In the budding yeast *Saccharomyces cerevisiae*, the Cdc42 effector Ste20 plays a crucial role in the regulation of filamentous growth, a response to nutrient limitation. Using the split-ubiquitin technique, we found that Ste20 forms a complex with Vma13, an important regulatory subunit of vacuolar H\(^+\)-ATPase (V-ATPase). This protein–protein interaction was confirmed by a pull-down assay and coimmunoprecipitation. We also demonstrate that Ste20 associates with vacuolar membranes and that Ste20 stimulates V-ATPase activity in isolated vacuolar membranes. This activation requires Ste20 kinase activity and does not depend on increased assembly of the V\(_1\) and V\(_0\) sectors of the V-ATPase, which is a major regulatory mechanism. Furthermore, loss of V-ATPase activity leads to a strong increase in invasive growth, possibly because these cells fail to store and mobilize nutrients efficiently in the vacuole in the absence of the vacuolar proton gradient. In contrast to the wild type, which grows in rather small, isolated colonies on solid medium during filamentation, hyperinvasive *vma* mutants form much bigger aggregates in which a large number of cells are tightly clustered together. Genetic data suggest that Ste20 and the protein kinase A catalytic subunit Tpk2 are both activated in the large number of cells are tightly clustered together.

Ste20 is an important regulatory subunit of vacuolar H\(^+\)-ATPase (V-ATPase). This protein–protein interaction was confirmed by a pull-down assay and coimmunoprecipitation. We also demonstrate that Ste20 associates with vacuolar membranes and that Ste20 stimulates V-ATPase activity in isolated vacuolar membranes. This activation requires Ste20 kinase activity and does not depend on increased assembly of the V\(_1\) and V\(_0\) sectors of the V-ATPase, which is a major regulatory mechanism. Furthermore, loss of V-ATPase activity leads to a strong increase in invasive growth, possibly because these cells fail to store and mobilize nutrients efficiently in the vacuole in the absence of the vacuolar proton gradient. In contrast to the wild type, which grows in rather small, isolated colonies on solid medium during filamentation, hyperinvasive *vma* mutants form much bigger aggregates in which a large number of cells are tightly clustered together. Genetic data suggest that Ste20 and the protein kinase A catalytic subunit Tpk2 are both activated in the *vma13*Δ strain. We propose that during filamentous growth, Ste20 stimulates V-ATPase activity. This would sustain nutrient mobilization from vacuolar stores, which is beneficial for filamentous growth.
In this work, we show evidence for Ste20 binding to Vma13 and for localization of Ste20 at vacuolar membranes. We also find that Ste20 positively regulates V-ATPase activity in vivo, and this activation is independent of V-ATPase assembly. Furthermore, deletion of VMA13 or other V-ATPase subunits leads to a strong increase in agar invasion, possibly due to defective nutrient storage and mobilization in these cells. We propose that during filamentous growth, Ste20 not only triggers a MAPK cascade, but, in parallel, activates the V-ATPase, facilitating mobilization of intracellular nutrient reserves.

**MATERIALS AND METHODS**

**Yeast strains, plasmids, and growth conditions.** All yeast strains used in this study are listed in Table 1. The strains are in the *S. cerevisiae* background (PPY966), with the exception of strains used for vacuolar assays. For vacuole isolation, wild-type SF838-3Aa was used. Yeast strains were constructed using PCR-amplified cassettes (18, 31) and were grown in 1% yeast extract, 2% peptone, 2% dextrose (YPD) or synthetic complete (SC) medium. For induction of the GAL1 promoter, yeast cells were grown in SC medium with 3% raffinose instead of glucose. Galactose (final concentration, 2%) was added to induce the GAL1 promoter. To compare the growth rates between strains, cells were grown overnight in SC medium. Serial dilutions starting from 10^6 cells were then spotted on YPD plates and incubated at 30°C for 2 days.

All constructs used in this work are listed in Table 2. To obtain 2 μm plasmids containing *STE20-3HA* and *STE20K649A-3HA* under the control of the *STE20* promoter, a C-terminal *STE20-3HA* fragment was amplified by PCR using pRS316-pGAL1-STE20-3HA and pRS316-pGAL1-STE20K649A-3HA, respectively, as templates and the following primers: 5′-TCCGCGGTGGAACATGA and 5′-ATGCGCCGCCCCACAGCTATGACCATGAT. PCR products were digested with SalI and ApaI and ligated into *SacI*/*ApaI*-cut pRS425-STE20.

**Split-ubiquitin technique.** The split-ubiquitin screen using *STE20* as bait is described by Tiedje et al. (57). For the interaction assays, 10^6 cells carrying the split-ubiquitin plasmids were spotted on SC medium lacking histidine and leucine to select for the plasmids or onto SC medium lacking histidine and leucine and supplemented with 0.5 g/liter 5-fluoroorotic acid (5-FOA) to monitor protein interactions. The 5-FOA plates also contained 2 mg/liter 5-FOA (5-FOA) to monitor protein interactions.

**TABLE 1 Yeast strains used in this study**

| Name | Genotype | Source or reference |
|------|----------|---------------------|
| MLY65 | PPY966 vma13Δ::His3MX6 | This study |
| MLY125 | PPY966 vma2Δ::His3MX6 | This study |
| MLY126 | PPY966 vma3Δ::His3MX6 | This study |
| MLY150 | PPY966 ste20Δ::hphNT1 vma2Δ::His3MX6 | This study |
| MLY151 | PPY966 ste20Δ::hphNT1 vma3Δ::His3MX6 | This study |
| MLY159 | PPY966 tpk2Δ::His3MX6 | This study |
| MLY161 | PPY966 tpk2Δ::His3MX6 ste20Δ::hphNT1 | This study |
| MLY166 | PPY966 tpk2Δ::KTRP1 vma13Δ::His3MX6 | This study |
| MLY167 | PPY966 tpk2Δ::KTRP1 ste20Δ::hphNT1 vma13Δ::His3MX6 | This study |
| MLY168 | PPY966 vma2Δ::His3MX6 st7Δ::kTRP1 | This study |
| MLY170 | PPY966 vma3Δ::hphNT1 | This study |
| MLY177 | PPY966 vma13Δ::His3MX6 ste11Δ::kTRP1 | This study |
| MLY208 | PPY966 STE20-3HA::His3MX6 | This study |
| PPY966 | MATa his::g leu2::hisG trp1::hisG ura3-52 | 57 |
| SHY26 | PPY966 stel1Δ::His3MX6 | This study |
| SHY45 | PPY966 stel1Δ::His3MX6 | This study |
| THY697 | PPY966 ste20Δ::hphNT1 | This study |
TABLE 2 Plasmids used in this study

| Name       | Genotype                        | Source or reference |
|------------|---------------------------------|---------------------|
| pAK6       | pADNIX carrying pADH1-NUbiquitin-VMA13 | This study          |
| pCT44      | pADNIX carrying pADH1-NUbiquitin-EXO70 | This study          |
| pKA86      | prS316 carrying pGAL1-STE20-3HA  | 57                  |
| pKK9      | prS316 carrying myc-VMA13       | 41                  |
| pTH197     | prS313 carrying pMET25-STE20-CUbiquitin-RURA3 | This study          |
| pTH1263    | pRS415 carrying STE20           | This study          |
| pTH380    | pRS415 carrying STE20-3HA       | This study          |
| pTH381    | pRS415 carrying STE20 <sup>kd</sup>-3HA | This study         |
| pTH383    | pADNIX carrying pADH1-NUbiquitin-VMA2 | This study          |
| pTH385    | prS313 carrying pMET25-STE14-CUbiquitin-RURA3 | This study |
| pTH386    | pADNIX carrying pADH1-NUbiquitin-VMA3 | This study          |
| MBP-Vma13  | pMALpAs carrying VMA13         | 7                   |

lacked methionine and cysteine to induce expression of the STE20 and STE14 fusion genes under the control of the MET25 promoter. The plates were grown for 2 days at 30°C.

**Biochemical interaction of Vma13 and Ste20.** Maltose-binding protein (MBP)-tagged Vma13 was expressed and purified from bacteria as described previously (7), but with the following modifications. Transformed cells were grown to an A<sub>600</sub> of 1 at 30°C. These cultures were then frozen in 1-mI stocks in 50% glycerol at −80°C, and 1 mL of frozen stock culture was added to 1 liter of LB containing 125 μg/mL ampicillin and 2 g glucose to grow the cells for MBP-Vma13 purification. The soluble fraction from the bacterial cell lysate was bound to a suspension of amylose resin (New England BioLabs), and the resin was washed with at least 4 volumes of cold TBSE (20 mM Tris-HCl, pH 7.2, 150 mM NaCl, 0.5 mM EDTA). Purified MBP-Vma13 was eluted in five 1-mL washes of TBSE containing 100 mM maltose.

Ste20-hemagglutinin (HA) was isolated from wild-type cells transformed with the plasmid pKA86 carrying HA-tagged STE20 under the control of the GAL1/10 promoter after 3 h of galactose induction. The cells were lysed, and cytosolic fractions were prepared as described previously (56). Each sample was incubated with 100 μL of protein A-Sepharose beads (a 40% suspension in phosphate-buffered saline-bovine serum albumin [PBS-BSA]) and 100 μg of anti-HA antibody (monoclonal antibody 16B12 from Covance Research Products) at 4°C for 1 h. Transformed cells that were not induced with galactose were treated in parallel as a negative control. The beads were washed with lysis buffer (50 mM Tris–HCl, 30 mM KCl, 30 mM NaCl, 0.3 mM EDTA) three times. Purified MBP-Vma13 was then incubated with Ste20-HA-bound beads (or beads from the uninduced control) at 4°C with gentle shaking for 3 h. The beads were washed three times with TBSE, and then the bound protein was eluted, separated by SDS-PAGE, and transferred to nitrocellulose as described previously (7). The level of MBP-Vma13 bound to the beads was detected on immunoblots with anti-MAb antibody (New England BioLabs).

For the coinmunoprecipitation experiment, 200 mL STE20-3HA cells carrying either prS316-myc-VMA13 (pKK19) or the empty prS316 were grown to an A<sub>600</sub> of 1. Cells were harvested, washed twice with PBS, and resuspended in 10 mL PBS. DSP (dithiobis [succinimidylpropionate]) was added from a freshly prepared 20 mM stock solution in dimethyl sulfoxide (DMSO) to a final concentration of 1 mM. The reaction mixture was incubated for 30 min at room temperature. The reaction was stopped by adding Tris, pH 7.5, to a final concentration of 20 mM and incubation for 15 min at room temperature. After washing with lysis buffer (20 mM Tris, pH 7.5, 100 mM NaCl, 10 mM EDTA, 1 mM EGTA, 5% glycerol, 1% Triton X-100), the cells were disrupted with glass beads in lysis buffer and clarified by centrifugation at 13,000 rpm for 5 min. The protein concentration was determined using Bradford protein assay solution. myc-Vma13 was immunoprecipitated by adding mouse monoclonal anti-myc antibody (9E10; Santa Cruz Biotechnology) and protein G-Sepharose (GE Healthcare). The resin was washed three times with lysis buffer, resuspended in 2× SDS sample buffer, and analyzed by immunoblotting. Mouse monoclonal anti-HA (12CA5) was obtained from Roche Diagnostics, and secondary antibodies were from Jackson Immunoresearch Laboratories.

**Filamentation assays.** For agar invasion assays, 10<sup>5</sup> cells of an overnight YPD culture were spotted on a YPD plate and grown for 3 days at 30°C. The plates were photographed before and after being rinsed under a stream of deionized water.

For the single-cell invasive-growth assay, cells were grown to stationary phase in SC medium, washed twice with water, and spread onto synthetic complete medium lacking glucose at a concentration of 10<sup>5</sup> cells/plate. The plates were incubated at 30°C for 18 h and analyzed microscopically with a Zeiss Axiovert 200 M fluorescence microscope. Images were captured using a Zeiss AxioCam MRm charged-coupled-device (CCD) camera.

The formation of cell clusters was analyzed by spotting 10<sup>3</sup> cells of an overnight YPD culture on a YPD plate and incubating the plate for 3 days at 30°C. The strains were examined microscopically after the plates were washed with water.

**Vacuole isolation and biochemical analysis.** Wild-type (SF383-5Ax) cells or the congenic ste20A::kanMX strain were transformed with a multicyclopis plasmid carrying STE20, STE20-3HA, or STE20<sup>kd</sup>-3HA (STE20<sup>kd</sup>-3HA). The cells were grown to log phase in SC buffered to pH 5 with 50 mM morpholinethanesulfonic acid (MES), converted to spheroplasts, and lysed, and then vacuolar vesicles were isolated by Ficoll density gradient centrifugation as described previously (48). ATPase activity was determined by coupled enzyme assay, and activity sensitive to 100 nM concanamycin A was taken as V-ATPase activity (48). For immunoblot analysis, vacuolar vesicles were solubilized, separated by SDS-PAGE, and transferred to nitrocellulose as described previously (56). V-ATPase subunits were detected with mouse monoclonal antibodies 10D7 (anti-V<sub>0</sub> a subunit), 8B1 (anti-V<sub>1</sub> A subunit), 13D11 (anti-V<sub>1</sub> B subunit), and 7 A2 (anti-V<sub>1</sub> C subunit) (22). STE20-HA and STE20<sup>kd</sup>-3HA were detected with monoclonal antibody 16B12 (Covance). Activities were compared between strains using the two-sample t test assuming equal variance in Microsoft Excel.

**RESULTS**

Ste20 interacts with the V-ATPase subunit Vma13. In an effort to identify regulators and targets of Ste20, we screened for proteins that bind to Ste20 using the split-ubiquitin system (57). This technique is based on the ability of the N- and C-terminal halves of ubiquitin to form a native-like ubiquitin (Fig. 2A) (19). Ubiquitin-specific proteases present in the cytosol and nucleus recognize the reconstituted ubiquitin, but not its halves, and cleave off a reporter protein, which had been linked to the C-terminal half of ubiquitin. The assay described here employs Ura3, a modified Ura3 with an arginine as the first amino acid, as the reporter (63). The freed RUra3 is rapidly degraded because arginine is a destabilizing residue in the N-end rule pathway. Therefore, interaction between two proteins fused to the N- and C-terminal halves of ubiquitin, respectively, results in nongrowth on medium lacking uracil. Conversely, growth on 5-FOA medium indicates a protein-protein interaction because 5-FOA is converted by Ura3 into uracil, respectively, results in nongrowth on medium lacking uracil. Mouse monoclonal anti-HA (12CA5) was obtained from Roche Diagnostics, and secondary antibodies were from Jackson Immunoresearch Laboratories.

For the coinmunoprecipitation experiment, 200 mL STE20-3HA cells carrying either prS316-myc-VMA13 (pKK19) or the empty prS316 were grown to an A<sub>600</sub> of 1. Cells were harvested, washed twice with PBS, and resuspended in 10 mL PBS. DSP (dithiobis [succinimidylpropionate]) was added from a freshly prepared 20 mM stock solution in dimethyl sulfoxide (DMSO) to a final concentration of 1 mM. The reaction mixture was incubated for 30 min at room temperature. The reaction was stopped by adding Tris, pH 7.5, to a final concentration of 20 mM and incubation for 15 min at room temperature. After washing with lysis buffer (20 mM Tris, pH 7.5, 100 mM NaCl, 10 mM EDTA, 1 mM EGTA, 5% glycerol, 1% Triton X-100), the cells were disrupted with glass beads in lysis buffer and clarified by centrifugation at 13,000 rpm for 5 min. The protein concentra-
To confirm the protein-protein interaction between Ste20 and Vma13 by an independent approach, Ste20-3HA was expressed from the inducible GAL1/10 promoter and immunoprecipitated from a yeast cell lysate. The immunoprecipitates bound to protein A-Sepharose beads were incubated with MBP-Vma13 purified from Escherichia coli. In this assay, MBP-Vma13 was specifically pulled down by Ste20-3HA in an induced sample but was not bound to the beads in an uninduced sample (Fig. 2C).

Finally, binding of HA-tagged Ste20 to myc-tagged Vma13 was confirmed by immunoprecipitation from yeast cell lysates. Ste20-3HA coprecipitated with myc-Vma13 but was not detected in immunoprecipitated protein from cells that do not express myc-Vma13 (Fig. 2D).

Thus, Ste20 forms a complex with Vma13 in vivo.

We next analyzed genetic interactions between STE20 and VMA13. As shown previously, the growth rate of vma13Δ cells was reduced in comparison to the wild type (Fig. 3A) (15). Notably, additional deletion of STE11 and STE7, respectively, does not affect the growth rate of the vma13Δ strain. Growth was assessed by serial dilutions as in panel A. (C) STE20 does not interact genetically with VMA2 or VMA3. Growth was assessed by serial dilutions as in panel A.

To confirm the protein-protein interaction between Ste20 and Vma13 by an independent approach, Ste20-3HA was expressed from the inducible GAL1/10 promoter and immunoprecipitated from a yeast cell lysate. The immunoprecipitates bound to protein A-Sepharose beads were incubated with MBP-Vma13 purified from Escherichia coli. In this assay, MBP-Vma13 was specifically pulled down by Ste20-3HA in an induced sample but was not bound to the beads in an uninduced sample (Fig. 2C).

Finally, binding of HA-tagged Ste20 to myc-tagged Vma13 was confirmed by immunoprecipitation from yeast cell lysates using anti-myc antibodies. Ste20-3HA coprecipitated with myc-Vma13 but was not detected in immunoprecipitated protein from cells that do not express myc-Vma13 (Fig. 2D). Thus, Ste20 forms a complex with Vma13 in vivo.

We next analyzed genetic interactions between STE20 and VMA13. As shown previously, the growth rate of vma13Δ cells was reduced in comparison to the wild type (Fig. 3A) (15). Notably, additional deletion of STE20 in the strain exacerbated this growth defect (Fig. 3A), suggesting that STE20 and VMA13 act in parallel to support normal cell growth. In contrast, there is no synthetic growth defect between vma13Δ and ste11Δ and ste7Δ, two mutants in the filamentation and pheromone response MAPK pathways activated by Ste20 (Fig. 1 and 3B). We also examined genetic interactions between STE20 and the V-ATPase subunit genes VMA2 and VMA3. Deletion of either VMA2 or VMA3 resulted in...
a growth defect comparable to that of the vma13Δ strain. However, the loss of STE20 in these strains had no effect on the growth rate (Fig. 3C). Thus, the genetic interaction between VMA13 and STE20 is remarkably specific.

Activation of V-ATPase by Ste20. Since Ste20 binds to Vma13, it is tempting to speculate that Ste20 regulates V-ATPase activity. To test this, we first isolated vacuolar vesicles from wild-type and ste20Δ cells and cells overexpressing STE20 and assessed concanamycin A-sensitive ATPase activity. STE20 expressed from its own promoter or the GAL1/10 promoter is active, allowing assessment of its activity in the absence of triggers, such as nutrient deprivation (43, 64). As shown in Fig. 4A, the specific ATPase activity in vacuolar vesicles isolated from a ste20Δ strain was not significantly different from the activity in wild-type vacuolar vesicles (P > 0.05). Overexpression of STE20 in the wild-type strain appeared to give higher ATPase activity, but the difference from wild-type activity was not statistically significant (P = 0.14). However, we also transformed ste20Δ cells with STE20-3HA and STE20K649A-3HA, a kinase-dead allele of Ste20 (64), and then isolated vacuolar vesicles. As shown in Fig. 4A, overexpressed STE20-3HA activates V-ATPase activity to an extent comparable to the untagged STE20; this activity was also significantly different from those of both the ste20Δ and wild-type strains (P ≤ 0.01 for each strain). In contrast, the inactive STE20K649A-3HA does not activate the V-ATPase. We next asked whether differences in V-ATPase activity in the ste20Δ mutant and STE20-overexpressing strains could be accounted for by differences in V-ATPase subunit levels at the vacuole. Immunoblotting of vacuolar vesicles from ste20Δ and STE20-3HA-overexpressing strains were probed for V0 subunit a and V1 subunits A, B, and C, as shown in Fig. 4B. There were no consistent differences in the levels of these subunits in the wild-type, ste20Δ, or STE20-overexpressing cells over several independent vacuole preparations. The ratio of V1 to V0 subunit levels at the vacuole provides an established measure of V-ATPase assembly (3, 8, 20), so these results also imply that Ste20 does not change V-ATPase activity by altering the level of V1-V0 assembly. The levels of alkaline phosphatase, a vacuolar membrane protein that is transported independently of the V-ATPase, also showed no significant differences (data not shown). In order to determine whether Ste20 is localized to the vacuole, we also probed immunoblots of vacuolar vesicles for Ste20-3HA (Fig. 4C). Both the wild-type Ste20-3HA and the kinase-dead mutant ste20K649A-3HA (ste20Δ-3HA) are localized to vacuolar membranes, even though the kinase-dead mutant does not support activation of the V-ATPase. These results indicate that Ste20 kinase activity is required for V-ATPase activation and that the kinase could exert its activity at the vacuolar membrane.

Finally, we tested whether direct phosphorylation of Vma13 could account for the activation of V-ATPase activity by Ste20. However, although bacterially expressed Vma13 could bind to Ste20 in pulldowns, there was no phosphorylation of Vma13 under these conditions. As a result, we cannot yet directly attribute V-ATPase activation to direct phosphorylation by Ste20.

V-ATPase activity and filamentous growth. Since STE20 and VMA13 interact genetically and at the protein level, we examined whether the V-ATPase contributes to processes that are regulated by Ste20. vma13Δ cells have normal morphology and form a mating projection in response to pheromone (data not shown). We also examined invasive growth. For this assay, cells are spotted at very high density on the rich YPD medium. Under these conditions, nutrients are locally used up quickly. As a consequence, filamentation is induced and cells penetrate the agar. This agar invasion can easily be assessed by washing off cells that grow only superficially. In the absence of VMA13, cells exhibited a marked increase in agar invasion (Fig. 5A). Presumably, vma13Δ cells are unable to store and/or mobilize nutrients in the vacuole efficiently due to the lack of V-ATPase activity. Therefore, these cells might be more sensitive to nutrient deprivation. We next asked whether this phenotype was specific to vma13Δ cells or characteristic of a general loss of V-ATPase activity. Like the vma13Δ strain, vma2Δ and vma3Δ cells displayed a growth defect and a hyperinvasive phenotype (Fig. 5A). Therefore, increased agar invasion seems to be a common feature of cells lacking V-ATPase activity and is not specific to vma13Δ.
The growth of vma cells was impaired under nutrient deprivation conditions. Cells of the indicated strains were spotted on YPD plates and grown for 3 days at 30°C. Bar, 10 μm.

![Figure 5](ec.asm.org)
a MAPK cascade by phosphorylation of the MAPK kinase Ste11 (30, 49, 62, 64). We reasoned that if Ste11 were the only Ste20 target, deletion of \( \text{STE11} \) would cause a defect in filamentous growth as severe as that observed in the \( \text{ste20} \) strain. Importantly, the agar invasion phenotype of \( \text{ste11} \) cells was much less pronounced than that of cells lacking \( \text{STE20} \) (Fig. 6C). Deletion of \( \text{STE7} \), which encodes the MAPK kinase that is activated by Ste11 during filamentous growth, similarly led to only a relatively moderate phenotype (Fig. 6C). Ste20 also activates Ste11 and Ste7 during mating. Notably, all three \( \text{STE} \) genes are essential for this process (data not shown) (14, 26). Thus, the difference in phenotype severity between the \( \text{ste} \) mutants is specific for filamentous growth and could be explained by additional functional targets of Ste20, such as the V-ATPase. This is in line with the regulation of V-ATPase activity by Ste20 as described above.

**DISCUSSION**

**Regulation of V-ATPase activity by Ste20.** The Cdc42 effector Ste20 is involved in multiple signaling pathways, including a MAPK cascade that triggers filamentation (30, 49). Here, using the split-ubiquitin technique, a pulldown assay, and coimmunoprecipitation, we showed that Ste20 specifically interacts with the V-ATPase subunit Vma13, demonstrating that these proteins bind to each other in vivo and in vitro. We further reported the stimulation of V-ATPase activity by Ste20. It is not clear how Ste20 regulates V-ATPase activity. \( V_0-V_1 \) assembly, a major control mechanism, is not affected by overexpression or deletion of \( \text{STE20} \). Vma13, which activates the assembled V-ATPase (15), does not seem to be a phosphorylation target of Ste20. V-ATPase activation by Ste20 requires Ste20 kinase activity, but Vma13 was not phosphorylated in our hands. Therefore, other V-ATPase subunits might be targets of Ste20, with Vma13 serving as a platform for binding.

Ste20 has previously been known to localize to the plasma membrane at sites of polarized growth in the cytoplasm and the nucleus (1, 25, 28, 43). Here, we show that Ste20 is also enriched in vacuolar membranes. This localization of Ste20 is in line with its
role in V-ATPase activation and is consistent with observations of other groups. Cdc42 and Bem1, a scaffold protein that brings Cdc42, its activator Cdc24, and Ste20 into close proximity, are also present at vacuolar membranes (9, 10, 37, 46, 66). Thus, it seems that not only Ste20 associates with vacuolar membranes, but all components of the Bem1-Cdc24-Cdc42-Ste20 cascade.

It has been reported that, together with Cla4, another Cdc42 effector and also a p21-activated kinase, Ste20 regulates vacuolar inheritance (2). Thus, Ste20 has at least two distinct vacuolar functions.

**Hyperinvasive growth is stimulated in the absence of V-ATPase activity.** Deletion of a number of V-ATPase subunits leads to strongly increased invasive growth relative to wild-type cells. This phenotype is not caused by increased cell elongation. Instead, it correlates with strong cell clustering, vma mutants probably experience acute nutrient deprivation under conditions where wild-type cells remain nutrient replete. The vacuolar pH gradient is required for both uptake and mobilization of nutrients (27, 51). The loss of vacuolar acidification in vma mutants causes defective nutrient storage and mobilization in these strains (54). Since depletion of certain nutrients induces filamentation, it seems most likely that the hyperinvasive phenotype of vma mutants is caused by the failure of these cells to maintain sufficient nutrient stores for times of starvation. As a consequence, cells lacking V-ATPase activity would be much more sensitive to nutrient limitation. Stronger invasive growth might increase the chance of finding extracellular nutrients and would therefore be beneficial for these cells.

Our genetic data suggest that **STE20** and **TPK2** are both required for the hyperinvasive phenotype of **vma13** cells, but it is not clear how a nutrient deprivation signal activates these pathways. This is most likely an intracellular signal. Possibly, cells are able to somehow sense the loss of nutrient storage, as suggested for autophagy-deficient yeast (34). In fact, a direct role for the V-ATPase in lysosomal amino acid sensing by mTORC1 in mammals was recently described (70), although this pathway involves Ragulator components not present in yeast. Alternatively, since the V-ATPase plays a crucial role in the homeostasis not only of vacuolar but also of cytosolic pH (35), the altered pH of these compartments may trigger filamentation.

The only function that has been attributed to Ste20 in filamentous growth is the phosphorylation and thereby activation of the MAPK kinase kinase Ste11 (30, 49, 62, 64) (Fig. 7). In this work, we show that **ste11Δ** and **ste7Δ** cells exhibit a rather mild invasive-
growth defect. In contrast, in cells lacking STE20, agar invasion is almost completely absent. This difference in phenotype severity is filamentation specific. STE20, STE11, and STE7 are all absolutely required for mating (14, 26). A likely explanation for the more pronounced phenotype of ste20Δ cells is that Ste20 has targets beyond Ste11 in filamentous growth. Here, we show that Ste20 can activate V-ATPase activity. Although this observation was obtained in yeast cells grown in nutrient-replete liquid medium, activation of the V-ATPase by Ste20 could be particularly important for filamentous growth on solid surfaces (Fig. 7). When nutrients are limited in cells growing on a solid surface, Ste20 triggers the Ste11-Ste7-Kss1 cascade. Together with other activated pathways, including PKA, this results in cell elongation, substratum invasion, and increased adhesion. Under these conditions, Ste20 could stimulate V-ATPase activity as well, sustaining vacuolar pH gradients during nutrient mobilization by vacuolar antiporters and allowing more efficient mobilization of intracellular reserves required for cell elongation. Thus, filamentous growth and vacuolar mobilization are complementary approaches to deal with starvation. Unfortunately, altered V-ATPase activity during true filamentous growth cannot easily be examined because the cells grow into the agar, precluding biochemical isolation of vacuoles.

Low glucose levels induce filamentation and also trigger disassembly and downregulation of V-ATPase (4, 6, 20). Here, we propose that V-ATPase activity might be increased during filamentous growth. These seemingly contradictory observations can be reconciled. The experiments demonstrating V-ATPase disassembly in response to glucose depletion were carried out in liquid medium (6, 20). Under these conditions, it is likely to be advantageous to reduce growth and to save energy by V-ATPase disassembly. In contrast, for cells growing on a solid medium it may become essential to increase V-ATPase activity and to invade the substratum because there is a chance of finding nutrients. Disassembly of the V-ATPase in response to glucose deprivation is suppressed at high extracellular pH (8), presumably in order to preserve organelle acidification under conditions of alkaline stress. Similarly, nutrient limitation under filamentous-growth conditions may prevent disassembly so that high V-ATPase activity can be maintained.

We found that STE20 deletion specifically exacerbates the growth defect of vma13Δ cells. This observation is somewhat puzzling, since mutations in V-ATPase subunits abolish all V-ATPase activity and generally result in similar growth phenotypes, and it is unlikely that Vma13 functions outside the V-ATPase. Vma13 does play a distinctive role in regulating V-ATPase activity, however, acting as an inhibitor of ATPase activity in cytosolic V1 complexes but as an activator of the intact V-ATPase (41). Because of this regulatory role, vma13Δ mutations show very specific genetic interactions with certain other vma mutations (47), and the genetic interaction with ste20Δ observed here could also be accounted for by an increased need for the silencing or activating properties of Vma13 in this mutant. Further investigation will be necessary to test this possibility.

In summary, we describe a link between V-ATPase and invasive growth and demonstrate the activation of V-ATPase activity by Ste20. Since these components are conserved from yeast to humans, it will be interesting to test whether V-ATPase activity is modulated in a similar way in higher eukaryotes.

ACKNOWLEDGMENTS

We thank Silke Horn, Maureen Tarsio, and Theodore Diakow for excellent technical support.

The project was supported by Deutsche Forschungsgemeinschaft grant HO 2098/3 to T.H. and NIH grant R01 GM50322 to P.M.K.

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