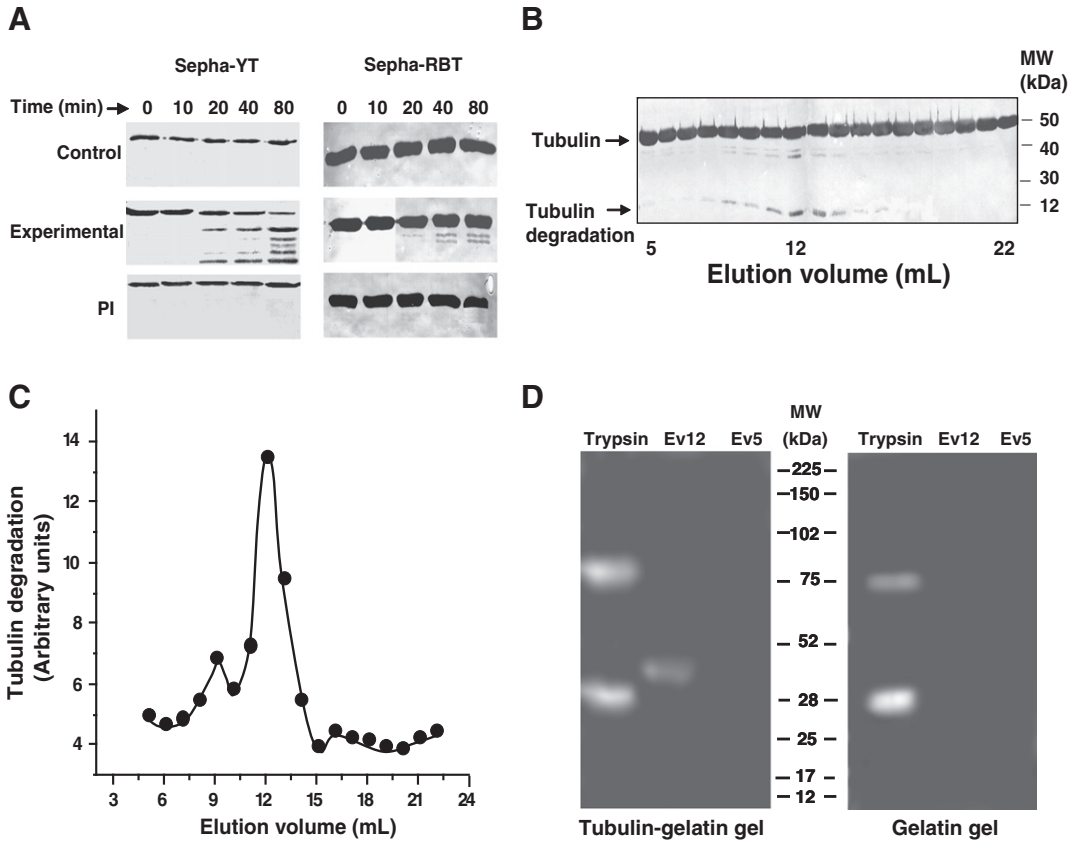


Fig. 3. Isolation of the tubulin-degrading protease and determination of its molecular mass. Equal amounts of tubulin purified from yeast or rat brain linked to Sepharose beads (Sepha-YT and Sepha-RBT, respectively) were added to a freshly obtained detergent-solubilized yeast preparation (see Materials and Methods) and incubated for 12 h at 4 °C with gentle agitation. The mixtures were centrifuged and the precipitated materials were washed 5 times with Mes/Tris buffer containing 0.1% Triton X-100. (A) Fractions (50 μL) of packed beads were incubated at 30 °C for the indicated times. Control: Mes/Tris buffer alone was used instead of total homogenate, PI: added with protease inhibitor cocktail. The samples were resuspended in 50 μL Laemmli sample buffer, heated at 50 °C for 15 min, and centrifuged. Aliquots (20 μL) of the soluble fractions were subjected to SDS-PAGE. Proteins were transferred to nitrocellulose sheets and tubulin content was determined by Western blotting using mAb6-11B-1as described in Materials and Methods. (B) A fraction (200 μL) of packed Sepha-YT with the protein precipitated from total homogenate was incubated with Tris-buffered saline (300 μL) at 4 °C for 30 min and centrifuged. The supernatant proteins were separated by SE-HPLC using a Biosep-sec-S-3000 column as stationary phase and 50 mM NaH_2PO_4 buffer, pH 6.8 as mobile phase. The flux was 1 mL/min. The tubulin-degrading activity was measured in each Ev (Elution volume) by Western blotting using mAb6-11B-1. (C) Quantification of the resulting tubulin fragments. (D) Proteins from Ev 5 and 12 were separated by semi-denaturing PAGE in slab gels in which acrylamide was copolymerized with purified tubulin (1.5 mg/mL) and gelatin (10 mg/mL) as a control for specific tubulin proteolysis. After the separation, the SDS was removed and the gels were incubated in 0.1 M glycine, pH 8.3 to allow the development of proteolytic activity. Amido Black negative stain was used to reveal the areas of proteolytic activity. Swine trypsin was used as a positive control.

activity was not retained. In the cation column (equilibrated with buffer at pH 7), the protease activity was retained, and treatment with 0.5 M NaCl was necessary to elute the activity from the column.

The proteins contained in the most active fractions eluted from the cation exchange column were concentrated and subjected to 2-dimensional gel electrophoresis (Fig. 4C). When tubulin was present in the gel during the non-reducing SDS-PAGE step, proteolytic activity was detected in the gel in the zone of pH 8–9 and 35–40 kDa. In a parallel experiment using gelatin instead of tubulin, no degradation zone was observed (data not shown).

To classify our protease in relation to the many known protease families, we investigated H^+-ATPase activation and tubulin degradation *in vivo* in the presence of various protease inhibitors, which has been shown previously to be cell permeable [28–33]. The effects of these inhibitors on pH_e (as an indirect measurement of H^+-ATPase activity) and on tubulin degradation are shown in Fig. 5. PMSF and to a lesser degree phenanthroline produced inhibition of H^+-ATPase activity that was correlated with no (or little) degradation of membrane tubulin (Fig. 5A, B). Bestatin had no effect on pH_e or on tubulin degradation (Fig. 5C). Other protease inhibitors (chymostatin, pepstatin, leupeptin, TLCK, E64) also had no effect on these parameters (data not shown). Analysis of the amount of H^+ pumped showed that treatment with PMSF and phenanthroline resulted in the reduction of H^+-ATPase activity to 18% and 60% of the control level, respectively (Fig. 5E). This reduction was also confirmed by measuring the ATP hydrolyzing activity of isolated plasma membranes in each condition (Fig. 5E). Tubulin degradation of 5% and 27% was observed in membrane of cells treated with PMSF and phenanthroline, respectively. Results similar to those for PMSF were obtained for antipain, another serine protease inhibitor (data not shown). PMSF and to a lesser degree phenanthroline inhibited rat brain tubulin degradation (Fig. 5D) catalyzed by the most active fraction eluted from the cation chromatography (Fig. 4B), suggesting that the protease isolated from the detergent-solubilized yeast preparation (Fig. 3) was the same as the membrane protease involved in the regulation of the H^+-ATPase through interaction with tubulin. Collectively, the above findings indicate that a serine protease is involved in H^+-ATPase activation by glucose in yeast.

3.3. Analyses of protease knockout yeast strains

The protease involved in the regulation of H^+-ATPase activity has the following characteristics, based on the results described above: (1) It is a serine protease whose catalytic function probably requires a divalent cation; (2) — It has a molecular mass ~40 kDa and an isoelectric point between 8 and 9; and (3) — It is capable of associating with tubulin. We performed searches of the Comprehensive Yeast Genome Database (http://mips.helmholtz-muenchen.de/genre/proj/yeast/) and the Saccharomyces Genome Database (http://www.yeastgenome.org/) based

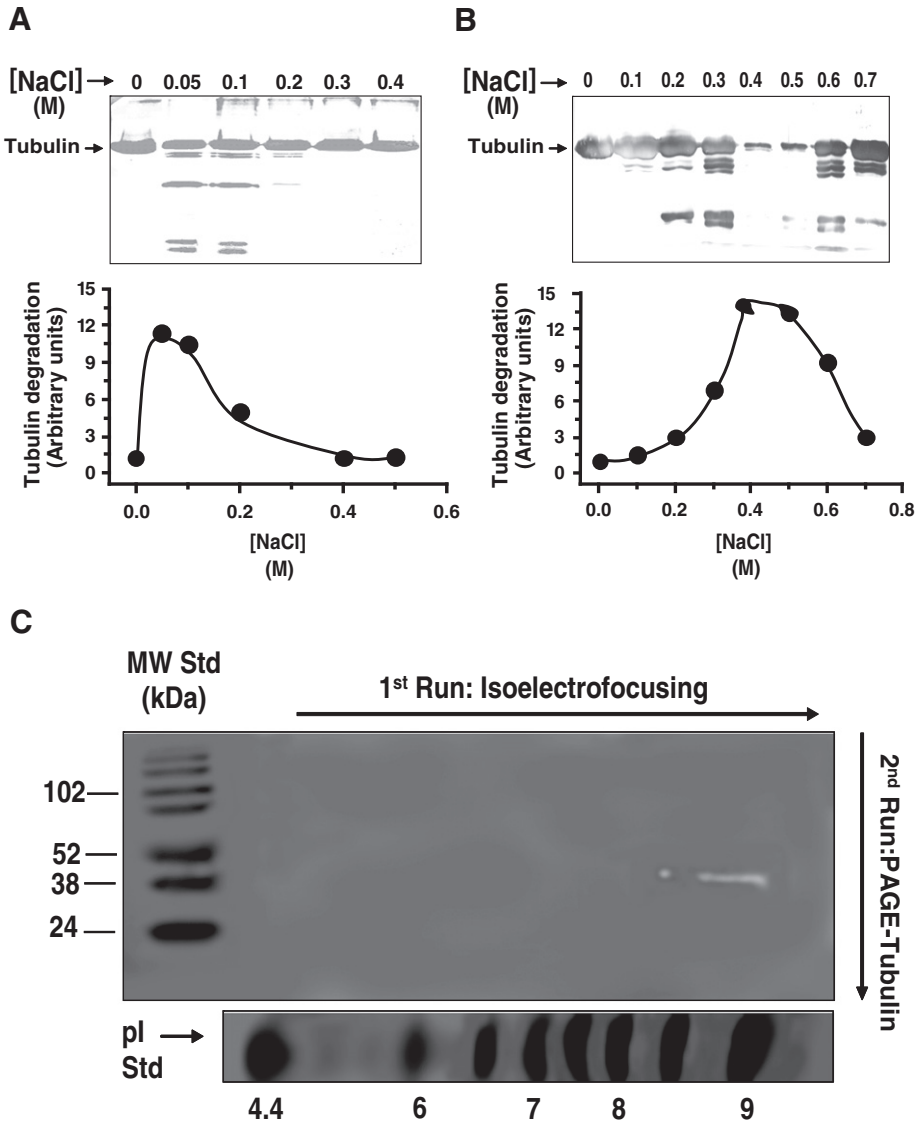
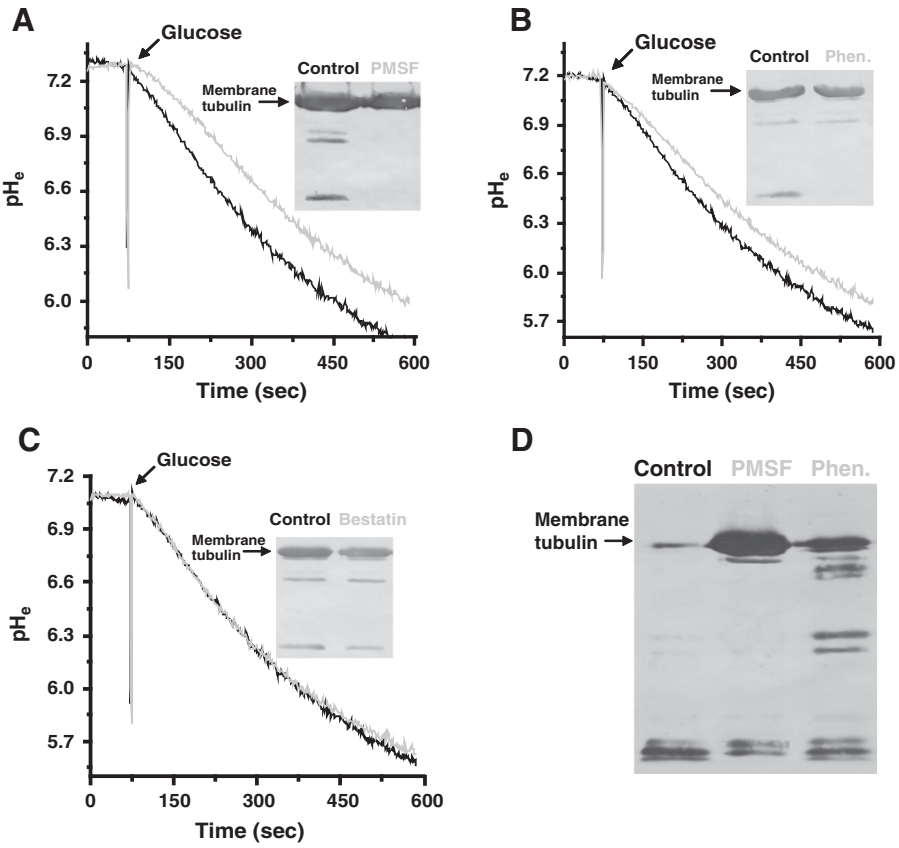


Fig. 4. Determination of the isoelectric point of the tubulin-degrading protease. (A) Proteins from Ev 12 isolated as described in Fig. 3B were applied to a 1 × 7 cm Q-Sepharose column equilibrated with Tris–HCl 10 mM, pH 7 (see Materials and Methods) and eluted with a discontinuous gradient of 0.05 to 1 M NaCl in the same starting buffer. Fractions (1 mL each) were collected and dialyzed against the starting buffer, and tubulin-degrading activity was assayed by Western blotting using mAb6-11B-1. Lower panel: quantification of resulting tubulin fragments. (B) Active fractions from A were combined, concentrated with a Centrivap concentrator to 0.7 mL, placed on a 1 × 8 cm cation exchange cellulose cellex-CM column equilibrated with Tris–HCl 10 mM, pH 7.5 and eluted with a discontinuous gradient of 0.05 to 1 M NaCl in the same starting buffer. Tubulin-degrading activity was assayed as described in A. (C) Active fractions from B (0.3 and 0.4 M NaCl) were dialyzed, concentrated and subjected to 2-dimensional electrophoresis. The first dimension isoelectric focusing step was performed using a 7.5% acrylamide, 1.8% 3–10 "carrier" ampholytes (Sigma) gel. pI markers with a 4.45–9.6 range (BioRad) were used. The electrophoresis was run at 200 V for 2.5 h. Following isoelectric focusing, a sample strip was incubated in equilibration buffer (75 mM Tris–HCl, pH 8.8, 30% glycerol, 2% SDS). Equilibrated strips were subjected to semi-native electrophoresis on SDS-PAGE in which purified tubulin (1.5 mg/mL) was copolymerized. Following protein separation and the removal of SDS, the gel was incubated in 0.1 M glycine-NaOH, pH 8.3 for 4 h to develop proteolytic activity, which was visualized by negative staining with Amido Black.

on these characteristics. The resulting candidate was the protein Lpx1p, coded by the YOR 084 open reading frame, which has molecular mass 44 kDa, isoelectric point 8.4, and ubiquitous subcellular localization. Based on fluorescence microscopy and sedimentation assays, Thoms et al. [34] described Lpx1p as a peroxisomal protein that displays *in vitro* acyl hydrolase and phospholipase A activities. Its delivery to the peroxisome depends on the QKL motif in its PTS1 (Peroxisomal Targeting Signal) domain. These authors also found Lpx1p remaining in cytosolic fractions. Other yeast proteins that contain the QKL motif are not targeted to peroxisomes [35,36]. To test whether Lpx1p is the same protease that is involved in the activation of H^+-ATPase by glucose *via* tubulin degradation, we determined the capacity of strain *lpx1Δ* to activate H^+-ATPase through membrane tubulin hydrolysis. H^+-ATPase activation (as estimated by change of pH_e) was detectable immediately after glucose addition in wild-type cells but was not detectable until ~4 min after glucose addition in *lpx1Δ* cells (Fig. 6A). Wild type cells displayed fast activation, and H^+-ATPase activity was decreasing slowly with the time (see the data from the light blue and pink region in Fig. 6A, analyzed in 6C). Interestingly, in *lpx1Δ* cells, H^+-ATPase activation was delayed but once it was activated (3 min after glucose addition), the rate of H^+ pumping was enhanced by a 50% if compared with the wild type. Astonishingly, at around 9 min after glucose addition, the amount of H^+ that was pumped out of the cell was almost the same, indicating that provably it is the maximum time available to the cell to regulate the pH before suffering stress related to poor regulation of intracellular pH. Membrane tubulin was rapidly degraded and released from the plasma membrane following glucose addition in wild-type cells but remained essentially intact in membrane for >9 min following glucose addition in *lpx1Δ* cells (Fig. 6B). There was an inverse correlation between H^+-ATPase activity and membrane tubulin content measured 5 min after glucose addition; the values for these two parameters for *lpx1Δ* cells were 5.9% and 98%, respectively, of the values for



E

Inhibitor	H^+-ATPase activity: nmol Pi hydrolyzed · min^{-1} · mg $protein^{-1}$	% of control	H^+-efflux * t^{-1} * 10^{-10} (M/sec)	% of control	Tubulin degradation: Arbitrary units	% of control
Control	20.14 ± 1.71	100 ± 11	9.3 ± 1.3	100 ± 14	44 ± 5.0	100.0± 11
PMSF	5.4 ± 0.41	26 ± 3	1.7 ± 0.3	18 ± 3	2 ± 0.4	4.6 ± 1
Phen.	10.73 ± 0.89	53 ± 4	5.6 ± 0.4	60 ± 4	12 ± 5.0	27.3 ± 11

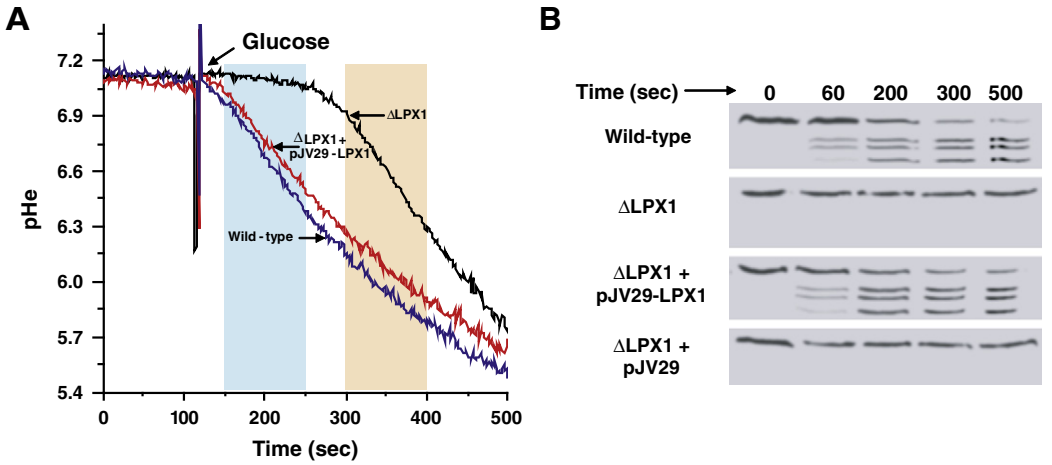
Fig. 5. *In vivo* and *in vitro* effects of protease inhibitors on H^+-ATPase activation and tubulin proteolysis. Starved yeast cells were incubated in the presence (gray lines) or absence (black lines) of 1 mM PMSF (A), 1 mM phenanthroline (B), or 0.1 mM bestatin (C) for 30 min at 30 °C. The cells were washed twice with 100 mM Mes–Tris, pH 6.8, and 5×10^7cells were loaded with 1.4 mL of a solution containing 0.9% NaCl, 5 mM $MgCl_2$, and 2 μM BCECF, pH 7 in a cuvette and placed in a thermostatically controlled spectrofluorometer. Extracellular pH values were calculated from BCECF ratio emission values at wavelength 530 nm with excitation at 440 and 500 nm and following the calibration curve showed in Fig. 1 A. Glucose (10 mM) was added (arrows), and 5 min later 4×10^7 cells were subjected to plasma membrane protein extraction. Membrane tubulin degradation was analyzed by Western blotting with mAb 6-11B-1 (inserts). (D) Purified tubulin from rat brain (7 μg protein) and proteins (5 μg) from the 0.3 M NaCl elution fraction of CM-methylcellulose chromatography (obtained as in Fig. 4) were mixed in Mes/Tris buffer (final volume 25 μL) in the presence or absence of 1 mM PMSF or 1 mM phenanthroline. The samples were incubated for 10 min at 30 °C, and tubulin proteolysis was determined by immunoblotting. (E) H^+-ATPase activity was either calculated as H^+ released per unit time (mol/s) for PMSF, phenanthroline, and control treatments, using the curves in A and B, or by the [γ-^{32}P]ATP hydrolysis method in isolated plasma membranes [17]. Tubulin degradation was quantified from bands of hydrolyzed tubulin in D by Western blotting with mAb6-11B-1; values are expressed in arbitrary units. Data from fluorescence assays, H^+-ATPase activity and tubulin band quantification are means ± SD from three independent experiments.

wild-type cells (Fig. 6C). Thus, the promoting effect of glucose on H^+-ATPase activity was delayed in *lpx1Δ* cells, presumably because of the absence of the protease that removes tubulin from the plasma membrane. Proteases other than Lpx1p (*e.g.*, Kex1p, Yuh1p, Ysp3p) with comparable biochemical behaviors were assayed but did not appear to be involved in H^+-ATPase activation (data not shown). The complementation of the *lpx1Δ* strain with the wild-type gene (*lpx1Δ* + pJV29-LPX1) caused the reversion of the mutant phenotype to the wild-type phenotype (Fig. 6). The vector was not responsible for this effect (see results for *lpx1Δ* + pJV29 [empty vector]). Collectively, these findings clearly indicate that Lpx1p is involved in the mechanism of H^+-ATPase activation in *S. cerevisiae*, through the direct or indirect degradation of plasma membrane tubulin.

4. Discussion

The activation of plasma membrane H^+-ATPase by glucose in *S. cerevisiae* was first described by Serrano et al. [1]. Several mechanisms for this activation process have been proposed, including Ptk2p-mediated phosphorylation of Pma1p at its C-terminus [1,37], an increase in PMA1 expression involving Rap1p and Gcr1p [38,39], and changes in kinetics parameters [40]. Our previous study showed that H^+-ATPase is inhibited by the interaction and complex formation with AcTub and that glucose addition causes dissociation of tubulin from the complex and restoration of H^+-ATPase activity [8].

We showed here that microtubule dynamism plays a role in pma1 activation by glucose. Both, Taxol and Nocodazole, modify MT's (MT:



C

	H⁺-ATPase activity				Membrane tubulin	
	H⁺-efflux * t⁻¹ * 10⁻¹⁰ (M/sec)	nmol Pi hydrolyzed * min⁻¹ * mg protein⁻¹	H⁺-efflux * t⁻¹ * 10⁻¹⁰ (M/sec)	nmol Pi hydrolyzed * min⁻¹ * mg protein⁻¹	Arbitrary units	% of control
Wild-type	24.3 ± 3.0	25.3 ± 2.8	16.3 ± 2	18.3 ± 1.3	5 ± 3	10 ± 5
ΔLPX1	2.94 ± 0.2	7.3 ± 1.05	34.3 ± 4.8	33.3 ± 2.2	55 ± 4	98 ± 11
ΔLPX1 + pJV29-LPX1	21.8 ± 0.2	21.3 ± 3.5	15.8 ± 1.8	16.8 ± 1.6	11 ± 5	20 ± 11
ΔLPX1 + pJV29	2.2 ± 0.3	5.8 ± 1.5	31.9 ± 2.7	29.6 ± 3.0	54 ± 4	9 ± 7

Fig. 6. H^+-ATPase activation and tubulin degradation in mutant yeast strain ΔLPX1. (A) Cells from strain ΔLPX1 (black), ΔLPX1 + pJV29-LPX1 (red) and wild-type (blue) were harvested, washed twice with 100 mM Mes–Tris, pH 6.8 and starved for 30 min. Cells (1×10^8) were loaded with 1.4 mL of a solution of 0.9% NaCl, 5 mM $MgCl_2$, 2 μM BCECF, pH 7 in a cuvette and placed in a thermostatically controlled spectrofluorometer. Extracellular pH values were calculated as in Fig. 5A–C. (B) Following glucose addition (arrow in panel A), 4×10^7 cells were subjected to plasma membrane protein extraction and membrane tubulin degradation was analyzed by Western blotting with mAb 6-11B-1.(C) Note: In order to make a more exhaustive analysis, the curves in A were split in two regions (light blue and pink) and data were analyzed independently. H^+-ATPase activity was either calculated as H^+ released per unit time (mol/s) based on the curves of A, or by the [γ-^{32}P]ATP hydrolysis method in isolated plasma membranes [17]. The membrane tubulin bands from B at 300 s incubation with 10 mM glucose were quantified (see Materials and Methods). The values were expressed as percentages relative to the wild-type value at time zero. Data from fluorescence assays, H^+-ATPase activity and tubulin band quantification are means ± SD from three independent experiments.

Microtubule) dynamism. The fact that both drugs favor pma1 activation (Fig. 1), installs the hypothesis that MT's dynamism plays a role in the glucose signaling that leads to pma1 activation. In conditions of low dynamism (Taxol or Nocodazole treatment) glucose activation is favored and in high dynamism condition (no drugs), glucose activation is a bit slower. Glucose *per se*, increases MT's dynamism because this molecule is an ATP precursor and ATP regulates MT's dynamism, being more dynamics at high ATP level and less dynamics at low ATP level [41]. All these isolated data, obtained in *S. cerevisiae* and other cells (see other papers from our group) suggest that MT's dynamism is involved in pma1 activation by glucose. But not only MT's dynamism is involved in pma1 activation. The present study shows that the mechanism of H^+-ATPase activation by glucose involves the dissociation of the AcTub/ATPase complex *via* degradation of tubulin, presumably by the action of Lpx1p.

Glucose addition to budding yeast causes a short-term decrease of intracellular pH because of glucose metabolism. The pH is restored to its normal value by the vacuolar accumulation of protons by the vacuolar V-ATPase [7] and proton release to the extracellular medium by plasma membrane H^+-ATPase (Pma1p). It has been proposed that the pH decrease is necessary for Pma1p activation because plasma membrane-permeable organic acids activate Pma1p even in the absence of glucose [2]. We showed previously that Pma1p activation correlates with its release from its complex with AcTub [8]. The present study shows that the dissociation of tubulin and the concomitant H^+-ATPase activation are enhanced by tubulin degradation. The degradation is caused by a protease that is localized specifically at the plasma membrane, as indicated by the fact that tubulin present in washed membranes was degraded during incubation without exogenous glucose addition (Fig. 2A). The treatment of yeast with protease inhibitors blocked tubulin degradation, and the reduction of H^+-ATPase activity following glucose addition was due to the impaired degradation of membrane tubulin (Fig. 5A, E). Tubulin degradation is pH-dependent and reaches a high level at pH 6.2 similar to the level found in cytoplasm following glucose addition. Higher levels of tubulin degradation were correlated with increases in H^+-ATPase activity (Fig. 2A). A proteolytic enzyme was precipitated using tubulin as an affinity ligand, indicating that these proteins interact, presumably as an enzyme-substrate complex (Fig. 3). This interaction was stabilized by the differing isoelectric points of the two proteins: tubulin is acidic and the protease is basic (Fig. 4).

The proteolytic degradation of an inhibitor protein is a potentially dangerous mechanism for activating an enzyme because proteases often have low specificity and could conceivably degrade several targets. In this case, however, the protease preferentially degraded yeast tubulin and had much less effect on mouse tubulin (Fig. 3). Gelatin degradation was not observed in the *in gel* tubulin degradation assays (Figs. 4 and 5), and Pma1p was not degraded when incubated with a protein fraction that was highly enriched in the putative tubulin protease (data not shown). Thus, tubulin degradation in the Pma1p activation pathway appears to be an accurately controlled process. Treatment of yeast with PMSF prevented plasma membrane degradation and H^+-ATPase activation by glucose (Fig. 5), suggesting that the protease involved is a serine protease. This idea was supported by the observation that a variety of inhibitors unrelated to serine-like proteases had no effect on H^+-ATPase

activation or tubulin degradation. These biochemical properties were helpful in our search for a yeast mutant that lacked this protease.

The delay of Pma1p activation following glucose addition in the *lpx1Δ* mutant as compared with the wild-type strain (Fig. 6A) suggested that Lpx1p is the membrane protease responsible for tubulin degradation. Consistently with this idea, the delay in Pma1p activation was not correlated with plasma membrane tubulin degradation (Fig. 6B). *lpx1Δ* cells that were transformed with a vector carrying the native LPX1 gene recovered the wild-type phenotype; *i.e.*, Pma1p activation and membrane tubulin degradation following glucose addition (Fig. 6B, C). A question remains regarding the relationship between the protease activity determined for Lpx1p in the present study and its acyl hydrolase and phospholipase A activities reported previously by Thoms et al. [34]. We demonstrated here that the activation of H^+-ATPase by glucose occurs through a mechanism that involves the dissociation of the AcTub/ATPase complex and that is accelerated by the degradation of tubulin in hands of lpx1. The fact that H^+-ATPase is activated (albeit with some delay) even in the absence of tubulin degradation (Fig. 6B, *lpx1Δ* cells) corroborates this phenomenon and indicates that other complementary (or redundant) mechanisms for activation of the proton pump also operate in yeast. Curiously, after the lag time showed in Fig. 6A, the rate of Pma1p activity in *lpx1Δ* mutant is faster than for the wild type. It could be possible that tubulin association to Pma1p is modified with no complete detachment. If one consider this last point, and the fact that the Km_{ATP} of Pma1p changes from 2 mM to 1 mM and V_{max} is increased a 100% when pH varies from 7 to 6 [42], the delay in Pma1p activation observed for *lpx1Δ* mutant in Fig. 6A could be generating the decrease in cytosolic pH that modifies the kinetic parameters that enhance Pma1p activity, explaining the faster rate observed in *lpx1Δ* cells at time 300 s in Fig. 6A. However, our preliminary measurement of cytosolic and vacuolar pH in wild type and *lpx1Δ* (not shown) cells after glucose addition is very similar, suggesting that metabolic generation of protons could be affected in *lpx1Δ* cells, an issue which, although is beyond the question of this paper, is being investigated in our lab and will be very useful to explain in more detail how the activation mechanisms operates.

Despite this, the H^+-ATPase activation in *S. cerevisiae* seems to be regulated by more than one mechanism that operates in a hierarchical mode. While in wild type cells the mechanism that operates by default to activate the H^+-ATPase is the dissociation of the AcTub-ATPase complex by proteolysis of tubulin mediated by Lpx1p, because the first response observed in *lpx1Δ* cells was a delay in the H^+-ATPase activation, in *lpx1Δ* cells, other mechanism (not necessarily totally different) of a less hierarchical level operates to activate the pump.

These results show that the degradation of tubulin is one of the earliest events for the activation of the pump, provably making the H^+-ATPase molecule more accessible for next modification for Pma1p activation, *i.e.* phosphorylation. However, in *lpx1Δ* cells activation still happen provably because tubulin presence is not that strong impediment and can be overcome after some time trough a secondary mechanism. It is relevant to point out that the dissociation of the AcTub-H^+-ATPase complex by Lpx1p is a primary mechanism because it leads to a rapid regulation of intracellular pH (20–30 s).

The present results demonstrate that tubulin degradation is a necessary step in H^+-ATPase activation and suggest that a decrease in intracellular pH triggers the activity of a membrane protease (Lpx1p) and consequent tubulin degradation. Based on our present and previous results [8], we propose that AcTub and Lpx1p are the two key players in the regulatory mechanism of H^+-ATPase in *S. cerevisiae*, as follows: the addition of glucose causes a decrease in cytosolic pH; this decrease triggers the activation of Lpx1p which degrades tubulin; this degradation causes the dissociation of the AcTub/H^+-ATPase complex and the consequent activation of ATPase. In the absence of glucose, the internal pH returns to a higher value, Lpx1p is inactivated, the AcTub/H^+-ATPase complex is reformed, and the enzyme activity is once more inhibited.

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