Regulated ubiquitination and degradation of signaling proteins have emerged as key mechanisms for modulating the strength and duration of signaling pathways. The reversible nature of the ubiquitination process as well as the large number and diversity of the deubiquitinating enzymes raise the possibility that signaling pathways might be modulated by specific deubiquitinating enzyme(s). Here we provide evidence that in the yeast *Saccharomyces cerevisiae*, the Pkc1-mediated signaling pathway that controls the cell wall integrity is negatively regulated by the deubiquitinating enzyme Ubp3. Disruption of the *UBP3* gene leads to an enhanced activation of the cell wall integrity pathway MAPK Slt2 when cells are challenged with a variety of pathway activation agents such as pheromone and Congo red. The *ubp3* deletion mutants accumulate high levels of Pkc1, suggesting potential regulation of Pkc1 by Ubp3. Consistent with this, Pkc1 and Ubp3 interact in vivo, and the stability of Pkc1 is markedly increased in the *ubp3* deletion mutants. Moreover, disruption of the *PKC1* gene, but not the genes that encode components downstream of Pkc1, completely suppresses other phenotypes displayed by the *ubp3* deletion mutants such as hyperactivation of the pheromone-responsive MAPK Fus3 (Wang, Y., and Dohlman, H. G. (2002) *J. Biol. Chem.* 277, 15766–15772). These findings demonstrate that Ubp3 can regulate Pkc1 by facilitating its destruction and provide the initial evidence that Pkc1 plays a positive role in modulating the parallel pheromone-signaling pathway.

All cells have the capacity to sense various environmental stimuli and then generate an appropriate intracellular response. One common mechanism for detecting and transmitting extracellular signals uses cell surface receptors coupled to intracellular heterotrimeric guanine nucleotide-binding proteins (G proteins). In this signaling system, a receptor captures an extracellular stimulus and switches on its coupled G proteins, which in turn activate the downstream effector proteins, such as mitogen-activated protein kinases (MAPKs) (1, 2). Activated MAPKs go on and activate many of their cellular targets, including nuclear transcription factors, cytoskeleton proteins, and other enzymes, to enable the cells to generate appropriate responses to the perceived stimulus (3).

Studies in signaling regulation typically focused on the behavior of individual signaling pathways. However, the cellular responses to a given stimulus often involve the actions of multiple parallel signaling pathways, and the mechanisms underlying their coordination are largely unknown (1). In the budding yeast *Saccharomyces cerevisiae*, pheromone stimulation primarily activates a signaling cascade that includes a heterotrimeric G protein (Gpa1/Ste4/Ste18) and a MAPK complex (Ste11/Ste7/Fus3) (Fig. 1), which is responsible for most of the cellular responses such as growth arrest and new gene transcription that are triggered by pheromone (1, 4). Pheromone stimulation also activates a parallel signaling pathway that primarily controls the cell wall synthesis and thereby maintains the cell integrity (5–7). The key components of the cell wall integrity pathway include the upstream kinase Pkc1, which is the sole protein kinase C homolog in yeast, and a MAPK cascade composed of the MAPK kinase kinase (MAPKKK) Bck1, the two redundant MAPK kinases (MAPKKs) Mkk1 and Mkk2, and the MAPK Mpk1/Slt2 (6) (Fig. 1). Signaling from the pheromone pathway to Mpk1/Slt2 is achieved via two distinct routes: one via Ste20-induced morphological change and the other via Ste12-mediated new gene transcription (5, 7) (Fig. 1). Whether activation of the cell wall integrity pathway may in turn contribute to modulation of the activity of the pheromone-responsive MAPK Fus3 is unclear.

Post-translational modification is one of the most widely used mechanisms for regulating the activities of signaling proteins. With regards to MAPK components, much of the effort has focused on phosphorylation. Recent studies indicate that MAPK components also undergo ubiquitination, a modification typically leading to protein degradation (8, 9). Similar to phosphorylation, the ubiquitination process is reversible. Removal of conjugated ubiquitin or ubiquitin chains from substrates is facilitated by deubiquitinating enzymes (10). In yeast, there are a total of 17 deubiquitinating enzymes; in human, genome analysis revealed the existence of 95 such enzymes (11). One remarkable characteristic of deubiquitinating enzymes is their diversity. Outside their catalytic domains, there are very few structural similarities (12). The large number and the diversity of those enzymes suggest that each might have a specific function in regulating biological processes (13). Consistent with this view, several deubiquitinating enzymes with specific substrates and regulatory functions have been identified. The most notable examples are HAUSP in mammals, which specifically regulates the stability of both p53 and Mdm2 (14, 15), and...
Ub3p in yeast, which has been demonstrated to have a specific role in regulating histone (16, 17).

Here we provide evidence that the yeast Pkc1-mediated signaling is modulated by the specific deubiquitinating enzyme Ubp3. Ubp3 interacts with Pkc1 and facilitates its destruction. We also demonstrate that a functional Pkc1 is required for maintaining the optimal activation of the parallel pheromone signaling pathway and thus reveal a previously unknown level of cross-talk between the cell wall integrity and pheromone response pathways.

**EXPERIMENTAL PROCEDURES**

**Strains and Plasmids**—Standard methods for the growth, maintenance, and transformation of yeast and bacteria and for the manipulation of DNA were used throughout. The yeast *S. cerevisiae* strains used in this study were BY4741 (MATa leu2Δ met15Δ ura3Δ), BY4741-derived mutants lacking MPK1 (SLT2), UBP1, UBP2, UBP3, DOA4 (UPB4, SSV7, NPI2, ASI7), UBP5, UBP6, UBP7, UBP8, UBP9, UBP11, UBP12, UBP13, UBP14, UBP15, and UBP16 (LPF12) (Research Genetics, Huntsville, AL); BY4741-derived mutants lacking both MPK1 (SLT2) and UBP3 (mpk1::KanMX ubp3::HIS3, this work); YPH499 (MATa ura3-52 lys2-801Δ ade2-101Δ trp1-Δ 63 his3-Δ 200 leu2-Δ 1); YPH499-derived mutants lacking UBP3 (ubp3::KanMX), and PKC1 (pkc1::LEU2, from J. Thorner, University of California, Berkeley, CA), and both UBP3 and PKC1 (ubp3::KanMX pkc1::LEU2, this work).

Expression plasmids used in this study that have been described previously are pRS316-GAL-STE4 and YCP50-STE11-4 (from George Sprague, University of Oregon), pYES-GAL-UBP3C469S (18), and BG1805-GAL-ROM1 and BG1805-GAL-BCK1 (purchased from Openbiosystems). Overexpression of His6-tagged PKC1 was achieved by PCR amplification and subcloning into pYES2.1/V5-His-TOPO (2 μm, IRA3, GAL1 promoter, CYC1 terminator) (Invitrogen). PCR primers were 5′-CCA GAA TGA GTT TTT CAC AAT TGG AGC-3′ and 5′-CCC AAG CTT TAA ATC CAA ATC ATC TGG C-3′. The PKCI allele encoding a constitutively active form of Pkc1 (Pkc1<sup>ES398A</sup>) was generated by site-directed mutagenesis using PKCI in pYES2.1 as the template (Qiagen). Mutagenesis primers were 5′-GCA GTT GAT GGG TGG ACT ACA TGC TGA TCG TGC TAT TAT CAA TAG G-3′ and 5′-CCT ATT GAT AAT AGC ACC ATG AGC ATG TAG TCC ACC CAT CAA CTG C-3′.

A triple-FLAG epitope tag was placed at the C terminus of Ubp3 (Ubp3-FLAG) by PCR amplification and subcloning into the XmaI site of pAD4M (2 μm, LEU2, ADH promoter and terminator) to yield plasmid pAD4M-UBP3-FLAG. PCR primers were 5′-TCC CCC CGG GCA GCT GCC AGA ATG AAC ATG CAA GAC GCT AAC AAA GAA G-3′ and 5′-TCC CCC CGG GCA GCT GCC AGA ATG AAC ATG CAA GAC GCT AAC AAA GAA G-3′.

**FIGURE 1.** The components of pheromone and cell wall integrity pathways. See Introduction for details.

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Growth, Transcription, Phosphorylation, and Degradation Bioassays—Growth and reporter transcription assays in response to pheromone was conducted as described previously (19). Phosphorylation of Fus3, Kss1, and Mpk1/Slt2 was monitored by immunoblotting of whole cell extracts following the same procedures described previously (20). To monitor the stability of Pkc1, mid-log cell cultures grown on galactose-containing medium (PKC1 under the control of the galactose-inducible promoter GAL1) were switched to glucose-containing medium for up to 120 min. Growth was stopped by the addition of 10 mM NaN<sub>3</sub> and transfer to an ice bath. Cells were washed and resuspended directly inboiling SDS-PAGE sample buffer (62.5 μl Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 1% 2-mercaptoethanol, 0.0005% bromphenol blue) for 10 min, subjected to glass bead homogenization, and clarified by microcentrifugation. Following SDS-PAGE and transfer to nitrocellulose, the membrane was probed with antibodies to phospho-p44/42 at 1:1000 (from Cell Signaling), Pgk1 at 1:75,000 (from J. Thorner), ubiquitin at 1:100 (Sigma), penta-His at 1:1000 (Qiagen), and Mpk1 (yC-20) at 1:500 and Pkc1 (yC-20) at 1:500 (Santa Cruz Biotechnology). The anti-Pkc1 antibody was precleared by incubating it with membranes containing yeast cell extracts from the pck1Δ cells. Immune-reactive species were visualized by enhanced chemiluminescence detection (Pierce) of horse-radish peroxidase-conjugated anti-rabbit IgG (Bio-Rad) or anti-goat IgG (Santa Cruz Biotechnology). Specificity of detection was established using mpk1Δ, fus3Δ, kss1Δ, and pck1Δ cell extracts as negative controls.

**Immunoprecipitation**—The association of Pkc1 and Ubp3 was examined by immunoprecipitation of FLAG-tagged Ubp3 and immunoblotting with anti-His antibodies for the detection of His-tagged Pkc1. Cells (50 ml) cotransformed with plasmids pYES-PKC1-His<sub>6</sub> and pAD4M-UBP3-FLAG were grown to A<sub>600 nm</sub> = 1, harvested, and resuspended in 550 μl of lysis buffer (50 mM NaPO<sub>4</sub>, pH 7.5, 400 mM NaCl, 0.1% Triton X-100, 10% glycerol, 0.5 mM dithiothreitol, 25 mM NaF, 25 mM glycerophosphate, 1 mM sodium orthovanadate, 10 mM N-ethylmaleimide, 5 mM phenylmethylsulfonyl fluoride, and one pellet of complete EDTA-free protease inhibitor mixture (Roche Applied Science)). This and all subsequent manipulations were carried out.
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at 4 °C. Cells were subjected to glass bead vortex homogenization for 30 s, repeated 8 times, and centrifuged twice at 6000 × g for 5 and 25 min. Lysates were incubated for 2 h with a bead volume of 10 μl of anti-FLAG M2 affinity resin (Sigma) equilibrated in lysis buffer. Immunoprecipitates were collected by centrifugation at 1000 × g for 30 s, and pellets were washed with 1 ml of lysis buffer for 3 min, repeated four times before final resuspension in 30 μl of 2× SDS-PAGE sample buffer. Each sample was resolved by 7.5% polyacrylamide gel electrophoresis and immunoblotting with anti-His monoclonal antibodies at 1:1000 or anti-FLAG monoclonal antibodies at 1:2000.

Data Quantification and Statistical Analysis—Band intensities of Western blot analysis were quantified using the Image J software from the National Institutes of Health. Where indicated, the data were statistically analyzed by t test with a p value of <0.050 considered significant.

RESULTS

Regulated ubiquitination and degradation have emerged as important mechanisms to modulate the strength and duration of cell signaling (8, 9). Similar to phosphorylation, the ubiquitination process is reversible. Removal of ubiquitin or ubiquitin chains from conjugated substrates is catalyzed by a family of deubiquitinating enzymes (21). Genome analysis in humans revealed the existence of more than 90 deubiquitinating enzymes (10); in yeast, a total of 17 such enzymes have been identified (11). Remarkably, other than the catalytic regions, there are limited similarities among all these enzymes. An appealing hypothesis to explain the large number and diversity of the deubiquitinating enzymes is that each of them might have a specific function in regulating a certain biological process. To test this hypothesis and to identify additional mechanisms for MAPK regulation, we examined the regulatory role of the specific deubiquitinating enzyme Ubp3 on MAPK signaling. We chose Ubp3 because our previous work demonstrated that disruption of UBP3, but not any other UBP genes, led to hyperactivation of pheromone signaling in yeast as measured by growth arrest and reporter transcription assays (19).

The signaling phenotypes displayed by the ubp3Δ mutants suggest that the activity of the pheromone-responsive MAPK Fus3 might be up-regulated in the mutants. Therefore we examined first whether this is indeed the case. For this purpose, we monitored the activation status of Fus3 in wild-type cells versus the ubp3Δ mutants treated with the mating pheromone α factor over time. The activation status of Fus3 can be conveniently monitored by immunoblotting with a phospho-specific antibody directed against dually phosphorylated (Thr202/Tyr204) p44/42 MAPK (22). As expected, phosphorylation and thus activation of both Fus3 and Kss1, a redundant pheromone-responsive MAPK, in the ubp3Δ mutants were substantially increased, especially in the later time points of pheromone treatment (Fig. 2A). Consistent with previous work (5, 23), pheromone stimulation also induced phosphorylation of Mpk1/Slt2, a MAPK primarily responsible for maintaining cell wall integrity (Fig. 2A). Interestingly, pheromone-induced phosphorylation of Mpk1/Slt2 occurred much faster and to a greater extent in the ubp3Δ mutants compared with wild type. These effects did not appear to be caused by an alteration of Mpk1 stability, as the abundance of Mpk1 were essentially the same in both wild-type and ubp3Δ mutant cells, with or without pheromone treatment (Fig. 2A, middle panel).

The rapid and enhanced activation of Mpk1/Slt2 with no change in its expression observed in the ubp3Δ mutants suggests that a certain component upstream of Mpk1/Slt2 might be negatively regulated by Ubp3. An expectation of this model would be that the ubp3Δ mutants should similarly augment the activation of Mpk1/Slt2 when treated with other stimuli that can activate the cell wall integrity pathway. To test this, we treated the mutant and wild-type cells with the commonly used cell wall-stressing agent Congo red (24, 25) and monitored the activation status of Mpk1/Slt2 using the same phosho-specific antibody. Once again, we observed an increase of Mpk1 phosphorylation in the ubp3Δ mutants compared with the wild-type cells (Fig. 2B). The ubp3Δ cells showed a growth defect in the presence of Congo red (Fig. 2C) similar to the phenotype displayed by the msg5Δ mutants, which lack a functional phosphatase for Mpk1/Slt2 (25). To examine whether any other ubp deletion mutants display a similar phenotype, we monitored the Congo red sensitivity of the following available ubp deletion mutants from the systematic yeast deletion collection: ubp1Δ, ubp2Δ, ubp4Δ, ubp5Δ, ubp6Δ, ubp7Δ, ubp8Δ, ubp9Δ, ubp11Δ, ubp12Δ, ubp13Δ, ubp14Δ, and ubp15Δ. None of these mutants displayed an abnormal sensitivity to Congo red (Fig. 2C and data not shown). Taken together, these findings indicate that Ubp3 negatively regulates the activation of Mpk1/Slt2, possibly at a step upstream of Mpk1/Slt2, and that this regulatory effect appears to be specific to Ubp3.

Both pheromone- and Congo red-induced phosphorylation of Mpk1/Slt2 requires Pck1, the sole homolog of mammalian protein kinase C in yeast. Previous work showed that compared with wild type, a constitutively active mutant of Pck1, i.e. Pck1R380A, was underproduced by a factor of 4 (26), raising the possibility that activated Pck1 might be unstable and that the deubiquitinating enzyme Ubp3 may facilitate its degradation. To test this idea, we initially compared the stability of Pck1 and Pck1R380A. For this purpose, we expressed both alleles under the control of the galactose-inducible promoter GAL1. Upon induction with galactose for 6–8 h, we turned off the expression of both alleles by switching the cells to noninducing glucose-containing medium and tracked the presence of the proteins over time. This method has been commonly used in estimating the half-life of proteins (27, 28). By this assay, Pck1 appears to be a fairly unstable protein with an estimated half-life of 70 min; as expected, the constitutively active Pck1R380A was significantly less stable (Fig. 3A). To examine whether Ubp3 facilitates the destruction of Pck1, we compared the stability of Pck1 in wild-type versus ubp3Δ mutant cells using the same approaches described above. As shown in Fig. 3B, disruption of UBP3 gene dramatically stabilized Pck1. To determine whether an increase in Pck1 stability would lead to an accumulation of the protein, we compared the steady-state levels of Pck1 in wild type versus ubp3Δ mutants by immunoblotting. Indeed, compared with wild type, the level of Pck1 was significantly increased in the ubp3Δ mutants (Fig. 3C). Moreover, immunoprecipitation of Ubp3 resulted in the copurification of Pck1 (Fig. 3D), further suggesting direct regulation of Pck1 by
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Ubp3. These findings indicate that the stability of Pkc1 is affected by its activation status and that proteolysis of Pkc1 is facilitated by Ubp3.

Regulation of Pkc1 stability by Ubp3 suggests that Pkc1 might be degraded by the ubiquitin-proteasome pathway. To test this possibility, we measured the abundance of Pkc1 in temperature-sensitive cim3-1 and cim5-1 mutants, which at the restrictive temperature have severely impaired proteasomal protease activity. Cim3 (also known as Rpt6) and Cim5 (also called Rpt1) are subunits of the proteasome 19 S regulatory particle, which confers specificity for ubiquitinated proteins for presentation to the proteasome proteolytic subunits. As expected, Pkc1 expression was significantly enriched in both cim3-1 and cim5-1 mutants in the pheromone treated cells, confirming that the abundance of Pkc1 protein is controlled by the proteasome (Fig. 4).

We reasoned that if Pkc1 is a functionally relevant target of Ubp3 in the cell wall integrity pathway, constitutive signaling achieved via overexpression of pathway components upstream or downstream of Pkc1 might be differentially affected by loss of Ubp3 activity. Specifically, constitutive signaling from upstream but not downstream components of Pkc1 would be further augmented in the ubp3Δ mutants. To test this predication, we first screened for the components in the pathway whose overexpression would lead to activation of Mpk1/Slt2. From this analysis, we found that overexpression of ROM1 or BCK1 activated Mpk1/Slt2 (Fig. 5A and data not shown). ROM1 encodes a guanine nucleotide exchange factor for the small G protein Rho1, which acts upstream of Pkc1, whereas BCK1 encodes the MAPKK Bck1, which is a direct target of Pkc1 (Fig. 1). Consistent with our model that Pkc1 is a functional target of Ubp3, constitutive signaling from upstream of Pkc1 (by overexpression of ROM1) but not from downstream of Pkc1 (by overexpression of BCK1) was further increased in the ubp3Δ mutants (Fig. 5, A and B). An increase in the abundance of either Rom1 or Rho1, two components immediately upstream of Pkc1, would give the same outcome in the ubp3Δ mutants. However, this was not the case, as the level of neither protein was increased in the ubp3Δ mutants (data not shown).

Having established a regulatory role of Ubp3 in Pkc1 proteolysis, we next sought to examine whether up-regulation of
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Pkc1 in the ubp3Δ mutant could account for its phenotype of hyperactivation of pheromone-responsive MAPks Fus3 and Kss1 (Fig. 2A). For this purpose, we constructed a yeast strain that lacks both PKC1 and UB P3 and monitored the kinetics of Fus3 and Kss1 activation in response to pheromone treatment.

Remarkably, the enhancement of Fus3 activation observed in the ubp3Δ mutants upon pheromone treatment was completely suppressed by an additional disruption of PKC1 (Figs. 2A and 6A). An obvious interpretation of this result is that abnormal up-regulation of Pkc1 itself is responsible for the per-
FIGURE 6. Enhanced activation of Fus3 in UBP3 disruptive mutants requires a functional Pkc1. A, pheromone-dependent activation of Fus3 in ubp3Δ and ubp3Δ pkc1Δ mutants was measured by immunoblotting (IB) with anti-phospho-p42/44 antibodies. Whole cell extracts were prepared from ubp3Δ and ubp3Δ pkc1Δ mutants treated with 3 μM pheromone α factor for the indicated times, resolved by 10% SDS-PAGE, and probed with anti-phospho-p42/44 (top panel) or anti-Pgk1 (bottom panel) antibodies to confirm equal loading of each lane. B and C, the effect of expressing a dominant-negative allele of UBP3, i.e. UBP3-C459S, on Fus3 activation was measured by immunoblotting with anti-phospho-p42/44 antibodies. Whole cell extracts from wild type, bck1Δ, mpk1Δ, or pkc1Δ mutants transformed with plasmids expressing UBP3-C459S or empty vector were resolved by 10% SDS-PAGE and probed with anti-phospho-p42/44 (top panel) or anti-Pgk1 (bottom panel) antibodies. The bar graph in C shows a comparison of the relative amount of activated Fus3 (phospho-Fus3 (P-Fus3)) in pheromone-treated pkc1Δ cells transformed with empty vector (Vector) or plasmids expressing UBP3-C459S. Relative amounts are normalized to the amount of phospho-Fus3 in cells transformed with empty vector. Values are averages from three independent experiments.

sistent activation of Fus3 in the ubp3Δ mutant cells. Alternatively, as Pkc1 is required for transmitting basal signaling to its downstream components, including Bck1 and Mpk1/Slt2, up-regulation of any of these proteins by Ubp3, which in turn regulates the activation status of Fus3, would potentially give the same outcome. To dissect these possibilities, we further analyzed the activation status of Fus3 in the UBp3 defective mutant cells that also lack BCK1 or MPK1. For these purposes, we made use of a dominant-negative allele of UBp3 (i.e. UBp3C459S (18)) to disrupt Ub3 function in wild-type versus mutant cells that lack PKC1, BCK1, or MPK1. As expected, expression of UBp3C459S in wild-type cells, but not in pkc1Δ mutant cells, significantly increased Fus3 phosphorylation (Fig. 6, B and C). However, phosphorylation of Fus3 in bck1Δ or mpk1Δ mutant cells was enhanced when the dominant-negative allele of UBp3 was expressed (Fig. 6B). Therefore, the effect of Ubp3 on the activation status of Fus3 requires Pkc1 but not its downstream component Bck1 or Mpk1.

The above data support a model in which an enhancement of Pkc1 activity in the ubp3Δ mutants leads to the persistent and augmented activation of Fus3 in response to pheromone stimulation. A logical implication of this model is that Pkc1 might have a previously unappreciated role in pheromone signaling, such as the maintenance of optimal activation of Fus3. To test this, we analyzed the status of pheromone-induced activation of Fus3 in cells lacking PKC1. As shown in Fig. 7A, disruption of PKC1 significantly diminished the strength of Fus3 activation,
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indicating that Fus3 or certain components upstream of Fus3 might be regulated by Pkc1.

To determine where in the pheromone pathway Pkc1 operates, we compared signaling in wild-type and pkc1Δ cells activated at different points downstream of the pheromone receptor. For this analysis, the pathway was activated in two distinct ways. In the first case, we overexpressed STE4, which leads to an increase in cellular Gβγ beyond that which can bind to Gα and as a consequence leads to constitutive signaling. In the second case, we used an activated allele of the MAPKKS STE11 (STE11-4) containing a single amino acid substitution (T596I) within the catalytic domain. Constitutive activation by STE11-4 is unaffected by mutations in the receptor (Ste2) or Gβ (Ste4) but is blocked by mutations in the downstream MAPKK (Ste7), or MAPK (Fus3, Kss1). Thus STE11-4 activates signaling at a well defined step in the pathway between Ste20 and Ste7. The pck1Δ mutant can diminish signaling by overexpression of STE4 (Fig. 7, B, left panel, and C) but not of STE11-4 (B, right panel, and C). These data indicate that Pkc1 regulates pheromone signaling at the step(s) between the Gβ Ste4 and the MAPKKK Ste11.

DISCUSSION

Many biological processes are regulated by protein kinase-mediated signaling pathways. A clear understanding of the mechanisms and regulation of these signaling pathways is essential in revealing new disease mechanisms and designing novel approaches for pharmaceutical intervention. Studies of the signaling regulation typically focused on phosphorylation and de-phosphorylation of signaling components. Recent studies indicate that an alteration of the abundance of many of the same components also plays an important role in modulating signaling strength and duration (8, 9). Examples can be found in both yeast and mammalian cells and include cell surface receptors, G proteins, MAPKKS, a MAPKK, and numerous transcription factors (9).

Here we provided evidence that the abundance of Pkc1, the sole protein kinase C homolog in yeast, is negatively regulated by the specific deubiquitinating enzyme Ubp3. Disruption of UBP3 dramatically stabilizes Pkc1 and consequently leads to a significant increase in its abundance. As a result, signaling mediated by Pkc1 is up-regulated in the ubp3Δ mutants. Pck1 and Ubp3 physically interact, suggesting a direct regulation of Pkc1 by Ubp3. In mammalian cells, protein kinase C is known to be regulated by ubiquitination-dependent proteolysis. It has been shown that activation leads to ubiquitination and subsequent degradation of protein kinase C in renal epithelial cells as well as in 3Y1 rat fibroblasts (29, 30). Thus it is not surprising that the stability of Pkc1 is dependent on its activation status. Our data further suggest that the degradation of activated Pkc1 is facilitated by a specific deubiquitinating enzyme, raising the possibility that such an additional layer of regulation might exist in mammalian cells. Genomic analysis indicates Usp10 as a Ubp3 homolog in humans; thus it would be interesting to examine whether disruption of Usp10 would result in a similar up-regulation of protein kinase C-mediated signaling in mammalian cells. Identification of a specific deubiquitinating enzyme as a regulator for protein kinase C will provide a new avenue to pharmacologically modulate signaling mediated by protein kinase C, as specific inhibition of a deubiquitinating enzyme is fairly easy to achieve.

A remaining challenge is to understand the mechanism by which Ubp3 destabilizes Pkc1. Our analysis clearly indicates that Pkc1 is a substrate of the proteasome, as its abundance is significantly increased in proteasome-deficient cin3-1 and cim5-1 mutants. Certain proteasome-associating deubiquitinating enzymes such as Rpn11 have been demonstrated to facilitate substrate proteolysis by removing the conjugated ubiquitin chain from the substrates that have been delivered to the proteasome (31). It is appealing to speculate that Ubp3 might play a similar role in facilitating selected substrates, including Pkc1, for degradation in the proteasome.

Pheromone-induced activation of the cell wall integrity pathway has been studied extensively (5, 7). However, whether the activated cell wall integrity pathway in turn regulates pheromone response has not been investigated fully. Here we provided evidence that a functional Pkc1 is required for maximal activation of the mating pathway-specific MAPK Fus3. Epistasis analysis using multiple well characterized strategies revealed that in the pheromone pathway the step(s) that integrate input from Pkc1 lies between the Gβ Ste4 and the MAPKK Ste11. One exciting possibility is that Pkc1 directly phosphorylates components in the pheromone pathway and as such to enhance the activation of such components. Based on the data of epistasis analysis, such a component could either be the MAPKKK Ste11 or its upstream component such as Ste50. Another possibility that may explain the phenotype is the competition of a common phosphatase by pheromone and cell wall integrity pathways. Although it is purely speculative, it is possible that there is a common phosphatase that inactivates both Ste11, the MAPKKS in the pheromone pathway, and Bck1, the corresponding kinase in the cell wall integrity pathway. Activation of Bck1 by Pkc1 might diminish the negative effect of such a phosphatase on the activated Ste11; conversely, lack of Pkc1 would favor the action of such a phosphatase on Ste11. Consequently, the pkc1Δ mutants would display a diminished activation of Fus3, as we have observed here. Regardless of the mechanism, our data clearly suggest a new connection between the pheromone and cell wall integrity pathways, which reinforces the idea that any biological process is a result of the coordination of parallel signaling pathways.

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