A Novel Role of the Yeast CaaX Protease Ste24 in Chitin Synthesis

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Ste24 is a membrane-integral CaaX metalloprotease residing in the endoplasmic reticulum (ER). In yeast, the only known substrate of Ste24 is the mating factor α precursor. A global screening for protein–protein interactions indicated that Ste24 interacts with chitin synthesis deficient (Chs3), an enzyme required for chitin synthesis. We confirmed this interaction by yeast two-hybrid analyses and mapped the interacting cytoplasmic domains. Next, we investigated the influence of Ste24 on chitin synthesis. In sterile (ste24Δ) mutants, we observed resistance to calcofluor white (CFW), which was also apparent when the cells expressed a catalytically inactive version of Ste24. In addition, ste24Δ cells showed a decrease in chitin levels and Chs3-green fluorescent protein localized less frequently at the bud neck. Overexpression of STE24 resulted in hypersensitivity to CFW and a slight increase in chitin levels. The CFW phenotype of ste24Δ cells could be rescued by its human and insect orthologues. Although Chs3 binds to Ste24, it seems not to be a substrate for this protease. Instead, our data suggest that Chs3 and Ste24 form a complex in the ER that facilitates protease action on prenylated Chs4, a known activator of Chs3 with a C-terminal CaaX motif, leading to a more efficient localization of Chs3 at the plasma membrane.

INTRODUCTION

Although chitin is only a minor constituent of the yeast cell wall (~2% dry weight), its synthesis is essential, because it has vital functions during cell division and sporulation. During cell division chitin is first concentrated in a ring-like structure at the site where the bud emerges, and then it is deposited as a disk, forming the primary septum (Lesage and Bussey, 2006). Chitin is also found in the ascospore wall, deposited as a disk, forming the primary septum (Lesage structure at the site where the bud emerges, and then it is remodelled by spore-specific chitin deacetylases and mediates spore wall resistance (Briza et al., 1988; Christodoulidou et al., 1999). Chitin synthesis in yeast is catalyzed by three chitin synthases encoded by the genes CHS1, CHS2, and CHS3 (Cabib et al., 1993). Chs1 acts as a repair enzyme that is involved in remodeling of the cell wall during cell division, and Chs2 forms the chitin in the central disk of the primary septum. Chs3 accounts for ~90% of the chitin produced in vivo (Shaw et al., 1991) and is required for chitin synthesis at the lateral cell wall and for the formation of the chitin ring at the bud neck (Cabib et al., 1996). Chs3 is a membrane-integral protein with its catalytic domain facing the cytosol, where it catalyzes the transfer of the sugar moiety of UDP-N-acetyl-glucosamine (UDP-GlcNAc) to the nonreducing end of the growing chitin chain. The transmembrane domains may be involved in the translocation of nascent chitin across the plasma membrane (Merzendorfer, 2006).

The precise number and topology of transmembrane helices and soluble domains of Chs3 are uncertain. Fluorescence microscopic studies revealed that Chs3 is localized in smaller amounts at the plasma membrane, in higher amounts in the membranes of post-Golgi vesicles, and during cell division in a ring-like structure at the bud neck of small-budded cells (Cabib et al., 1993). Chs3 is synthesized by ribosomes and inserted into the lipid bilayer of the endoplasmic reticulum (ER), where it eventually attains its native conformation. This process requires the presence of the ER chaperone Chs7, which seems to prevent Chs3 aggregation (Trilla et al., 1999). After processing of Chs3 in the ER and Golgi apparatus, including glycosylation and palmitoylation (Santos and Snyder, 1997; Lam et al., 2006), it is transported from the trans-Golgi network to the cell surface, a process that requires Chs5 and Chs6, which are part of an exomer coat-complex (Wang et al., 2006). At the bud neck, Chs3 is linked to septins via Chs4 and Bni4 (DeMarini et al., 1997). Bni4 also recruits the catalytic subunit of protein phosphatase 1 (Glc7) to the bud neck in a temporal and spatial restricted manner, a process that assists in recruiting Chs3 by a yet unidentified substrate (Larson et al., 2008). Chs3 is not degraded in vacuoles but accumulates in chitosomes, which are specific secretory vesicles for chitin synthase transport to the plasma membrane (Ruiz-Herrera et al., 1977). They seem to act also as a trans-Golgi reservoir that is replenished by the endocytotic turnover of the enzyme (Ziman et al., 1996). Endocytotic turnover seems to be impaired by Chs4, which binds to Chs3 at the plasma membrane and regulates chitin synthase activity (Trilla et al., 1997; Reyes et al., 2007). Chs4 is not only required for Chs3 activity but also for the interaction between Chs3 and Bni4.
which promotes chitin synthesis and proper chitin localization (DeMarini et al., 1997). Chs4 is transported to the plasma membrane independently of the Chs3 trafficking route (Reyes et al., 2007). Two groups have reported that Chs4 is a prenylated protein. However, the role of this prenylation is controversial. Although Grabinska et al. (2007) suggest that Chs4 prenylation is required for Chs3 activity and chitin biosynthesis but not for membrane association, Reyes et al. (2007) concluded that prenylation is required for its membrane association and lateral diffusion but not for its biological function in chitin biosynthesis. The latter conclusions were mainly drawn by analyzing yeast mutants expressing Chs4 with an altered C-terminal CaaX motif. It is the cysteine of the CaaX motif that becomes prenylated by the farnesyltransferase. Prenylation is a prerequisite for the subsequent steps of CaaX processing, which include prenylation-dependent cleavage of the CaaX motif by a CaaX protease and carboxy-methylation by a methyltransferase (Wright and Philips, 2006).

To gain more insight into the role of post-prenylation steps of CaaX processing of Chs4, we investigated the CaaX protease Ste24, which we showed to bind to Chs3 and hence seems related to chitin synthesis. Ste24 is a metalloprotease that resides in the membranes of the ER, and it is, in addition to Rce1, the only CaaX protease identified in yeast (Huyer et al., 2006). The only substrate of Ste24 in yeast so far known is the precursor of the mating factor a (MFa). Ste24 cleaves the MFa precursor at the N terminus, and at the C terminus in a prenylation-dependent manner (Tam et al., 2001). CaaX processing of the MFa precursor (prenylation by Ram1/Ram2, cleavage by Rce1/Ste24 and carboxy-methylation by Ste14) is essential for the nonconventional export of MFa by an ATP-binding cassette transporter Ste6 (Chen et al., 1997). In this study, we provide evidence for a new function of Ste24 in modulating chitin synthesis by delocalization of Chs3, possibly involving CaaX cleavage of Chs4.

**MATERIALS AND METHODS**

**General**

All chemical reagents were of analytical grade and purchased from local distributors. Primers (Supplemental Table S1) were synthesized by Eurofins. Molecular Biology of the Cell

**Table 1. Strains**

| Yeast strain | Genotype / description | Source or reference |
|--------------|------------------------|---------------------|
| BY4741       | MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 | Euroscarf (Winzeler et al., 1999) |
| BY4742       | MATa his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 | Euroscarf (Winzeler et al., 1999) |
| BY4741 chs3Δ | As for BY4741, chs3Δ::kanMX4 | Euroscarf (Winzeler et al., 1999) |
| BY4741 ste24Δ | As for BY4741, ste24Δ::kanMX4 | Euroscarf (Winzeler et al., 1999) |
| BY4741 chs4Δ | As for BY4741, chs4Δ::kanMX4 | Euroscarf (Winzeler et al., 1999) |
| BY4742 chs4Δ | As for BY4742, chs4Δ::kanMX4 | Euroscarf (Winzeler et al., 1999) |
| BY4741 rce1Δ | As for BY4741, rce1Δ::kanMX4 | Euroscarf (Winzeler et al., 1999) |
| BY4742 ss22Δ | As for BY4742, ss22Δ::kanMX4 | Euroscarf (Winzeler et al., 1999) |
| CEN.PK2-1D   | MATa ura3-52 trp1-289 leu2-3,112 his3Δ1 MAL2-8°C SUC2 | Euroscarf (Entian et al., 1999) |
| CEN.EN13-3C ste24Δ | As for CEN.PK2, YJR117w::HIS3 | Euroscarf (Entian et al., 1999) |
| Ste24TAP     | MATa ade2 arg4 leu2-3,112 trp1-289 ura3-52 | CellZone AG (Heidelberg, Germany) |
| Chs3GFP      | As for BY4741, CHS3-GFP::HIS3 | Invitrogen (Huh et al., 2003) |
| Ste24GFP     | As for BY4741, STE24-GFP::HIS3 | Invitrogen (Huh et al., 2003) |
| Chs3GFP ste24Δ | As for BY4741, CHS3-GFP::HIS3 | This study |
| Chs3GFP chs4Δ | As for BY4741, CHS3-GFP::HIS3, chs4Δ::kanMX4 | This study |
| rce1Δ ste24Δ | As for BY4741, rce1Δ::kanMX4, ste24Δ::HIS3 | This study |
| AH109        | MATa trp1-901 leu2-3,112 trn3-52 his3-200 gal4Δ gal80Δ lys2Δ::GAL1-UAS-GAL1-TATA-HIS3 GAL1-UAS-GAL2-TATA-ADE2 URA3::MEL1-UAS-MEL1-TATA::HIS3 MEL1-UAS-MEL1-ter::MEL1 | Clontech (James et al., 1996) |
| BY4741 CHS4G693S      | As for BY4741, CHS4C693G chs4-1 terminator::kanMX4 | This study |

**Strains**

All strains used in this study are listed in Table 1. BY4741 (mating type [MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0]; its isogenic knockout strains ste24Δ, chs3Δ, chs4Δ, rce1Δ, and CEN.PK2-1D [MATa ura3-52 trp1-289 leu2-3,112 his3Δ1 MAL2-8°C SUC2]), with its knockout strain ste24Δ, were obtained from Euroscarf (Frankfurt, Germany). Green fluorescent protein (GFP)-fusion protein strains (Chs3GFP and Ste24GFP) were obtained from Invitrogen (Carlsbad, CA). The genomic point mutation chs4E269G was generated by homologous recombination using the primers chs4E269G-F and chs4E269G-R (Supplemental Table S1) and the vector pRS415-GFP(S65T)-KanMX4 as a template (McElver and Weber, 1992). After transformation of BY4741 cells and selection on appropriate growth media, the point mutation was verified by sequencing of a polymerase chain reaction (PCR)-generated fragment. The double mutants ste24Δ chs4Δ were constructed by mating of the strains CEN.EN13-3C ste24Δ, BY4741 rce1Δ, Chs3GFP, BY4742 ste24Δ, BY4742 chs4Δ, and BY4741chs4E269G. Resulting diploid strains were sporulated, and segregants carrying double mutations were selected on SD-His + G418 plates. The double mutations and mating types were confirmed by direct PCR (Huxley et al., 1990).

**Plasmids**

All plasmids used in this study are listed in Supplemental Table S2. The open reading frames encoding Chs3, Chs4, Ste24, zinc metalloprotease Ste24 homologue (ZmpSte24), and TcSte24 were amplified from yeast genomic DNA, or human and Tribolium cDNAs by PCR using specific primers (Supplemental Table S1) and cloned into centromeric vectors pAG503, pRS415, pREG576, or the 2µ vector pJH71 under the control of the GAL1 promoter (Raben et al., 1995). The nucleotide sequences of the GAL1 promoter or the 3'MYC epitope tag were amplified by PCR using specific primers (Supplemental Table S1) and cloned into the respective vectors. Yeast strains were transformed via electroporation (1 pulse, 1.5 kV, 25 μF, 200 Ω) with 1 μg of plasmid DNA per transformation performed in 50 μl of 1 M sorbitol. Plasmids were assayed for complementation of chs3Δ, chs4Δ, or ste24Δ on the basis of the CFW resistance phenotype or by CFW staining. The pRS415ste24E269G plasmid was created by site directed mutagenesis using the QuickChange kit (Stratagene, Cedar Creek, TX) and pRS415ste24Δ as a template. The point mutation was confirmed by nucleotide sequencing. For generation of the STE24 strain expressing STE24, the plasmid pAG503 GAL1-STE24 was placed under the control of the GAL1 promoter to be transformed BY4741 cells.
CFW Serial Drop Dilution Assay
Each 5 μl of a yeast suspension was spotted at different concentration (10^4-10^6 cells/ml) onto solid rich medium (YPD or YPG) plates containing 50 μg/ml CFW. After incubation at 30°C for 3 d, colony growth was documented using a Versa Doc Imaging System (Bio-Rad Laboratories, Hercules, CA) and the Quantity One, version 4.6 (Bio-Rad Laboratories).

Fluorescent Calcofluor White Assay
To estimate chitin levels, a CFW fluorescence assay was used according to a modified assay published by Lam et al. (2006). In brief, each 5 μl of a yeast suspension (concentrated to 10^6 cells/ml) was spotted onto solid rich medium (YPD or YPG) plates containing 50 μg/ml CFW. After incubation at 30°C for 3 d, the fluorescence was quantified densitometrically using the Versa Doc imaging system (λex = 356 nm, 520LP filter; Bio-Rad Laboratories) and Quantity One, version 4.6 (Bio-Rad Laboratories). The optical densities from constant areas within the spots were averaged over 15–40 independent experiments and corrected for the local background. The mean optical density averaged over 40 spots of wild-type cells was set to 100%, whereas the mean optical density averaged over 40 spots of chs3Δ cells was set to 0%. Relative chitin amounts (RCA; ± 5%) from various mutants were calculated according to RCA = x·chs3Δ/WT·chs3Δ, with x is the mean optical density averaged over 20 spots of the respective yeast mutant.

Measurement of the Chitin Content
The chitin content of different yeast cell strains was determined by the Morgan-Ellson method as described in Bulik et al. (2003), with some minor modifications. KOH-treated cell pellets were incubated for 48 h with 5 μl of Streptomyces griseus chitinase (Sigma-Aldrich). Colorimetric determination of GlcNAc was performed in microtiter plates, of which each slot was loaded with 150 μl of the samples treated with Ehrlich’s reagent.

Microscopy
Cells were grown in YPD or SD media until the early logarithmic phase was reached. When gene expression was controlled by a GAL1 promoter, cells were grown in glucose-free medium containing 1% (wt/vol) raffinose as a carbon source and analyzed in early logarithmic phase 3 h after induction of gene expression by adding 2% (wt/vol) galactose to the medium. For CFW staining, yeast cells were incubated in 0.02% (wt/vol) CFW solution for 30 min at room temperature and washed three times with deionized water. Microscopy performed with a 100x oil-immersion objective (numerical aperture 1.36) and an IX70 fluorescence microscope (Olympus, Hamburg, Germany). Fluorescence was excited with a U-RFL-burner (Olympus), and appropriate filter cubes were used to set excitation and emission wavelengths.

RESULTS

Ste24 Interacts with Chs3 through Cytoplasmic Domains
Several studies have suggested that insect and fungal chitin synthases including yeast Chs3 are produced aszymogens requiring proteolytic cleavage for activation (reviewed in Merzendorfer (2006)). However, until now, no protease has been identified that cleaves thezymogenic form. Therefore, we screened different databases (BioGRID, BOND, BioPIXIE, DIP, and Yeast RC two-hybrid) for proteases of any kind that might interact with yeast Chs3. Among 95 proteins that potentially interact with Chs3, we detected only one likely protease, Ste24. Ste24 is a membrane-integral metalloprotease of the ER, which was identified to interact with Chs3 in a large-scale split ubiquitin screen (Miller et al., 2005). To confirm this interaction with an independent method and to map the interacting regions, we performed a yeast two-hybrid analysis testing different soluble domains of Chs3 and Ste24. As a positive control, we included Chs4, a known activator of Chs3, which was reported previously to interact with Chs3 in two independent studies (DeMarini et al., 1997; Ono et al., 2000).

We amplified the cDNA sequences encoding four soluble Chs3 domains, three soluble Ste24 domains, and the complete coding sequence of Chs4 (Figure 1A), and we ligated each of them into pGADT7-AD and pGBK7-BD of the Matchmaker yeast two-hybrid system, to allow switching of bait and prey inserts. After negatively testing all constructs for endogenous activation of reporter gene activity (Figure 1B, examples shown in the two bottom rows), we tested the C1, C3, C4, and C7 domains of Chs3 for their ability to bind Chs4. In doing so, we could confirm the previously reported interaction between Chs3 and Chs4 and identify the C3 domain (amino acid positions 226–452) as the region binding to Chs4 (Figure 1B). No other Chs3 domains or Ste24 domains interacted with Chs4 (data not shown). Subsequently, we tested the C1, C3, C4, and C7 domains of Chs3 for their ability to interact with the cytosolic S2, S5, and S8 domains of Ste24 (Tam et al., 2001). We found that the C3 domain of Chs3 interacts with an interface of Ste24 formed at the cytoplasmic side by the S2 and S8 domains (Figure 1B). The observation that the C3 domain of Chs3 interacts with regions of proteins known to be exposed to the cytoplasm strongly supports a topology model placing this domain at the cytoplasmic site of the membrane (Figure 1A).

Ste24 Affects Chitin Levels but Not Its Cellular Distribution
CFW is a fluorescent dye that binds primarily to chitin (Roncero and Duran, 1985). Because this compound is toxic buffer, pH 7.0, containing 20 mM NaCl including Complete Protease Inhibitor Cocktail (Roche, Basel, Switzerland). After adding the same volume of glass beads, cells were vortexed for 5 min at 4°C. Subsequently, the suspension was centrifuged for 3 min at 3000 × g and 4°C, and the resulting pellet was resuspended in Laemmli buffer and boiled for 1 min (Laemmli, 1970).

Other Methods
Protein concentrations were determined by the Amido Black method (Wieczorek et al., 1990), and SDS-polyacrylamide gel electrophoresis was performed according to Laemmli (1970). Semidry electroblotting onto nitrocellulose membranes (Millipore, Schwalbach, Germany) was carried out essentially as described by Kyhse-Andersen (1984), with the modification that the buffers were supplemented with 20% (vol/vol) methanol. Blot membranes were stained with 0.02% (vol/vol) Ponceau S (Sigma-Aldrich; Taka-Kitchen, Germany). Immunoblots were performed as described previously (Zimoch and Merzendorfer, 2002). Primary antibodies were used polyclonal anti-Myc antibodies (1:100; AbD Serotec, Dusseldorf, Germany), and secondary antibodies were anti-mouse antibodies conjugated to alkaline phosphatase (1:50,000; Sigma-Aldrich).

CFW Affects Chitin Levels but Not Its Cellular Distribution
CFW is a fluorescent dye that binds primarily to chitin (Roncero and Duran, 1985). Because this compound is toxic...
to yeast cells, mutants with reduced chitin levels exhibit a CFW resistance phenotype. In vivo, the majority of chitin deposited in the yeast cell wall is synthesized by Chs3 (Shaw et al., 1991). Correspondingly, chs3Δ cells that produce significantly less chitin are CFW resistant. Therefore, changes in CFW resistance can be correlated with changes in Chs3 levels. To test whether Ste24 affects chitin levels, we analyzed wild-type and different mutant strains for their sensitivity to CFW. As expected, wild-type cells were hypersensitive toward CFW, whereas chs3Δ or chs4Δ cells exhibited increased resistance to CFW (Figures 2 and 4). We observed a clear, but moderate resistance to CFW, comparable with that of chs3Δ cells (2 ± 1.4%; n = 20). To evaluate the results from the CFW fluorescence assay, we measured chitin amounts independently by the method of Morgan–Elson (Figure 3B). Wild-type cells exhibited a chitin content of 5.2 nmol GlcNAc/mg cells (±0.2; n = 6), which was significantly reduced to 1.8 nmol GlcNAc/mg cells (±0.1; n = 6) in chs3Δ cells. Both values are in good agreement with previous measurements (Bulik et al., 2003). In line with the CFW fluorescence assay the chitin content in ste24Δ was reduced to 4.4 nmol GlcNAc/mg cells (±0.2; n = 6), which are ~76% of the chitin produced by Chs3. As observed in the

Figure 1. Putative domain architectures of Chs3, Chs4, and Ste24 and yeast two-hybrid analysis to identify interacting domains. (A) Horizontal bars at the top represent extracellular domains, horizontal bars at the bottom intracellular domains, and vertical bars transmembrane helices. Soluble domains tested in the yeast two-hybrid analysis are marked with black squares. (B) AH109 cells were co-transformed with bait and prey vectors. Cells grown overnight in liquid SD-Leu–Trp medium were diluted with water to a final concentration of 1 × 10⁶ cells/ml. Five microliters of each suspension and three subsequent 10-fold serial dilutions were individually spotted onto SD–Leu–Trp, SD–Leu–Trp–His and SD–Leu–Trp–His–Ade–Xα-Gal plates for selection. Cells were incubated at 30°C for 2 d.

Figure 2. Calcofluor white resistance phenotypes of different yeast strains. Wild-type, chs3Δ, ste24Δ, rce1Δ, chs4Δ, and STE24 Δ cells were grown overnight in liquid YPD medium and diluted with water to a final concentration of 1 × 10⁷ cells/ml. Five microliters of each suspension and three subsequent 10-fold serial dilutions were spotted onto YPD or YPD plates with or without 50 μg/ml CFW. Cells were incubated at 30°C for 3 d.
CFW fluorescence assay, STE24-overexpressing cells increased chitin levels of 6.4 GlcNAc/mg cells (±0.3; n = 6), which corresponds to 135% of the chitin produced by Chs3 and is somewhat higher than estimated from CFW fluorescence measurements. The chitin content of chs4Δ cells was with 2.0 nmol GlcNAc/mg cells (±0.3; n = 6) in the same range as that of chs3Δ cells. Overall, the chemical determination of chitin content was in good agreement with the estimate based on CFW fluorescence.

To visualize chitin deposition in growing wild-type, ste24Δ, chs3Δ, and STE24 ↑ cells, we performed a CFW staining and monitored the cells under a fluorescence microscope. The results shown in Figure 3A more or less reflect the chitin levels as they were determined by the CFW fluorescence and Morgan–Elson assays. To test for possible differences in chitin distribution, we measured local fluorescence intensities at the bud neck and in the cell walls and calculated their ratios for wild-type and mutant cells. The fluorescence intensity ratios were 1.7 (±0.3; n = 10) in wild-type, 1.5 (±0.5; n = 8) in chs3Δ, 1.4 (±0.2; n = 9) in ste24Δ and 2.0 (±0.5; n = 10) in STE24-overexpressing cells. Thus, although chitin content in various mutant cells differed significantly from that of wild-type cells its distribution did not change significantly as indicated by analysis of variance (ANOVA) tests (honestly significant difference [HSD] 0.05 = 0.50; HSD 0.01 = 0.63) of the combined data (Figure 3A).

Catalytically Inactive Ste24 Does Not Restore CFW Sensitivity in ste24Δ Cells
To examine whether the observed effects on chitin levels depend on the catalytic activity of Ste24, we generated a catalytically inactive mutant Ste24E298G and tested whether it can restore CFW sensitivity in ste24Δ cells. After cloning STE24 into a centromeric vector and placing it under the control of its endogenous promoter, we mutated the catalytic glutamate within the conserved HExxH motif and changed it to a glycine residue. MATA cells carrying mutations in this motif are unable to process the MFA precursor and hence are mating-deficient (Fujimura-Kamada et al., 1997). To assess whether a mutant expressing a catalytically inactive form of Ste24 had been successfully generated, we performed a halo assay described in Trueblood et al. (2000). In this assay, the growth of MATA cells is suppressed when active MFA is produced by cleavage of the MFA precursor by active Ste24 producing a halo. The halo will be absent if Ste24 is not active. Because rce1Δ and ste24Δ single mutants still exhibit a halo due to the activity of the remaining CaaX protease, they are almost indistinguishable from wild-type cells (Supplemental Figure S1). However, in rce1Δ ste24Δ double deletion mutants, no halos were visible. When we transformed the rce1Δ ste24Δ double deletion cells with a vector carrying STE24, we could restore the halo phenotype of wild-type cells. In contrast, transformation of ste24Δ cells with a vector carrying ste24ΔE298G showed no halo, indicating that the catalytic activity of Ste24 was abolished (Supplemental Figure S1). Next, we tested CFW resistance in ste24Δ cells expressing either Ste24 or the catalytically inactive Ste24E298G. As shown in Figure 4A, transformation with the wild-type STE24 vector restored CFW sensitivity of ste24Δ cells, whereas transformation with the catalytically inactive ste24ΔE298G vector did not. Thus, the observed effect on chitin synthesis in ste24Δ mutants depends on the catalytic activity of Ste24.

Human and Insect Homologues of STE24 Restore CFW Sensitivity in ste24Δ Cells
Ste24 is a conserved CaaX protease and homologues can be found ubiquitously in eukaryotes. Despite a rather low amino acid sequence identity of ~36% between yeast Ste24 and its human homologue ZmpSte24, Leung et al. (2001) demonstrated that human ZmpSte24 can restore the halo phenotype in the rce1Δ ste24Δ cells. The insect Ste24 homologue from the red flour beetle, Tribolium castaneum (TcSte24) fully restores the halo phenotype in the rce1Δ ste24Δ cells (Supplemental Figure S1). To test whether the STE24 homologues could restore the CFW phenotype, we expressed ZmpSTE24 and TcSTE24 in ste24Δ cells and plated them on plates containing CFW. These growth tests indicated that ste24Δ cells expressing human or Tribolium STE24 exhibit the same sensitivity to CFW as wild-type cells (Figure 4A), suggesting that they are functional orthologues of yeast STE24.
Membrane Association of Chs4 Requires Prenylation but Is Independent of Further CaaX Processing

To further analyze the role of Chs4 prenylation and CaaX cleavage, we performed localization studies using GFP-tagged proteins. To investigate the contribution of prenylation and cleavage independently, we expressed Chs4-GFP and Chs4C693S-GFP in wild-type, ste24Δ and ste24Δ rce1Δ cells. As shown in Figure 5, no significant difference in Chs4-GFP localization was detectable in wild-type, ste24Δ, or ste24Δ rce1Δ suggesting that Ste24-mediated cleavage of the CaaX motif is not required for membrane association of Chs4. However, Chs4C693S-GFP showed distinct differences in localization compared with wild-type Chs4-GFP. The majority of Chs4C693S-GFP localized in the cytoplasm and no signal could be observed at the plasma membrane (Figure 5). The deletion of the CaaX proteases had, as expected, no influence on Chs4C693S-GFP localization. Hence, membrane association of Chs4 depends on prenylation but not on subsequent steps of CaaX processing.

Deletion of ste24 Leads to Reduced Levels of Chs3 at the Bud Neck

Finally, we addressed the question whether the different chitin levels in the cell walls of wild-type, ste24Δ, chs3Δ, and STE24Δ cells result from perturbations of the intracellular transport of Chs3. For this purpose, we examined the subcellular localization of Chs3-GFP in these strains, after we have shown that Chs3-GFP can restore the wild-type CFW phenotype in chs3Δ cells and thus is fully functional (data not shown). In all four strains, Chs3-GFP localizes mainly to vesicles (~90% of examined cells; Figure 6), although in wild-type cells as well as in cells overexpressing STE24Δ, ~40% of all examined cells showed Chs3-GFP was located at the bud neck, but in ste24Δ cells, which produce significantly less chitin, only ~20% of all examined cells showed Chs3-GFP at the bud neck (Figure 6). In chs3Δ cells, the Chs3-GFP signal was more diffuse than in the other examined strains and Chs3-GFP was less frequently at the bud neck as in the case of ste24Δ cells (~20% of all examined cells). Thus our data indicate that the effects of ste24 on chitin synthesis may be mediated by delocalization of Chs3.

Immunobots Do Not Support That Chs3 Is a Substrate for Ste24

Ste24 has dual roles in protein processing. In addition to its function of cleaving the C-terminal CaaX motif, it is also known to cleave the N terminus of the MFα precursor (Trueblood et al., 2000). Because Chs3 lacks a C-terminal CaaX motif, Ste24 evidently does not cleave the C terminus of Chs3. Therefore, we addressed the question whether Ste24 cleaves Chs3 at all. For this purpose, we prepared total cellular extracts from wild-type and ste24Δ cells expressing Chs3-13Myc from centromeric plasmids and performed Western blot analysis. In both cell extracts, we could detect two protein bands of ~100 and 160 kDa, both of which did not shift in response to deletion of STE24 (Supplemental Figure S2). This result suggests that Chs3 is not cleaved by Ste24 and further substantiates the findings of Cos et al. (1998), who also observed no differences in the migration behavior of Chs3 tagged with HA-epitopes either at the N or C terminus. However, we cannot totally exclude the possibility that a small peptide is cleaved off by Ste24 at the N or C terminus.

Prenylation of Chs4 Is Required for Ste24 Effects on Chitin Synthesis

The results described above imply that the proteolytic activity of Ste24 is required to maintain wild-type chitin levels. Although Chs3 binds to Ste24, direct processing of Chs3 by the CaaX protease turned out to be unlikely. However, Ste24 may process another protein required for chitin synthesis. An obvious candidate is Chs4, a known activator of Chs3 (DeMarini et al., 1997; Trilla et al., 1997). Unlike Chs3, Chs4 possesses a C-terminal CaaX motive (CVIM). Thus, Chs4 fulfills all sequence requirements to be cleaved by Ste24 (Trueblood et al., 2000). Furthermore, Chs4 is known to be prenylated, which is a prerequisite for removal of the C-terminal tripeptide by the CaaX protease. Direct biochemical proof corroborating that Chs4 is a substrate of Ste24 is difficult to obtain, because protein analysis is hampered by prenylation and the fact that only a tripeptide is removed yielding only small differences in molecular masses. Therefore, we used a genetic approach to address this issue based on the observation that mutants expressing a nonprenylatable version of Chs4, in which the cysteine of the CaaX motif is replaced by a serine (Chs4C693S), exhibit a CFW resistance phenotype (Grabinska et al., 2007). When we repeated this experiment we could confirm the CFW resistance phenotype. CFW resistance of chs4C693S cells was more pronounced than that of ste24Δ cells but less pronounced than that of chs4Δ cells (Figure 4B). The chitin content in chs4C693S cells was reduced by 33%, which was 11% more than the reduction of the chitin content in ste24Δ cells (Figure 3B). If Ste24-mediated cleavage of the CaaX motif of Chs4 is required for chitin synthesis, the CFW phenotype of cells expressing a nonprenylatable form of Chs4 should be unchanged when STE24 is overexpressed or deleted. As shown in Figure 4B, Chs4C693S cells exhibit the same CFW phenotype as ste24Δ Chs4C693S or STE24 overexpressing Chs4C693S cells.

Figure 4. Calcofluor white sensitivity in different yeast strains. (A) Functional tests of a catalytically inactive Ste24 mutant, and human and insect orthologues. Wild-type, ste24Δ, ste24Δ pRS415ste24, ste24Δ pRS415ste24ΔC693S, ste24Δ pJJH71-ZMPSTE24, and ste24Δ pJJH71-TcSTE24 cells were grown overnight in liquid YPD medium and diluted to a final concentration of 1 × 10⁶ cell/ml in water. Five microliters of each suspension and three subsequent 10-fold serial dilutions were spotted onto YPD plates with or without 50 μg/ml CFW. Cells were incubated at 30°C for 3 d. (B) Calcofluor white sensitivities in mutants defective in Chs4 prenylation. Wild-type, chs4Δ, chs4Δ pJPJH71-ZMPSTE24, chs4Δ pJJH71-TcSTE24, and pJJH71-STE24 cells were grown and diluted as described above.
DISCUSSION

Protein interaction between Chs3 and Chs4 had been reported previously by classical yeast two-hybrid analysis (DeMarini et al., 1997; Ono et al., 2000), whereas that between Chs3 and Ste24 was detected in a large-scale split ubiquitin screening (Miller et al., 2005). DeMarini et al. (1997) further demonstrated that a 700-amino acid domain making up the N-terminal region of Chs3 mediates binding to Chs4, and homodimerization of Chs3. In this study, we were able to confirm these interactions and to narrow down the binding site of Chs3 to a region between amino acid positions 226–452 (C3 domain). Computer-based prediction of Chs3 topology with different programs yielded inconclusive results with six to nine postulated transmembrane helices and a C3 domain that is exposed either to the cytoplasm or to the extraplasmic space. Our yeast two-hybrid results strongly suggest that the C3 domain is located intracellularly: 1) Chs4 is a cytoplasmic protein and therefore can only interact with the C3 domain facing the cytoplasm. 2) The C3 domain interacts with a cytoplasmic interface of Ste24 formed by the S2 and S8 domains (Tam et al., 2001). 3) Lam et al. (2006) demonstrated that Chs3 is palmitoylated by Pf4, whose catalytic domain faces the cytoplasm, and that this post-translational modification is one prerequisite for its exit from the ER. Screening for palmitoylation sites with CSS-Palm2.0 identified only two putative type III motifs in Chs3, both located in the C3 domain (amino acid positions 227–231, 446–451; Ren et al., 2008), additionally supporting its cytosolic location. PhoA fusion studies performed with the rhizobial chitin synthase NodC showed that the catalytic domain of this β-glycosyltransferase is exposed to the cytoplasm (Barny et al., 1996). Therefore, we propose a topology model in which the C3 domain precedes the catalytic domain, both facing the cytoplasm (Figure 1A). Further studies have to be carried out to determine the correct topology and function of the remaining Chs3 domains.

Chs3 is synthesized at the rough ER and transported via the Golgi network and trans-Golgi vesicles to the plasma membrane of the bud neck (Lesage and Bussey, 2006). Ste24 and Rce1 are only known CaaX protease in yeast. Ste24 carries a C-terminal dilysine motif (KKXX) and thus is retained in the ER (Tam et al., 2001). An interaction between Chs3 and Ste24 can only take place at the ER, because it is the only membrane compartment in which both proteins are at least transiently located. Ste24 is also found at the inner nuclear membrane (Barrowman et al., 2008). In vertebrates, Ste24 orthologues cleave the nuclear filament lamin A, and mutations in the Zmpste24 gene have been linked to progeroid syndromes (Bergo et al., 2002; Pendas et al., 2002; Fong et al., 2004). A nuclear substrate of Ste24, however, has not been reported yet in yeast. The only proven substrate of Ste24 in yeast is the MFα precursor, which is cleaved by Ste24 at the C-terminal CaaX motive in a prenylation-dependent manner and at an undefined sequence at the N terminus (Tam et al., 1998). The sequence specificities for CaaX cleavage by Ste24 and Rce1 have been determined by site-directed mutagenesis and both proteases have overlapping but distinct substrate specificities (Trueblood et al., 2000).
Genome analysis identified 98 yeast proteins that are potentially cleaved by CaaX proteases at their C termini, 35 of which were postulated to be cleaved by Ste24 (Trueblood et al., 2000). Because Chs3 does not possess a CaaX motif it is obviously not a substrate for prenylation-dependent cleavage by Ste24. Moreover, Western blot analysis performed in this work and by Cos et al. (1998) yielded no evidence for cleavage of Chs3 at the N terminus. How then does Ste24 modulate chitin synthesis? A plausible answer is that another protein involved in chitin synthesis might be a substrate for Ste24. Among the 35 CaaX proteins that theoretically can be cleaved by Ste24 we could identify only one which is linked to chitin synthesis. This protein is Chs4, a known activator of Chs3 (Trilla et al., 1997), which possesses a C-terminal CaaX motif recognized by Ste24 (Trueblood et al., 2000). How precisely Chs4 activates Chs3 is still unclear; however, this process requires an interaction between both proteins (DeMarini et al., 1997; Ono et al., 2000). Chs4 is required for Chs3 activity and/or its recruitment to the bud neck (DeMarini et al., 1997; Trilla et al., 1997). DeMarini et al. (1997) further hypothesized that Bni4 functions as a linker protein between Chs4 and septins of the bud neck. However, further analysis of this process yielded a more complex picture involving also the catalytic subunit of a type 1 serine/threonine protein phosphatase (Glc7), which is necessary for Chs3 recruitment to the bud neck (Kozubowski et al., 2003; Lesage et al., 2005; Larson et al., 2008).

Does Ste24 cleave Chs4 at its CaaX motif and why does Ste24 bind to Chs3? Because CaaX cleavage depends on prenylation, we generated a mutant in which the cysteine of the CaaX motif was replaced by serine and examined its CFP phenotype in ste24Δ or STE24 overexpressing cells. Although yeast cells expressing wild-type Chs4 showed CFW resistance or hypersensitivity in response to deletion of STE24, we observed no effects in cells expressing nonprenylated Chs4C693S. Because prenylation of Chs4 by the Ram1/Ram2 complex may be required also for ER tethering. Prenylation of Chs4 by the Ram1/Ram2 complex seems not to be involved in membrane association. Nevertheless, deletion of ste24 affects proper localization of Chs3-GFP at the bud neck and decreases chitin levels. Therefore, next to prenylation subsequent steps of CaaX processing of Chs4 may contribute to the correct localization and activation of Chs3. However, deletion of ste24 moderately affects chitin synthesis and does not result in a complete loss of Chs3-GFP at the bud neck. Therefore, yeast cells may have mechanisms that partially compensate for the deficiency in Ste24 mediated CaaX cleavage, which may furthermore process additional substrates involved in chitin synthesis.

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