Rts1-protein phosphatase 2A antagonizes Ptr3-mediated activation of the signaling protease Ssy5 by casein kinase I

Deike J. Omnus and Per O. Ljungdahl
Department of Molecular Biosciences, Wenner-Gren Institute, Stockholm University, S-106 91 Stockholm, Sweden

ABSTRACT Ligand-induced conformational changes of plasma membrane receptors initiate signals that enable cells to respond to discrete extracellular cues. In response to extracellular amino acids, the yeast Ssy1-Ptr3-Ssy5 sensor triggers the endoproteolytic processing of transcription factors Stp1 and Stp2 to induce amino acid uptake. Activation of the processing protease Ssy5 depends on the signal-induced phosphorylation of its prodomain by casein kinase I (Yck1/2). Phosphorylation is required for subsequent Skp1/Cullin/Grr1 E3 ubiquitin ligase–dependent polyubiquitylation and proteasomal degradation of the inhibitory prodomain. Here we show that Rts1, a regulatory subunit of the general protein phosphatase 2A, and Ptr3 have opposing roles in controlling Ssy5 prodomain phosphorylation. Rts1 constitutively directs protein phosphatase 2A activity toward the prodomain, effectively setting a signaling threshold required to mute Ssy5 activation in the absence of amino acid induction. Ptr3 functions as an adaptor that transduces conformational signals initiated by the Ssy1 receptor to dynamically induce prodomain phosphorylation by mediating the proximity of the Ssy5 prodomain and Yck1/2. Our results demonstrate how pathway-specific and general signaling components function synergistically to convert an extracellular stimulus into a highly specific, tuned, and switch-like transcriptional response that is critical for cells to adapt to changes in nutrient availability.

INTRODUCTION Cells sense and respond to a multitude of extracellular and intracellular cues. Highly specific signaling pathways ensure that distinct stimuli evoke appropriate responses. Deciphering mechanisms that govern the fidelity of signaling pathways is fundamental to understanding how cells control growth and proliferation. The Ssy1-Ptr3-Ssy5 (SPS) signaling pathway in Saccharomyces cerevisiae provides a useful paradigm. In response to extracellular amino acids, this pathway induces the expression of a limited set of genes encoding broad-specificity amino acid permeases that function at the plasma membrane to facilitate amino acid uptake (reviewed in Ljungdahl and Daignan-Fornier, 2012). Accordingly, this pathway is essential for growth of auxotrophic cells lacking the ability to synthesize amino acids. An interesting characteristic of the SPS signaling pathway is that a highly specific output is generated by the orchestrated recruitment of general signaling components, including the plasma membrane–localized casein kinases Yck1 and Yck2, the Skp1/Cullin/F-box (SCF)Grr1 E3 ubiquitin ligase complex, and the 26S proteasome, which participate in diverse regulatory events within cells (Gross and Anderson, 1998; Willems et al., 2004; Wolf, 2004; Knippschild et al., 2005; Jonkers and Rep, 2009). The inherent constitutive and promiscuous activity of general factors raises fundamental questions regarding how tight, stimulus-dependent regulation of signal transduction can be achieved. The SPS pathway triggers the induced expression of amino acid permeases via the endoproteolytic activation of two latent transcription factors, Stp1 and Stp2 (Stp1/2; Andréasson and Ljungdahl,
2002). Accordingly, at the heart of this pathway lies the Stp1/2-activating protease Ssy5 (Abdel-Sater et al., 2004; Andréasson et al., 2006; Poulsen et al., 2006; Pfirrmann et al., 2010). Ssy5 exhibits homology to chymotrypsin-like serine proteases and is expressed as a zymogen. On folding, Ssy5 cleaves itself into an N-terminal prodomain and a C-terminal catalytic (Cat) domain. Of importance, the prodomain and Cat domain remain noncovalently associated, and the prodomain functions as an inhibitor that effectively masks the transcription factor processing activity of the Cat domain (Andréasson et al., 2006; Pfirrmann et al., 2010). On amino acid induction, a degron motif within the prodomain is phosphorylated at multiple serine/threonine residues by the pleiotropic Yck1/Yck2 kinases (Yck1/2; Omnus et al., 2011). The hyperphosphorylated degron is recognized by the SCFGrr1 ubiquitin E3 ligase complex, leading to prodomain ubiquitylation (Abdel-Sater et al., 2011; Omnus et al., 2011). As a consequence, the polyubiquitylated prodomain is degraded by the 26S proteasome. Thus the phosphorylation-dependent degradation of the inhibitory prodomain induced by extracellular amino acids unMASKS the endoproteolytic activity of the Ssy5 Cat domain (Pfirrmann et al., 2010; Omnus et al., 2011).

Protein phosphorylation is often reversible; hence a change of protein function can be controlled by the opposing activities of protein kinases and phosphatases. Thus, in principle, signaling events can modulate the activity of either a kinase or a phosphatase to protein kinases and phosphatases. Thus, in principle, signaling events can modulate the activity of either a kinase or a phosphatase.

RESULTS
Constitutive Rts1-targeted PP2A sets a threshold for phosphorylation-induced Ssy5 activation
Deletion of RTS1 results in constitutive endoproteolytic processing of Stp1/2 in a manner that retains a strict requirement for a functional SPS sensor (Eckert-Boulet et al., 2006). According to the current understanding of SPS-sensor signaling, the Stp1/2 processing protease Ssy5 is activated by phosphorylation-induced degradation of its inhibitory prodomain (Omnus et al., 2011). We therefore examined whether Rts1 directly controls the phosphorylation status of the Ssy5 prodomain and, as a consequence, its stability. In comparison to wild-type (WT) cells, prodomain levels in rts1Δ cells grown under noninducing (–leu) condition and 30 min after induction with leucine (+leu) were significantly lower (Figure 1A, compare lane 1 with lane 3, and lane 2 with lane 4, respectively). These results indicate that Rts1 participates in governing prodomain stability, likely by maintaining the hypophosphorylated state of the Ssy5 phosphodegron in the absence of bona fide amino acid–induced signaling.

To test this possibility, we monitored the status of prodomain phosphorylation under conditions in which phosphorylated forms of prodomain accumulate, that is, in cells lacking Grr1 (Abdel-Sater et al., 2011; Omnus et al., 2011). Grr1 is a component of the SCFGrr1 complex that ubiquitylates the phosphorylated prodomain, targeting it for proteasomal degradation (Omnus et al., 2011). In the absence of Grr1, prodomain degradation is blocked, enabling the accumulated phosphorylated species to be visualized. In rts1Δ grr1Δ double-mutant cells, and in contrast to grr1Δ cells, slower-migrating phosphorylated prodomain species were readily observed under noninducing conditions (Figure 1B, compare lane 3 with 1). On leucine induction, phosphorylated and extensively hyperphosphorylated prodomain species accumulated in both grr1Δ and rts1Δ grr1Δ cells (Figure 1B, lanes 2 and 4, respectively). Of note, in comparison to grr1Δ cells, the levels of hyperphosphorylated prodomain that accumulate were significantly higher in rts1Δ grr1Δ cells, indicating that Rts1 mediates an antagonistic dampening effect even under signaling conditions. These results demonstrate that Rts1 contributes to maintaining the hypophosphorylated state of the prodomain in the absence of signaling and that amino acid–induced signaling increases kinase access to the prodomain phosphodegron in a manner that overrides the constitutive Rts1-dependent phosphatase.

Ptr3 and Rts1 coordinate Ssy5 activation | 1481
Rts1 acts as a specificity factor that guides catalytic PP2A subunits Pph21 and Pph22 to their substrates (Zhao et al., 1997). We examined whether Rts1 can physically associate with Ssy5 by employing a directed two-hybrid assay. Only when introduced together did the bait DNA-binding domain (DBD)-RTS1 and the prey activation domain (AD)-SSY5 constructs facilitate robust growth of the host strain on media selective for expression of the two-hybrid interaction reporters, that is, \( P_{AGP1}\)-lacZ and \( P_{AB1} \) (Figure 1C). These observations suggest that Rts1 and Ssy5 interact, a finding consistent with the known role of Rts1 in defining the substrate specificity of PP2A.

Next we examined whether inactivation of the catalytic PP2A components would result in constitutive Stp1 processing; we monitored \( \beta \)-galactosidase (\( \beta \)-gal) expression from the Stp1-regulated \( G_{AGP1}\)-lacZ (Figure 1D, compare lanes 1–4). Next we tested whether the slowermigrating phosphorylated form of the prodomain was only observed in leucine-induced cells harboring Ptr3 (PTR3) and not in cells lacking Ptr3 (vector control [vc]; Figure 2B, compare lanes 1–4). Next we tested whether the previously described loss-of-function mutant of Ptr3 (ptr3-T525A), which harbors a threonine-to-alanine substitution at position 525 in the C-terminal WD40-like domain (Figure 2A; Liu et al., 2008), affected promodain phosphorylation. As in cells lacking Ptr3, we could not detect phosphorylated prodomain species in cells expressing ptr3-T525A (Figure 2B, lane 6). These results indicate that Ptr3 facilitates promodain phosphorylation in response to leucine induction and that the TS25A mutation interferes with this function.

The ability to homo-oligomerize is essential for Ptr3 function

Next we examined whether the loss of function resulting from the TS25A mutation could be linked to the inability of mutant protein to associate with knownPtr3 interaction partners, that is, the N-terminal cytoplasmic domain of Ssy1, Ssy5, and itself (Bernard and André, 2001) (Figure 2C, dilution 4). Consistent with previous findings (Bernard and André, 2001), the Ptr3 bait construct interacted with both Ssy5 and Ptr3; robust growth was observed on media selective for expression of the two-hybrid interaction reporters (Figure 2C, dilutions 4 and 6). Of importance, Ssy1\(_{NT}\) interacted with the ptr3-T525A mutant protein as well as the wild-type Ptr3 (Figure 2C, dilution 8). Also consistent with previous findings (Bernard and André, 2001), thePtr3 bait construct interacted with Ssy5 and wild-typePtr3 (Figure 2C, dilutions 12 and 14). Strikingly, thePtr3 bait construct did not interact with the mutant ptr3-T525A (Figure 2C, dilution 16), indicating that the TS25A mutation abolishes the ability of Ptr3 to interact with itself. This and the fact that

**FIGURE 1:** Ssy5 prodomain phosphorylation is modulated by Rts1-P2PA. (A) Immunoblot analysis of extracts from BY4741 (WT) and BY4741 (rts1Δ) carrying plasmids pCJ353 (SSY5-42-HA), pAB1 (to enable prototrophic growth), and pPbA1 (to enable prototrophic growth). (B) Immunoblot analysis of extracts from BY4741 (grr1Δ) and BY4741 (grr1Δ rts1Δ) carrying plasmids as in A. Immunoreactive forms of phosphorylated and nonphosphorylated Ssy5 prodomain species are indicated at their corresponding positions of migration. (C) Directed two-hybrid analysis of Ssy5 interactions. Plasmid pDO185 (DBD-RTS1) or pGBKT7 (DBD, AD), together with pACTII (AD, AD), or pACTII-SSy5 (AD-SSy5), was introduced into AH109. Growth of transformants was assayed on nonselective and selective media. (D) BY4741 (WT), BY4741 (rts1Δ), and BY4741 (reg1Δ) carrying pAGP1-lacZ and pAB1, as well as BY4742 (WT) and DC152 (pph21, pph22) carrying pAGP1-lacZ, pAB1, and pRS317, were grown in SD medium with or without leucine (leu). The levels of \( \beta \)-gal in permeabilized cells were assessed by X-Gal staining.
the T525A mutation abolishes Ssy5 prodomain phosphorylation suggested that the ability of Ptr3 to homo-oligomerize is essential for signal transduction.

**Ptr3 and Ssy5 form a constitutive complex**

To assess the role of Ptr3 in facilitating signal-induced prodomain phosphorylation, we first used a split ubiquitin assay to evaluate interactions between Ptr3 and Ssy5. This assay is specifically designed to detect in vivo interactions between proteins without altering their intracellular location, and importantly, allows the dynamic nature of signal-induced interactions to be assessed (see Materials and Methods). We fused the N-terminal fragment of ubiquitin (N_{Ub}) harboring the I13A mutation to Ptr3 (N_{Ub}-PTR3) and to the oligomerization deficient ptr3-T525A (N_{Ub-ptr3-T525A}) and individually coexpressed these constructs with full-length SSY5, SSY5 prodomain (SSY5_{pro}), or catalytic domain (SSY5_{cat}) fused to the C-terminal fragment of ubiquitin linked to a glutathione S-transferase (GST)-hemagglutinin (HA) reporter module (C_{Ub}-GST-HA; Figure 3A). The cleaved reporter (GST-HA), indicative of a physical interaction, was detected when N_{Ub} (vc) was coexpressed with SSY5-C_{Ub}-GST-HA (Figure 3A, lane 3). The absence of the cleaved reporter in cells expressing either the prodomain (ssy5_{pro}) or catalytic domain (ssy5_{cat}), lanes 5–8 suggests that only the correctly folded Ssy5 holoenzyme is capable of interacting with Ptr3. The confirmation that the mutant protein encoded by the ptr3-T525A allele, which cannot homo-oligomerize, retains the ability to interact with Ssy5 indicates that oligomerization of Ptr3 is not necessary for the interaction with Ssy5. Taken together, these results suggest that Ptr3 and Ssy5 constitutively interact and do so even in the absence of the upstream component Ssy1.

The constitutive nature of the Ptr3 and Ssy5 interaction suggested that signal-transducing events within the SPS sensor do not regulate this interaction. To test this, we fused Ptr3 to the C-terminus of Ssy5 and thereby forced their spatial proximity (Figure 3B). The chimeric construct was introduced into double-mutant ptr3Δ ssy5Δ and triple-mutant ssy1A ptr3Δ ssy5Δ strains, and Stp1 processing was monitored by immunoblotting (Figure 3B, left) and growth on yeast extract/petone/dextrose (YPD) in the presence of 2-[[4-(methoxy-6-methyl)-1,3,5-triazin-2-yl]-amino]carbonyl][amino][sulfonyl]-benzoic acid (MM; right). MM is an inhibitor of branched-chain amino acid synthesis, and, consequently, the ability to grow in YPD media containing MM requires Stp1/2-induced expression of high-affinity permeases for leucine, isoleucine, and valine (Jørgensen et al., 1998). Growth on YPD plus MM provides a highly sensitive readout of Ssy5 activity; low amounts of Stp1/2 processing result in readily detectable growth (Pfirrmann et al., 2010). The Ssy5–Ptr3 chimera is fully functional and subject to proper regulation in the context of SPS-sensor signaling; Ssy5–Ptr3 supported amino acid–induced Stp1 processing (Figure 3B, left, compare lanes 1 and 2) and robust growth on YPD plus MM (Figure 3B, dilution 1). The activity of the Ssy5–Ptr3 chimera was dependent on Ssy1; in ssy1A ptr3Δ ssy5Δ cells, leucine induction did not result in Stp1 processing (Figure 3B, left, lane 4) or growth on YPD plus MM (Figure 3B, right, dilution 2). Taken together, these results show that the mere placement of Ptr3 in the vicinity of Ssy5 is not sufficient for its activation. The fact that fusion of Ptr3 to Ssy5 is compatible with regulated signaling is consistent with the possibility that Ptr3 and Ssy5 interact constitutively.

The **Ptr3–Ssy5 subcomplex undergoes a conformational change that triggers Ssy5 activation**

The finding that Ptr3 and Ssy5 constitutively associate raised the possibility that Ptr3 facilitates Ssy5 phosphorylation by mediating amino acid–induced interactions with Yck1/2. Consistent with this possibility, Ptr3 is itself phosphorylated by Yck1/2 upon amino acid induction in a strictly Ssy1-dependent manner, and Ptr3 phosphorylation...
The results indicate that the Ssy1-independent activity of Ssy5-E131A requires Ptr3 for its activation. The results indicate that the Ssy1-independent activity of Ssy5-E131A exhibits a strict requirement for the presence of Ptr3; ssy1Δ ptr3Δ ssy5Δ cells harboring SSY5-E131A only grew on YPD plus MM when Ptr3 was coexpressed (Figure 3C, dilution 4). Taken together, these findings support a model in which amino acid binding to Ssy1 stabilizes a signaling conformation of Ptr3 that provides a binding surface for Yck1/2. Thus Ptr3 has a critical role in recruiting the kinase into spatial proximity of Ssy5. In addition, Ptr3 and Ssy5 conformations and conformational changes induced upon signaling appear to be tightly coupled, consistent with the finding that these proteins form a constitutive subcomplex within the SPS sensor.

**Spatial proximity of Yck1 to Ptr3 constitutively activates Ssy5**

To address whether amino acid–induced signaling triggers a physical interaction between Yck1/2 and Ptr3, we asked whether the forced spatial proximity of one of these functionally redundant kinases (Robinson et al., 1992; Wang et al., 1992) to Ptr3 would suffice to activate Ssy5. We constructed YCK1-PTR3, which encodes a fusion protein with the soluble catalytic domain of Yck1 fused to the N-terminus of Ptr3 (Figure 4A). This fusion construct was introduced into ptr3Δ and ssy1Δ ptr3Δ strains and Stp1 processing activity was assayed. Strikingly, the expression of YCK1-PTR3 led to constitutive, Ssy1-independent processing (Figure 4, A, center, lanes 1, 5, and 6, and C, lanes 3, 5, and 6) and supported growth on YPD plus MM (Figure 4B, right, dilutions 1, 3, 5, and 6). Of importance, the constitutive activity of Ssy5 in cells harboring Yck1-Ptr3 was fully dependent on the kinase activity of the chimeric protein; the introduction of the yck1-PTR3 allele carrying the kinase-inactivating K98R mutation (Wang et al., 1992) prevented constitutive Stp1 processing (Figure 4A, center, lanes 3, 7, and 8) and did not support growth on YPD plus MM (Figure 4B, right, dilution 4). Of note, the yck1-PTR3 allele expresses a functional Ptr3; it fully complemented the amino acid–induced signaling defects when introduced into a ptr3Δ strain (Figure 4A, center, lane 4; right, dilution 2).

We observed that the amount of processed Stp1 triggered by expression of YCK1-PTR3 in cells harboring a functional SPS sensor increased upon induction of signaling (Figure 4, A, center, compare lane 1 with lane 2, and C, compare lane 3 with lane 4). The enhanced Stp1 processing indicates that even in the context of the fusion to an active kinase, Ptr3 retains its ability to transmit amino acid–induced signals toward Ssy5, likely due to its ability to interact with endogenous Yck1/2. Consistently, the constitutive activity of Ssy5 resulting from expression of YCK1-PTR3 was the consequence of phosphorylation-induced and ubiquitylation-dependent degradation of its prodomain. In the absence of Grr1, the Yck1–Ptr3 chimera did not support growth on YPD plus MM (Figure 4B, dilution 7). Taken together, our results demonstrate that the placement of ocurs even in the absence of Ssy5 (Liu et al., 2008). In addition, a selection for mutants in SSY5 that bypass the requirement of Ssy1 identified the SSY5-E131A allele (Pfirrmann et al., 2010), which, in contrast to wild-type SSY5, triggers constitutive Stp1 processing and supports growth of a ssy1Δ strain on YPD plus MM (Figure 3C, compare dilutions 2 and 3). Significantly, although independent of Ssy1, the constitutive activity of the mutant SSY5-E131A protein retains the requirement for Yck1/2 and SCF{sup *Grr1} (Omnus et al., 2011). Thus Ssy5-E131A follows the phosphorylation- and ubiquitylation-dependent activation mechanisms of wild-type Ssy5. Because Ssy5-E131A does not require Ssy1 for its phosphorylation-dependent activation, it is unlikely that Ssy1 directly mediates the physical interaction of Yck1/2 and Ssy5.

To test whether Ptr3 mediates interactions between Yck1/2 and Ssy5, we examined whether Ssy5-E131A requires Ptr3 for its activation. The results indicate that the Ssy1-independent activity of Ssy5-E131A exhibits a strict requirement for the presence of Ptr3;
PTR3 and Rts1 coordinate Ssy5 activation

Volume 24 May 1, 2013

Ptr3 and Rts1 coordinate Ssy5 activation | 1485

Yck1 in proximity to Ptr3 suffices to trigger Ssy5 activation, consistent with SPS-sensor signaling regulating the spatial proximity of Yck1/2 to the Ptr3–Ssy5 subcomplex.

The N-terminal domain of Ptr3 mediates interactions with Ssy5

The observation that fusion of Yck1 to Ptr3 constitutively activates Ssy5 independent of Ssy1 allowed us to identify and assign specific functions to elements in Ptr3 required for Ssy5 activation. The T525A loss-of-function mutation in the WD40-like domain of Ptr3 abrogates amino acid–induced Ssy5 phosphorylation; however, the mutant protein retains the ability to interact with Ssy5 (Figure 2, B and C; Liu et al., 2008). The latter finding raised the possibility that elements other than the WD40-like domain mediate Ptr3 and Ssy5 interactions, which we proceeded to test. First, we introduced the T525A mutation into the YCK1–PTR3 allele and examined whether the modified fusion protein would retain the ability to direct Yck1 to Ssy5 and promote constitutive signaling. Expression of YCK1–PTR3-T525A resulted in constitutive Stp1 processing independent of amino acid induction and Ssy1 (Figure 4C, right, lanes 7–10). Stp1 processing depended on an active kinase; in the context of chimera with an inactive kinase (yck1–ptr3), Stp1 was not processed

(Figure 4C, right, lanes 9 and 10). Next we examined whether the entire WD40-like domain is dispensable for mediating Ptr3 and Ssy5 interactions; we constructed a Yck1 chimera containing only the first 210 amino acids of Ptr3 (YCK1–PTR3ΔCT). Strikingly, expression of this chimera with the heavily truncated Ptr3 protein resulted in constitutive Stp1 processing independent of Ssy1 at levels indistinguishable from that of the YCK1–PTR3-T525A chimera (Figure 4D, center, lanes 5 and 6 and lanes 1 and 2, respectively) and robust growth on YPD plus MM (Figure 4D, right, dilutions 7 and 8 and dilutions 4 and 5, respectively). These data are consistent with the possibility that the first 210 amino acids of Ptr3 mediate interactions with Ssy5.

Ptr3 possesses a conserved LFA motif required for function

To identify sequence elements within the N-terminal domain of Ptr3 that mediate interactions with Ssy5, we compared the amino acid sequences of a set of fungal Ptr3 orthologues (Figure 5A, top). Our analysis identified four regions in the first 210 amino acids of Ptr3 containing stretches of conserved amino acid residues. The biological relevance of these sequence motifs was tested by creating a series of N-terminal deletion constructs lacking the first 35, 70, 150, and 210 amino acids, which successively remove the four regions of sequence similarity (Figure 5A). The four N-terminal truncation mutants were evaluated for their effects on Ssy5 activation, as assessed by Stp1 processing and growth on YPD plus MM. Similar to full-length Ptr3, the Δ35, Δ70, and Δ150 truncations resulted in properly regulated Stp1 processing (Figure 5B, compare lanes 1 and 2 and lanes 3 and 4, 5 and 6, and 7 and 8, respectively) and robust growth on YPD plus MM (Figure 5D, compare dilutions 1 and 2–4, respectively). In contrast, the Δ210 truncation resulted in a nonfunctional protein that did not support Stp1 processing and growth (Figure 5, B, lanes 9 and 10, and D, dilution 5). Apparently, the fourth region of conservation, between residues 150 and 210, is essential for Ptr3 function.

A closer examination of this region revealed a relatively small but highly conserved sequence motif composed of hydrophobic amino acids between amino acids 160 and 180 (Figure 5A, middle). On the basis of the amino acid composition, we designated this motif, we deleted 9 amino acids (aa 167–175), including the LFA helix (Figure 5A, bottom). To test the biological significance of this motif, we deleted 9 amino acids (aa 167–175), including the LFA motif (Figure 5A, bottom). To test the biological significance of this motif, we deleted 9 amino acids (aa 167–175), including the LFA motif. (Figure 5A). The four N-terminal truncation mutants were evaluated for their effects on Ssy5 activation, as assessed by Stp1 processing and growth on YPD plus MM. Similar to full-length Pan3, the Δ35, Δ70, and Δ150 truncations resulted in properly regulated Stp1 processing (Figure 5B, compare lanes 1 and 2 and lanes 3 and 4, 5 and 6, and 7 and 8, respectively) and robust growth on YPD plus MM (Figure 5D, compare dilutions 1 and 2–4, respectively). In contrast, the Δ210 truncation resulted in a nonfunctional protein that did not support Stp1 processing and growth (Figure 5, B, lanes 9 and 10, and D, dilution 5). Apparently, the fourth region of conservation, between residues 150 and 210, is essential for Pan3 function.

A closer examination of this region revealed a relatively small but highly conserved sequence motif composed of hydrophobic amino acids between amino acids 160 and 180 (Figure 5A, middle). On the basis of the amino acid composition, we designated this sequence the LFA motif, which is predicted to fold into an amphipathic α-helix (Figure 5A, bottom). To test the biological significance of this motif, we deleted 9 amino acids (aa 167–175), including the L, F, and A residues (ΔLFA; Figure 5A, middle, red bar) in the context of a myc-tagged Pan3. The ΔLFA mutant proteins were expressed at levels similar to those of wild-type Pan3 (Figure 5C, compare lanes 3 and 4 and lanes 1 and 2), but the mutant protein failed to support signaling; no Stp1 processing (Figure 5C, lane 4) and no growth on YPD plus MM (Figure 5D, dilution 8) was observed. These results indicate that the LFA motif of Pan3 is important for Ssy5 activation.

FIGURE 4: A Yck1–Ptr3 fusion protein constitutively induces the Stp1-processing activity of Ssy5. Schematic representation of the Yck1–Ptr3 fusion proteins carrying an internal HA tag (HA). Red arrows indicate the positions of the kinase-inactivating K98R mutation (yck1Δ) and the T525A mutation within the WD40-like domain of Ptr3. (A) Immunoblot analysis of extracts from HKY31 (ptr3Δ; HindIII) and HKY33 (ptr3Δ; ssy1Δ) carrying plasmids pCA204 (STP1-MYC) and pDO84 (YCK1–PTR3) or pDO86 (yck1–ptr3). (B) Growth of strains as in (A) and of HKY31 (ptr3Δ), HKY33 (ptr3Δ; ssy1Δ), and CAY272 (ptr3Δ; grr1Δ) carrying plasmids pCA204 (STP1-MYC) and pDO84 (YCK1–PTR3) (right) on YPD and YPD plus MM media. (C) Immunoblot analysis of extracts from HKY31 (ptr3Δ; lanes 1–4, 7 and 8, and 11 and 12) and HKY33 (ptr3Δ; ssy1Δ; lanes 5 and 6, and 9 and 10) carrying plasmids pCA204 (STP1-MYC) and pRS316 (vc), pDO84 (YCK1–PTR3), pDO95 (YCK1–PTR3–T525A), or pDO98 (yck1–ptr3). (D) Immunoblot analysis of extracts from HKY31 (ptr3Δ; lanes 1 and 2 and lanes 5 and 6) and HKY33 (ptr3Δ; ssy1Δ; lanes 3 and 4 and lane 7) carrying plasmids pCA204 (STP1-MYC) and pDO95 (YCK1–PTR3–T525A), pDO98 (yck1–ptr3), or pDO162 (YCK1–PTR3ΔCT). Immunoreactive forms of the fusion proteins and of Stp1 (full length and processed) are indicated. (E) Growth of strains as in (D) and of strain HKY31 (ptr3Δ) carrying plasmids pCA204 (STP1-MYC) and pDO86 (yck1–PTR3) on YPD and YPD plus MM media.
The LFA domain of Ptr3 suffices to mediate the interaction with Ssy5

To test whether the LFA domain, defined as amino acids 151–210, enables Ptr3 to physically associate with Ssy5, we used a split ubiquitin approach. We fused NUB to Ptr3 (NUB-PTR3), the first 210 amino acids of Ptr3 (NUB-ptr3ΔCT), and Ptr3 lacking the LFA motif (NUB-ptr3-LFA). These constructs were individually coexpressed with SSYS-CΔ5-GST-HA, and interactions were scored by the presence of the cleaved reporter (GST-HA; Figure 6A). The NUB constructs containing either full-length Ptr3 or the C-terminal deletion, both with an intact LFA domain, interacted with Ssy5 in a constitutive manner; the cleaved GST-HA reporter was detected independent of amino acid induction (Figure 6A, lanes 3 and 4 and lanes 7 and 8, respectively). Identical to NUB alone (vc), the NUB construct lacking the LFA motif failed to interact with Ssy5, and no cleaved reporter was detected (Figure 6A, lanes 1 and 2 and lanes 5 and 6, respectively). The data clearly demonstrate that an intact LFA domain is required to facilitate interactions between Ptr3 and Ssy5.

We posited that the loss of function resulting from the deletion of the LFA motif (Figure 5, C and D) could be suppressed in the context of the Ssy5–Ptr3 fusion protein (Figure 3B). We constructed and expressed an SSYS-PTR3ΔLFA chimera lacking the LFA motif in ptr3Δ ssy5Δ cells and found that it supported Stp1 processing and growth on YPD plus MM, as well as SSYS-PTR3 (Figure 6B, compare lanes 1–4 and dilutions 3 and 1). In contrast, the loss of function resulting from the T525A mutation within the WD40-like domain, which perturbs the ability of Ptr3 to engage in self-interactions (Figure 2C), was not suppressed by the forced Ssy5–Ptr3 interaction; the chimeric protein carrying the T525A mutation encoded by the Ssy5-ptr3 allele did not facilitate Stp1 processing and thus failed to support growth on YPD+MM (Figure 6B, lanes 5 and 6 and dilution 2). These results indicate that the LFA domain is important for mediating interactions with Ssy5, and that Ptr3 homooligomerization, via its WD40-like domain, is critically required for Ssy5 activation.

Finally, to unambiguously test the role of the LFA domain of Ptr3 as the binding site for Ssy5, we assessed whether the LFA domain fused to Yck1 would suffice to direct the kinase activity toward the Ssy5 prodomain. Strikingly, amino acids 151–210 harboring the complete LFA domain fused to Yck1 (YCK1-PTR3(151-210)) triggered constitutive Ssy5 activation, leading to efficient Stp1 processing (Figure 7C, lanes 6–8) and robust growth on YPD plus MM (Figure 7B, dilutions 4 and 5). In contrast, deletion of the 9 core amino acids of the LFA motif (aa 167–175), in the context of otherwise full-length Ptr3 (YCK1-ptr3ΔLFA) or in the isolated LFA domain (YCK1-PTR3(151-210ΔLFA)), did not support growth on YPD plus MM (Figure 7B, dilutions 3 and 7, respectively). The lack of growth reflected the lack of Stp1 processing (Figure 7, C, lanes 4 and 5, and D, lanes 3 and 4, respectively). These results confirm that the LFA domain of Ptr3 possesses the necessary structural information to support interactions with Ssy5.

DISCUSSION

We defined the signaling events within the SPS sensor that couple the amino acid–stabilized signaling conformation of the Ssy1 receptor to the phosphorylation-dependent degradation of the Ssy5 prodomain. Prodomain degradation is the first irreversible and thus committing step in the cellular response to extracellular amino acids. Specifically our results illuminate the mechanisms governing the phosphorylation status of the Ssy5 prodomain. We show that phosphorylation is tuned through interactions of Ssy5 with the general and constitutively active Rts1-PP2A and the pathway specific factor Ptr3. Rts1 associates with Ssy5 and directs PP2A to maintain the stable hypophosphorylated inhibitory state of the prodomain in the absence of bona fide amino acid induction. Hence the constitutive
Vol. 24, May 1, 2013

Ptr3 and Rts1 coordinate Ssy5 activation

The negative control exerted by Rts1-PP2A on prodomain phosphorylation is important for two reasons. First, the subsequent SPS signaling events involve proteolytic events that are not reversible, that is, prodomain degradation and the endoproteolytic activation of Stp1/Stp2. Second, SPS-sensor signaling is amplified since the endoproteolytic activity of the Ssy5 is catalytic (Andréasson et al., 2006). Consequently, Ssy1 signaling is sensitive to both external and internal levels of amino acids; the SPS sensor only induces gene expression when the levels of external amino acids are higher than the levels of free amino acids in cytoplasmic pools, that is, conditions that stabilize the outward-facing conformation. Of note, in its amino acid–free state, the Ssy1 receptor is able to switch between its two conformations. Hence, even under conditions in which no stimulus is provided, a population of Ssy1 will transiently adopt the signaling conformation and can inappropriately initiate downstream signaling events. Consistent with this notion, in the absence of amino acid induction, cells lacking a functional PP2A exhibit constitutively induced SPS-sensor-regulated gene expression (Figure 1, C and D), the consequence of reduced steady-state levels of prodomain (Figure 1A). Accordingly, phosphorylated species of prodomain accumulate in rats1Δ cells, when prodomain degradation is blocked (Figure 1B). Thus constitutive Rts1-mediated dephosphorylation antagonizes prodomain phosphorylation and is essential to set the threshold for SPS-sensor activation required to prevent spurious signaling when the Ssy1 receptor transiently adopts the signaling conformation.

The Ssy1 receptor is a unique member of the amino acid permease family of proteins that does not catalyze amino acid uptake (Didion et al., 1998; Iraqui et al., 1999; Klasson et al., 1999). However, Ssy1 undergoes transporter-like conformational changes between an outward-facing (signaling) and an inward-facing (nonsignaling) conformation (Wu et al., 2006). In contrast to functional transporters, amino acid binding to a single substrate-binding site imposes a reaction barrier that inhibits the conversion between these conformations (Wu et al., 2006). Consequently, Ssy1 signaling is sensitive to both external and internal levels of amino acids; the SPS sensor only induces gene expression when the levels of external amino acids are higher than the levels of free amino acids in cytoplasmic pools, that is, conditions that stabilize the outward-facing conformation. Of note, in its amino acid--free state, the Ssy1 receptor is able to switch between its two conformations. Hence, even under conditions in which no stimulus is provided, a population of Ssy1 will transiently adopt the signaling conformation and can inappropriately initiate downstream signaling events. Consistent with this notion, in the absence of amino acid induction, cells lacking a functional PP2A exhibit constitutively induced SPS-sensor-regulated gene expression (Figure 1, C and D), the consequence of reduced steady-state levels of prodomain (Figure 1A). Accordingly, phosphorylated species of prodomain accumulate in rats1Δ cells, when prodomain degradation is blocked (Figure 1B). Thus constitutive Rts1-mediated dephosphorylation antagonizes prodomain phosphorylation and is essential to set the threshold for SPS-sensor activation required to prevent spurious signaling when the Ssy1 receptor transiently adopts the signaling conformation.
Our study demonstrates how a dedicated adapter protein facilitates the regulated activation of an intracellular signaling protease in response to conformational states of a plasma membrane–localized receptor. It is intriguing that the mechanisms governing SPS-sensor signaling exhibit striking similarity to those controlling NFκB activation. NFκB transcription factors are retained in the cytoplasm by association with inhibitory IkB proteins. Stimulus-dependent IkB phosphorylation leads to its ubiquitylation and subsequent proteasomal degradation, enabling translocation of NFκB to the nucleus (Karin, 1999). Like Ptr3 in the activation of Ssy5, NEMO functions as an adapter that facilitates IkB phosphorylation by recruiting the catalytic IkB kinase components of IKKα and IKKβ (Schrofelbauer et al., 2012). It has been reported that induced oligomerization of NEMO, IKKα, or IKKβ leads to activation of NFκB (Inohara et al., 2000; Poyet et al., 2000). Oligomerization of NEMO may induce oligomerization of the IKKα/IκB kinase and, consequently, induction of its activity (Poyet et al., 2000). Similarly, as described here, we found that Ptr3 multimerizes via its WD40-like domain and that homo-oligomerization is essential for its role in Ssy5 activation (Figure 2C); the functional ptr3-T525A carrying a mutation affecting the WD40-like domain does not oligomerize but retains the ability to interact with the N-terminal domain of Ssy1 (Figure 2C) and Ssy5 (Figure 3A). Presumably, oligomerization of Ptr3 is required for the amino acid–induced juxtaposition of Yck1/2 and Ssy5 prodomain. The concept of the requirement of protein multimerization to facilitate signal transduction has been well established, and examples include signaling induced by G protein–coupled receptors (Lee et al., 2003; Maggio et al., 2007) and receptor tyrosine kinases (Ullrich and Schlessinger, 1990; Elion, 2003; Lamson et al., 2010). Furthermore, the significance and implications of the observed ability of the scaffold protein Ste5 to multimerize is under discussion in studies focused on mitogen-activated protein kinase signaling (Yablonski et al., 1996; Wang and Elion, 2003; Lamson et al., 2006).

Several findings indicate that Ptr3 functions as an adapter protein that couples conformational information reflecting signal perception by Ssy1 to the degradation of the Ssy5 prodomain, a requisite for Ste1/Ste2 processing. We confirmed that Ptr3 interacts with the N-terminal cytoplasmic domain of Ssy1 (Figure 2C), includes the N-terminal cytoplasmic domain of Ssy1 (Figure 2C) and Ssy5 (Figure 3A). Presumably, oligomerization of NEMO, IKKα, or IKKβ leads to activation of NFκB (Inohara et al., 2000; Poyet et al., 2000). Oligomerization of NEMO may induce oligomerization of the IKKα/IκB kinase and, consequently, induction of its activity (Poyet et al., 2000). Similarly, as described here, we found that Ptr3 multimerizes via its WD40-like domain and that homo-oligomerization is essential for its role in Ssy5 activation (Figure 2C); the functional ptr3-T525A carrying a mutation affecting the WD40-like domain does not oligomerize but retains the ability to interact with the N-terminal domain of Ssy1 (Figure 2C) and Ssy5 (Figure 3A). Presumably, oligomerization of Ptr3 is required for the amino acid–induced juxtaposition of Yck1/2 and Ssy5 prodomain. The concept of the requirement of protein multimerization to facilitate signal transduction has been well established, and examples include signaling induced by G protein–coupled receptors (Lee et al., 2003; Maggio et al., 2007) and receptor tyrosine kinases (Ullrich and Schlessinger, 1990; Elion, 2003; Lamson et al., 2010). Furthermore, the significance and implications of the observed ability of the scaffold protein Ste5 to multimerize is under discussion in studies focused on mitogen-activated protein kinase signaling (Yablonski et al., 1996; Wang and Elion, 2003; Lamson et al., 2006).

MATERIALS AND METHODS

Yeast strains and plasmids

The S. cerevisiae strains and plasmids used in this work are listed in Tables 1 and 2, respectively. The yeast strains are isogenic descendents of the S288C-derived strain AA255/PLY115 (Antebi and Fink, 1992) or by BY4741 and BY4742, with the exception of the two-hybrid strain AH109. Strain AH109 is a derivative of strain PJ69-2A and includes the ADE2 and His3 markers (James et al., 1996). The sequences of mutagenic oligonucleotides and PCR primers for homologous recombination are available upon request.

Media

Standard media, including YPD medium, ammonia-based synthetic minimal dextrose (SD) medium, supplemented as required to

FIGURE 7: Fusion of Yck1 to the LFA domain constitutively activates Ssy5. (A) Schematic representation of the Yck1–Ptr3 fusion proteins with wild-type and mutant forms of Ptr3 fused to the C-terminus of Yck1. (B) Growth of HKY31 (pt3Δ; dilutions 1, 3 and 4, and 7), HKY33 (ptr3Δ ssy1Δ; dilutions 2, 5, and 8) and CAY285 (ssy1Δ ptr3Δssy5Δ; dilution 6) carrying plasmids pCA204 (STP1-MYC) and pDO84 (YCK1-Ptr3), pDO145 (YCK1-ptr3ΔLFA), pDO176 (YCK1-Ptr3ΔLFA), or pDO177 (YCK1-ptr3ΔLFAE131A) on YPD and YPD plus MM media. (C) Immunoblot analysis of extracts from HKY31 (ptr3Δ; lanes 1 and 2 and lanes 4–7) and HKY33 (ptr3Δ ssy1Δ; lanes 3 and 8) carrying plasmids pCA204 (STP1-MYC) and pDO84 (YCK1-Ptr3), pDO145 (YCK1-ptr3ΔLFA), or pDO176 (YCK1-Ptr3ΔLFA). (D) Immunoblot analysis of extracts from HKY31 (ptr3Δ; lanes 1 and 3) and HKY33 (ptr3Δ ssy1Δ; lanes 2 and 4) carrying plasmids pCA204 (STP1-MYC) and pDO176 (YCK1-Ptr3ΔLFA) or pDO177 (YCK1-ptr3ΔLFAE131A). Immunoreactive forms of Yck1–Ptr3 chimeras and of Stp1 (full length and processed) are indicated at their corresponding positions of migration.
enable growth of auxotrophic strains, and ammonia-based synthetic complete dextrose (SC) were prepared as described (Andréasson and Ljungdahl, 2002). Sensitivity to MM (100 μg/ml) was monitored complete dextrose (SC) were prepared as described (Andréasson and Ljungdahl, 2002). Sensitivity to MM (100 μg/ml) was monitored complete dextrose (SC) were prepared as described (Andréasson and Ljungdahl, 2002). Sensitivity to MM (100 μg/ml) was monitored complete dextrose (SC) were prepared as described (Andréasson and Ljungdahl, 2002). Sensitivity to MM (100 μg/ml) was monitored complete dextrose (SC) were prepared as described (Andréasson and Ljungdahl, 2002). Sensitivity to MM (100 μg/ml) was monitored

Directed yeast two-hybrid assay

Two-hybrid interactions between Gal4 DBD and Gal4 AD fusion proteins were tested in strain AH109. Growth of transformants on SC media lacking tryptophan and leucine (nonselective) and SC media lacking tryptophan, leucine, histidine, and adenine (selective) were incubated at 30°C for 2–3 d and photographed.

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

| Strain   | Genotype                                      | Reference/source                      |
|----------|-----------------------------------------------|---------------------------------------|
| AH109    | MATa trp1-901 leu2-3, 112 ura3-52 his3-200,   | James et al. (1996), A. Holtz           |
|          | gal4Δ gal80Δ LYS2::GAL1, UAS::GAL1, TATA::        | (unpublished data)                     |
|          | HIS3 GAL2::ADE2 URA3::MEL1, TATA::lacZ          | EUROSCARF                             |
| BY4741   | MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0             | EUROSCARF                             |
| BY4741 rts1Δ | MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 rts1::kanMX4 | ResGen/Invitrogen (Carlsbad, CA)      |
| BY4741 reg1Δ | MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 reg1::kanMX | ResGen/Invitrogen                     |
| BY4742   | MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0             | ResGen/Invitrogen                     |
| CAY272   | MATα lys2Δ201 ura3-52 ptr3Δ15::hisG grr1Δ50::hphMX4 | Ljungdahl laboratory                   |
| CAY285   | MATα lys2Δ201 ura3-52 ptr3Δ15::hisG ssy1Δ13::hisG ssy5Δ2::hisG | Pfirrmann et al. (2010)               |
| CAY307   | MATα lys2Δ201 ura3-52 ptr3Δ15::hisG ssy5Δ2::hisG grr1Δ50::hphMX4 | Ljungdahl laboratory                   |
| DC152 (BY4742 phh21Δ phh22Δ) | MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 phh21Δ::KanMX phh22Δ::kanMX | Castermans et al. (2012)             |
| DOY03 (BY4741 grr1Δ) | MATα his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 grr1::natMX | This study                            |
| DOY04 (BY4741 rts1Δ grr1Δ) | MATα his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 rts1::kanMX4 grr1::natMX | This study                            |
| HKY31    | MATα lys2Δ201 ura3-52 ptr3Δ15::hisG            | Klasson et al. (1999)                  |
| HKY33    | MATα lys2Δ201 ura3-52 ssy1Δ13::hisG ptr3Δ15::hisG | Klasson et al. (1999)                  |
| HKY85    | MATα lys2Δ201 ura3-52 ptr3Δ15::hisG ssy5Δ2::hisG | Forsberg and Ljungdahl (2001)         |
| Plasmid    | Description                                                                 | Reference/source                                      |
|-----------|-----------------------------------------------------------------------------|-------------------------------------------------------|
| pAB1      | pRS313 carrying HIS3, MET15, and LEU2                                         | This study                                            |
| pACTII    | Yeast two-hybrid plasmid (LEU2) carrying Gal4 activation domain              | Clontech (Mountain View, CA)                         |
| pACTII-Ssy5| pACTII (LEU2) carrying the SSY5 ORF                                          | Liu et al. (2008)                                    |
| pACTII-Ptr3| pACTII (LEU2) carrying the PTR3 ORF                                          | Liu et al. (2008)                                    |
| pAGP1-lacZ | YCpAGP1-lacZ (URA3)                                                          | Irai et al. (1999)                                   |
| pCA122    | pRS317 (LYS2) carrying STP1-3HA                                              | Andréasson and Ljungdahl (2004)                       |
| pCA204    | pRS317 (LYS2) carrying STP1-MYC-kanMX                                        | Andréasson et al. (2006)                             |
| pCJ353    | YCp-SSY5-42-HA6 (URA3)                                                       | Abdel-Sater et al. (2011)                            |
| pDO61     | pRS316 (URA3) containing Nub-PTR3                                            | This study                                            |
| pDO79     | pRS316 (URA3) containing HA-SSY5-PTR3                                        | This study                                            |
| pDO84     | pRS316 (URA3) containing YCK1<sub>2-527</sub>-HA<sub>i</sub>-PTR3           | This study                                            |
| pDO85     | pRS317 (LYS2) containing SSYS-Cub-GST-HA                                      | This study                                            |
| pDO86     | pRS316 (URA3) containing yck1<sub>2-527</sub>-K98R-HA<sub>i</sub>-PTR3      | This study                                            |
| pDO88     | pRS316 (URA3) containing Nub                                                | This study                                            |
| pDO94     | pRS316 (URA3) containing Nub<sub>i</sub>-ptr3-T525A                         | This study                                            |
| pDO95     | pRS316 (URA3) containing YCK1<sub>2-527</sub>-HA<sub>i</sub>-PTR3-T525A    | This study                                            |
| pDO96     | pRS316 (URA3) MYC<sub>i</sub>-ptr3-T525A                                    | This study                                            |
| pDO98     | pRS316 (URA3) containing yck1<sub>2-527</sub>-K98R-HA<sub>i</sub>-ptr3-T525A | This study                                            |
| pDO131    | pRS316 (URA3) containing PTR3Δ35                                              | This study                                            |
| pDO132    | pRS316 (URA3) containing PTR3Δ70                                              | This study                                            |
| pDO133    | pRS316 (URA3) containing PTR3Δ150                                             | This study                                            |
| pDO134    | pRS316 (URA3) containing ptr3Δ210                                             | This study                                            |
| pDO138    | pRS316 (URA3) MYC<sub>i</sub>-ptr3ΔLA                                       | This study                                            |
| pDO139    | pRS316 (URA3) containing HA<sub>i</sub>-SSY5-PTR3ΔLFA                      | This study                                            |
| pDO145    | pRS316 (URA3) containing YCK1<sub>2-527</sub>-HA<sub>i</sub>-ptr3ΔLFA       | This study                                            |
| pDO147    | pRS316 (URA3) containing Nub<sub>i</sub>-ptr3ΔLFA                          | This study                                            |
| pDO148    | pRS317 (LYS2) containing GST-HA-PTR3                                        | This study                                            |
| pDO149    | pRS317 (LYS2) containing GST-HA-PTR3-T525A                                  | This study                                            |
| pDO151    | pRS316 (URA3) containing HA-SSY5-ptr3-T525A                                 | This study                                            |
| pDO162    | pRS316 (URA3) containing YCK1<sub>2-527</sub>-HA<sub>i</sub>-PTR3<sub>1-210</sub>ΔCT | This study                                            |
| pDO174    | pRS316 (URA3) containing Nub-PTR3<sub>1-210</sub>ΔCT                       | This study                                            |
| pDO176    | pRS316 (URA3) containing YCK1<sub>2-527</sub>-HA<sub>i</sub>-PTR3<sub>151-210</sub>ΔCT | This study                                            |
| pDO181    | pRS317 (LYS2) containing ssy<i>Cat</i>-CUS<sub>i</sub>-GST-HA (Cat = aa 382-699) | This study                                            |
| pDO182    | pRS317 (LYS2) containing ssy<i>Pro</i>-CUS<sub>i</sub>-GST-HA Pro (Pro = aa 1–381) | This study                                            |
| pDO185    | pGBKKT7 (TRP1) carrying RTS1 ORF                                            | This study                                            |
| pDO186    | pGBKKT7 (TRP1) carrying PTR3 ORF                                            | This study                                            |
| pDO189    | pACTII (LEU2) carrying ptr3-T525A                                            | This study                                            |
| pGBKKT7   | Yeast two-hybrid plasmid (TRP1) carrying Gal4 DNA-binding domain            | Clontech                                              |
| pGBKKT7-SSY1N| pGBKKT7 (TRP1) carrying sequence encoding N-terminal amino acid residues 2–273 of Ssy1 | Liu et al. (2008)                                    |
| pHK017    | pRS316 (URA3) containing PTR3                                               | Klasson et al. (1999)                                |
| pHK019    | pRS316 (URA3) MYC<sub>i</sub>-PTR3 (3x myc, between aa 157 and 158)        | Ljungdahl laboratory                                 |
| pHK048    | pRS316 (URA3) containing MYC-SSY5                                           | Forsberg and Ljungdahl (2001)                         |
| pRS316    | pRS316 (URA3)                                                              | Sikorski and Hieter (1989)                           |
| pRS317    | pRS317 (LYS2)                                                              | Sikorski and Hieter (1989)                           |
| pSH120    | pRS316 (URA3) containing HA<sub>i</sub>-SSY5-GST                            | Pfirrmann et al. (2010)                              |
| pTP115    | pRS316 (URA3) containing HA<sub>i</sub>-SSY5-E131A-GST                     | Pfirrmann et al. (2010)                              |

**TABLE 2: Plasmids used in this study.**
indicating interactions was assessed. Tenfold dilutions of cultures were spotted on nonselective and selective media, and plates were photographed 2–4 d after incubation at 30°C.

Split ubiquitin protein–protein interaction assay

The split ubiquitin protein–protein interaction assay is based on two findings: ubiquitin can be divided into two halves that spontaneously reassemble when coexpressed (Johnsson and Varshavsky, 1994), and ubiquitin that is fused to proteins is rapidly cleaved off by ubiquitin-specific proteases (Bachmair et al., 1986). These properties can be experimentally exploited to detect protein–protein interactions by creating proteins fused to either half of ubiquitin; when the C-terminal fragment of ubiquitin (NUb) is expressed as a fusion with an immunologically detectable reporter protein (e.g., GST-HA), the reporter protein is cleaved away if the N-terminal fragment of ubiquitin (NUb) is presented in a context that brings it into close proximity of the CUb fragment. The stringency of the assay is greatly enhanced if a variant of NUb carrying an alanine instead of isoleucine at position 13 is used; the 13A mutant NUb exhibits significantly decreased spontaneous association with CUb (Johnsson and Varshavsky, 1994). Immunoblot analysis of extracts prepared from strains coexpressing NUb and CUb constructs was performed; the detection of the cleaved-off GST-HA reporter protein reflects an interaction between the two proteins of interest.

β-Galactosidase activity assay

The β-gal activity was determined with N-lauroyl-sarcosine–permeabilized cells (Kippert, 1995). Semiquantitative measurements of β-galactosidase activity used equally turbid cell suspensions (OD600 = 1) diluted 1:1 in 0.4 M potassium phosphate buffer (pH 7) containing 0.1% β-mercaptoethanol, 0.1 mg/ml 5-bromo-4-chloro-3-indolyl β-galactopyranoside (X-gal). Cell suspensions were incubated at 30°C.

ACKNOWLEDGMENTS

We thank Thorsten Pfirrmann and the members of the Andréasson, Ott, and Ljungdahl laboratories for constructive comments throughout the course of this work. We acknowledge Anna Schick and Hansa Forsberg for constructing plasmids and thank Bruno André (Université Libre de Bruxelles, Belgium) and Zhengchang Liu (University of New Orleans, New Orleans, LA) for plasmids and Claes Andréasson and Johan Thevelein (KU Leuven, Leuven, Belgium) for yeast strains. This research was supported by funding from the Swedish Research Council (P.O.L.).

REFERENCES

Abdel-Sater F, El Bakkoury M, Urrestarazu A, Vissers S, André B (2004). Amino acid signaling in yeast: casein kinase I and the Ssy5 endoproteinase are key determinants of endoproteolytic activation of the membrane-bound Stp1p transcription factor. Mol Cell Biol 24, 9771–9785.

Abdel-Sater F, Jean C, Merhi A, Vissers S, André B (2011). Amino-acid sensing in yeast: activation of the Ssy5 protease is associated with its phosphorylation-induced ubiquitylation. J Biol Chem 286, 12006–12015.

Andréasson C, Heessen S, Ljungdahl PO (2006). Regulation of transcription factor activity by receptor-activated proteolysis. Genes Dev 20, 1563–1568.

Andréasson C, Ljungdahl PO (2002). Receptor-mediated endoproteolytic activation of two transcription factors in yeast. Genes Dev 16, 3158–3172.

Andréasson C, Ljungdahl PO (2004). The N-terminal regulatory domain of Stp1p is modular and, fused to an artificial transcription factor, confers full Syg1p-Pub3p-Syg5p sensor control. Mol Cell Biol 24, 7503–7513.

Antebi A, Fink GR (1992). The yeast Ca2+-ATPase homologue, PMR1, is required for normal Golgi function and localizes in a novel Golgi-like distribution. Mol Biol Cell 3, 633–654.

Bachmair A, Finley D, Varshavsky A (1986). In vivo half-life of a protein is a function of its amino-terminal residue. Science 234, 179–186.

Barnes D, Lai W, Breslav M, Naider F, Becker JM (1998). PRR3, a novel gene mediating amino acid-inducible regulation of peptide transport in Saccharomyces cerevisiae. Mol Microbiol 29, 297–310.

Bernard F, André B (2001). Genetic analysis of the signalling pathway activated by external amino acids in Saccharomyces cerevisiae. Mol Microbiol 41, 489–502.

Cartier A, Parent A, Labrecque P, Laroche G, Parent JL (2011). WDR36 acts as a scaffold protein tethering a G-protein-coupled receptor, Galphaq and phospholipase Cbeta in a signalling complex. J Cell Sci 124, 3292–3304.

Casteurman D, Somers I, Kriel J, Louwert W, Wera S, Versele M, Janssens V, Thevelein JM (2012). Glucose-induced posttranslational activation of protein phosphatases PP2A and PP1 in yeast. Cell Res 22, 1085–1077.

Dition T, Regenberg B, Jørgensen MU, Knippschild U, Gocht A, Wolff S, Huber N, Lohler J, Stoter M (2005). The yeast Snf1 protein kinase Snf1p regulates fructose 1,6-bisphosphatase. J Biol Chem 280, 12015–12022.

Domingo-Sananes MR, Kapron O, Hunt T, Novak B (2011). Switches and latches: a biochemical tug-of-war between the kinases and phosphatases that control mitosis. Philos Trans R Soc Lond B Biol Sci 366, 3584–3594.

Eckert-Boulet N, Larsson K, Wu B, Poulsen P, Regenberg B, Nielsen J, Kielland-Brandt MC (2006). Deletion of RTS1, encoding a regulatory subunit of protein phosphatase 2A, results in constitutive amino acid signaling via increased Stp1p processing. Eukaryot Cell 5, 174–179.

Forsberg H, Ljungdahl PO (2001). Genetic and biochemical analysis of the yeast plasma membrane Syg1p-Pub3p-Syg5p sensor of extracellular amino acids. Mol Cell Biol 21, 814–826.

Garnier J, Girbat JF, Robson B (1996). GOR method for predicting protein secondary structure from amino acid sequence. Methods Enzymol 266, 540–553.

Good M, Tang G, Singleton J, Remyena A, Lim WA (2009). The Ste5 scaffold directs mating signaling by catalytically unlocking the Fus3 MAP kinase for activation. Cell 136, 1085–1097.

Gross SD, Anderson RA (1998). Casein kinase I: spatial organization and positioning of a multifunctional protein kinase family. Cell Signal 10, 699–711.

Harvey SL, Enciso G, Dehpoure N, Gygi SP, Gunawardena J, Kellogg DR (2011). A phosphatase threshold sets the level of Cdk1 activity in early mitosis in budding yeast. Mol Cell Biol 22, 3595–3608.

Inohara N, Kosugi H, Kiyokawa E, Ihara K, Ishidoh K, Nunez G (2000). An induced proximity model for NF-kappaB activation in the Nod1/RICK and RIP signaling pathways. J Biol Chem 275, 27823–27831.

Iraqui I, Vissers S, Bernard F, de Craene JO, Boles E, Urrestarazu A, André B (1999). Amino acid signaling in Saccharomyces cerevisiae: a permease-like sensor of external amino acids and F-Box protein Grr1p are required for transcriptional induction of the AGP1 gene, which encodes a broad-specificity amino acid permease. Mol Cell Biol 19, 989–1001.

James P, Halliday J, Craig EA (1996). Genomic libraries and a host strain designed for highly efficient two-hybrid selection in yeast. Genetics 144, 1425–1436.

Johnsson N, Varshavsky A (1994). Split ubiquitin as a sensor of protein interactions in vivo. Proc Natl Acad Sci USA 91, 10340–10344.

Jonkers W, Rep M (2009). Lessons from fungal F-box proteins. Eukaryotic Cell 8, 677–695.

Jørgensen MU, Bruun MB, Didion T, Kielland-Brandt MC (1998). Mutations in five loci affecting GAP1-independent uptake of neutral amino acids in Saccharomyces cerevisiae. Mol Microbiol 29, 297–310.

Karin M (1999). How NF-kappaB is activated: the role of the IkappaB kinase (IKK) complex. Oncogene 18, 6678–6874.

Kippert F (1995). A rapid permeabilization procedure for accurate quantitaive determination of beta-galactosidase activity in yeast cells. FEMS Microbiol Lett 128, 201–206.

Klasson H, Fink GR, Ljungdahl PO (1999). Syg1p and Pub3p are plasma membrane components of a yeast system that senses extracellular amino acids. Mol Cell Biol 19, 5405–5416.

Knipschild U, Gocht A, Wolff S, Huber N, Lohler J, Stoter M (2005). The casein kinase 1 family: participation in multiple cellular processes in eukaryotes. Cell Signal 17, 675–689.

Lahsen RE, Takahashi S, Winters MJ, Pryciak PM (2006). Dual role for membrane localization in yeast MAP kinase cascade activation and its contribution to signaling fidelity. Curr Biol 16, 618–623.
Lee SP, O’Dowd BF, George SR (2003). Homo- and hetero-oligomerization of G protein-coupled receptors. Life Sci 74, 173–180.

Lemmon MA, Schlessinger J (2010). Cell signaling by receptor tyrosine kinases. Cell 141, 1117–1134.

Liu Z, Thornton J, Spirek M, Butow RA (2008). Activation of the SPS amino acid-sensing pathway in Saccharomyces cerevisiae correlates with the phosphorylation state of a sensor component, Prr3. Mol Cell Biol 28, 51–63.

Ljungdahl PO, Daignan-Fornier B (2012). Regulation of amino acid, nucleotide, and phosphate metabolism in Saccharomyces cerevisiae. Genetics 190, 885–929.

Maggio R, Innamorati G, Parenti M (2007). G protein-coupled receptor oligomerization provides the framework for signal discrimination. J Neurochem 103, 1741–1752.

Malleshaiah MK, Shahrezaei V, Sutani T, Shirahige K, Lucchini G, Piatti S (2010). The RSC chromatin-remodeling complex influences mitotic exit and adaptation to the spindle assembly checkpoint by controlling the Cdc14 phosphatase. J Cell Biol 191, 981–997.

Marchal C, Dupre S, Urban-Grimal D (2002). Casein kinase I controls a late step in the endocytic trafficking of yeast uracil permease. J Cell Sci 115, 217–226.

Moriya H, Johnston M (2004). Glucose sensing and signaling in Saccharomyces cerevisiae through the Rgt2 glucose sensor and casein kinase I. Proc Natl Acad Sci USA 101, 1572–1577.

Neer EJ, Schmidt CJ, Nambudripad R, Smith TF (1994). The ancient regulatory-protein family of WD-repeat proteins. Nature 371, 297–300.

Omnus DJ, Pfirrmann T, Andréasson C, Ljungdahl PO (2011). A phosphode

Prachiel T, Thornton J, Liu Z (2012). TORC2 signaling is antagonized by protein phosphatase 2A and the Far complex in Saccharomyces cerevisiae. Genetics 190, 1325–1339.

Ritterhoff S, Farah CM, Grabitzki J, Lochnt G, Skurat AV, Schmitz ML (2010). The WD40-repeat protein Han11 functions as a scaffold protein to control HIPK2 and MEKK1 kinase functions. EMBO J 29, 3750–3761.

Robinson LC et al. (1992). Yeast casein kinase I homologues: an essential gene pair. Proc Natl Acad Sci USA 89, 28–32.

Rossio V, Galati E, Ferrari M, Pellicioli A, Sutani T, Shirahige K, Lucchini G, Piatti S (2010). The RSC chromatin-remodeling complex influences mitotic exit and adaptation to the spindle assembly checkpoint by controlling the Cdc14 phosphatase. J Cell Biol 191, 981–997.

Schroffelbauer B, Polley S, Behar M, Ghosh G, Hoffmann A (2012). NEMO ensures signaling specificity of the pleiotropic IKKbeta by directing its kinase activity toward IkappaBalpha. Mol Cell 29, 297–310.

Sikorski RS, Hieter P (1989). A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in Saccharomyces cerevisiae. Genetics 122, 19–27.

Silve S, Volland C, Garnier C, Jurnd R, Chevallier MR, Haugener-Tsapis R (1991). Membrane insertion of uracil permease, a polytopic yeast plasma membrane protein. Mol Cell Biol 11, 1114–1124.

Sneddon AA, Cohen PT, Stark MJ (1990). Saccharomyces cerevisiae protein phosphatase 2A performs an essential cellular function and is encoded by two genes. EMBO J 9, 4339–4346.

Ulrich A, Schlessinger J (1990). Signal transduction by receptors with tyrosine kinase activity. Cell 61, 203–212.

Wang PC, Vancura A, Mitcheson TG, Kuret J (1992). Two genes in Saccharomyces cerevisiae encode a membrane-bound form of casein kinase-1. Mol Biol Cell 3, 275–286.

Wang Y, Elion EA (2003). Nuclear export and plasma membrane recruitment of the Ste5 scaffold are coordinated with oligomerization and association with signal transduction components. Mol Biol Cell 14, 2543–2558.

Willems AR, Schwab M, Tyers M (2004). A hitchhiker's guide to the cullin ubiquitin ligases: SCF and its kin. Biochim Biophys Acta 1695, 133–170.

Wolf DH (2004). From lysosome to proteasome: the power of yeast in the dissection of proteinase function in cellular regulation and waste disposal. Cell Mol Life Sci 61, 1601–1614.

Wu B, Ottow K, Poulsen P, Gaber RF, Albers E, Kielland-Brandt MC (2006). Competitive intra- and extracellular nutrient sensing by the transporter homologue Ssy1p. J Cell Biol 173, 327–331.

Yablonski D, Marbach I, Levitzki A (1996). Dimerization of Ste5, a mitogen-activated protein kinase cascade scaffold protein, is required for signal transduction. Proc Natl Acad Sci USA 93, 13864–13869.

Zhao Y, Boguslawski G, Zitomer RS, DePaoli-Roach AA (1997). Saccharomyces cerevisiae homologs of mammalian B and B' subunits of protein phosphatase 2A direct the enzyme to distinct cellular functions. J Biol Chem 272, 8256–8262.

D. J. Omnus and P. O. Ljungdahl Molecular Biology of the Cell