Identification of Novel Pheromone-response Regulators through Systematic Overexpression of 120 Protein Kinases in Yeast

Scott A. Burchett‡, Anisa Scott§, Beverly Errede§, and Henrik G. Dohlman¶

From the Department of Pharmacology, Yale University School of Medicine, New Haven, Connecticut 06538 and the $Department of Biochemistry and Biophysics, University of North Carolina, Chapel Hill, North Carolina 27599

Protein kinases are well known to transmit and regulate signaling pathways. To identify additional regulators of the pheromone signaling apparatus in yeast, we evaluated an array of 120 likely protein kinases encoded by the yeast genome. Each kinase was fused to glutathione S-transferase, overexpressed, and tested for changes in pheromone responsiveness in vivo. As expected, several known components of the pathway (YCK1, STE7, STE11, FUS3, and KSS1) impaired the growth arrest response. Seven other kinases also interfered with pheromone-induced growth arrest; in rank order they are as follows: YKL116c (renamed PRR1) = YDL214c (renamed PRR2) > YJL141c (YAK1, SRA1) > YNR047w = YCR091w (KIN82) = YIL095w (PRK1) > YCL024w (KCC4). Inhibition of pheromone signaling by PRR1, but not PRR2, required the glutathione S-transferase moiety. Both kinases inhibited gene transcription after stimulation with pheromone, a constitutively active kinase mutant STE11-4, or overexpression of the transcription factor STE12. Neither protein altered the ability of the mitogen-activated protein kinase (MAPK) FUS3 to feedback phosphorylate a known substrate, the MAPK kinase STE7. These results reveal two new components of the pheromone-signaling cascade in yeast, each acting at a point downstream of the MAPK.

Many cell-signaling processes require the action of receptors, G proteins, and protein kinases that propagate the signal. In humans, G protein-coupled receptors respond to hormones, neurotransmitters, odors, taste, and light. In the yeast Saccharomyces cerevisiae, G protein-linked pheromone receptors trigger events required for mating and cell fusion. Upon activation of the receptor (Ste2 or Ste3 in yeast), GTP binds to the G protein α subunit (Gpa1), which triggers its dissociation from the G protein βγ subunits (Ste4/Ste18). Gγy in turn activates a protein kinase cascade comprised of Ste20, Ste11, Ste7, and FUS3 (1). Ste5 promotes signaling, through the assembly and recruitment of Ste11, Ste7, and FUS3, to the activating G protein, as well as to effector targets within the nucleus (2). Sst2 promotes signal inactivation by accelerating Gpa1 GTPase activity and promoting subunit reassembly (3). The casein kinase I isoforms (Yck1, Yck2) promote signal inactivation by phosphorylating receptors, triggering their uptake and degradation (4–6). Notably, most (if not all) of the signaling components must exist as dimers to function. Prominent examples include the receptor (7, 8) Ste5 and its associated kinases (9–11), as well as the nuclear transcription factor Ste12 (12–15).

A central player in the kinase cascade is Fus3, a member of the MAPK family. In mammals, MAPKs can respond to a variety of signaling molecules and chemical or physical stresses (temperature, salt, etc.) (16). MAPKs in turn regulate almost all aspects of cell growth and homeostasis. In yeast, the MAPK Fus3 phosphorylates and regulates proteins required for pheromone detection (Ste3), kinase scaffolding and activation (Ste5) (17), morphological and cytoskeletal changes (Far1) (18–20), cell division arrest (Far1) (19–21), transcriptional activation (Ste12) (18, 22, 23), transcription inhibition (Dig1, Dig2) (24–26), and feedback inhibition (Sst2) (27). In most cases, phosphorylation by Fus3 leads to enhanced signal transduction, but in a few instances phosphorylation promotes signal attenuation or desensitization. For instance, phosphorylation of the receptor Ste3 leads to its endocytosis and degradation (28). Phosphorylation of Sst2 leads to its stabilization and to further inactivation of the G protein (27). Thus Fus3 allows a coordinated response to pheromone in preparation for cell fusion, with both positive and negative outcomes for signaling.

The completion of the yeast genome sequence has yielded a complete inventory of 6144 open reading frames. Of these, 123 are likely to encode protein kinases, many of which have not been characterized. Given the prominent role that kinases have in G protein signaling and desensitization, we undertook a comprehensive screen of these proteins for those affecting the pheromone response in yeast. Here we describe two novel regulators of the G protein/MAPK cascade, which we have designated Prr1 and Prr2.

**EXPERIMENTAL PROCEDURES**

**Strains, Plasmids, and Mutagenesis—**Standard methods for the growth, maintenance, and transformation of yeast and bacteria, and for the manipulation of DNA, were used throughout (29). The yeast *S. cerevisiae* strains used in this study were EJ 758 (MATa his3-200, leu2-3, 112, ura3-52, pep4::URA3 (30), C699–32 (MATa bar1::HisG FUS1::lacZ::LEU2 ade2-1 ura3-52 trp1 leu2-3 his3 ura3-31 can1), BY4741 (MATa leu2::met15 ura3-A), and BY4741-derived mutants lacking FUS3 (YBL018w), PRR1 (YKL116c), and PRR2 (YIL214c; all from Research Genetics, Huntsville, AL).

The following steps were used for construction by gene replacement of strain C699–32 from strain C699 (identical to strain W303–1A)

1. The abbreviations used are: MAPK, mitogen-activated protein kinase; GST, glutathione S-transferase; PIPEPS, 1,4-piperazinediethanesulfonic acid; NLS, nuclear localization signals.
B. Errede, unpublished data.

To identify novel proteins that regulate the mating pathway, we screened an expression array of 120 (of 123 total) protein kinases encoded by the yeast genome. Each open reading frame was fused to GST and overexpressed in a high copy plasmid, using the copper-inducible promoter from \textit{CUP1}. Most of the 6144 open reading frames are available in this format and are typically tested in arrays pools containing a defined set of gene products (30). Because we were primarily interested in finding novel kinase regulators of the pathway, we elected to test this subset of genes individually. Our approach has significant advantages over conventional genetic screening strategies. First, it is quite rapid; each round of screening can be conducted in about 2 weeks. Second, it is highly systematic, allowing us to test nearly all of the likely kinases encoded by the yeast genome, and provides immediate identification of the gene responsible for a particular activity. Third, it is sensitive, because each kinase is strongly overexpressed. Fourth, each open reading frame is fused to GST, which will facilitate eventual purification and biochemical analysis of the encoded kinase.

The following strategy was used to identify kinases that promote or inhibit pheromone signaling. Transformed cells were grown to saturation and plated on solid medium containing copper sulfate to induce kinase expression. \(a\)-Factor pheromone was spotted onto the nascent lawn, and the resulting zone of growth inhibition was documented after 2–3 days (halo assay). After screening all 120 clones in duplicate, the vast majority yielded no difference in growth. None caused an increase in halo size, whereas a few produced halos that were smaller and/or more turbid than normal. Of these, 12 were selected for further analysis and validation.

The following five of the 12 clones encode known kinases of the pathway: \textit{YCK1}, \textit{STE7}, \textit{STE11}, \textit{FUS3}, and \textit{KSS1} (a MAPK that can partially complement \textit{FUS3}). The only other known kinase in the pathway, \textit{STE20}, was not included in the screen, because it is one of three genes that could not be cloned into the yeast expression vector. Our ability to identify essentially all of the known kinase components in the pathway indicates that our screening strategy produced few false negatives. Further, recovery of all the expected kinases reinforces the idea that this set is functionally relevant and that the seven other clones identified in the screen are likely to have some role in pheromone signaling. These are as follows, in rank order of inhibitory potency: \textit{YKL116c} = YDL214c > YJL141c (YAK1, \textit{SRA1}) > YNR047w = YCR091w (\textit{KIN92}) – YIL095w (\textit{PRK1}) – YCL024w (\textit{KCC4}). Notably, the screen does not reveal whether the other clones recovered here might have a positive or negative role in pheromone signaling. For instance, \textit{Ste11} and \textit{Ste7} are primarily needed to transmit the pheromone signal, yet each inhibits the response in this overexpression method (see below).

Pheromone-induced growth inhibition experiments with representative kinases from the above set are shown in Fig. 1. In this and all subsequent figures, \textit{FUS3} is used as a positive control, whereas an irrelevant kinase or empty vector is used as a negative control. The two most potent inhibitors were renamed \textit{PRR1} (\textit{YKL116c}) and \textit{PRR2} (YDL214c), for pheromone response regulators 1 and 2. Because they had not previously been implicated in the pheromone-response pathway, we focused our attention on their further characterization.

The halo assay is a convenient but fairly qualitative measure of pheromone sensitivity. A more quantitative measure of pher-
Pheromone Response Regulatory Kinases in Yeast

**FIG. 1. Overexpression of kinase-GST fusions leads to diminished pheromone-dependent growth arrest response.** Cells were transformed with FUS3, PRR1, PRR2, YNR047w, KIN82, or PTK2, all in the vector pYEX-4T-2, plated, and exposed to sterile filter discs spotted with α-factor (clockwise from bottom: 0, 15, 25, 50, 60, and 75 μg) for 48 h and then photographed. Depicted are the four unknown protein kinases recovered in the screen, Prr1, Prr2, Ynr047w, and Kin82. Fus3 is the positive control. Ptk2 is the negative control. Rare colonies within the zone of growth inhibition are spontaneous sterile mutants.

Pheromone response can be obtained from analysis of transcriptional induction, using a reporter comprised of the FUS1 promoter and lacZ (β-galactosidase) gene. As shown in Fig. 2, overexpression of either PRR1 or PRR2 results in a marked inhibition of pheromone-dependent transcription. Compared with the vector control, overexpression of Prr1 or Prr2 reduced the maximum response at 30 μM α-factor by 1.24- and 2.77-fold, respectively, with no change in EC₅₀. Overexpression of our positive control kinase Fus3 yielded an even larger (11.7-fold) decrease in activity, as shown previously (44).

As noted above, the overexpression assays employed here do not distinguish whether a given kinase has a positive or negative role in pheromone-induced signaling. It was shown previously through systematic gene disruption analysis that neither PRR1 nor PRR2 is essential for cell viability (45). Therefore, the transcription response of deletion mutant strains could be evaluated over a range of α-factor concentrations. As shown in Fig. 3, the pheromone response in either the prr1Δ or prr2Δ single mutant is identical to that of the wild-type strain. By contrast, the pheromone response is diminished with the control fus3Δ mutant. Although there is substantial precedent and several reasons for single deletion mutants failing to give a phenotype, this outcome meant that different strategies would be needed to establish whether PRR1 and PRR2 act as positive or negative regulators (see below).

To determine where in the pathway Prr1 and Prr2 operate, we examined their ability to inhibit signaling at different points downstream of the pheromone receptor. For this analysis, the pathway was activated in two alternative ways. In the first case, we used an allele of the MAPK kinase kinase STE11 (STE11-4), containing a single amino acid substitution (T596I) within the catalytic domain. Cells expressing STE11-4 exhibit a significant increase in basal signal pathway activity (46). Constitutive activation by STE11-4 is unaffected by mutations in the receptor (Ste2) or Gβ (Ste4) but is blocked by mutations in the downstream MAPK kinase (Ste7), MAPK (Fus3, Kss1), or transcription factor (Ste12) (46). Thus STE11-4 activates signaling at a well defined step in the pathway, between Ste20 and Ste7. In the second case, we activated the pathway through expression of STE12 under the control of the inducible GAL1 promoter. STE12 overexpression leads to transcriptional induction in the absence of any upstream components or added pheromone (47).

As shown in Fig. 4, Prr1 and Prr2 are both potent inhibitors of transcriptional induction, in response to either STE11-4 (top panel) or STE12 overexpression (bottom panel). In agreement with previous reports, our positive control Fus3 is also a potent inhibitor of transcriptional induction (48–50). When overexpressed, a portion of Fus3 is presumably in the unphosphorylated and catalytically inactive state. This pool may interfere with the ability of any activate Fus3 to transmit a signal to Ste12 (48). These data show that Prr1 and Prr2 (like Fus3) regulate signaling downstream of Ste11, most likely at the level of Ste12.

To determine whether signal inhibition requires the GST
moiety, each open reading frame was polymerase chain reaction-amplified and cloned into another vector lacking any fusion protein. As shown in Fig. 5, overexpression of Prr2 or Fus3 reduced the maximum response at 30 sion protein. As shown in Fig. 5, overexpression of Prr2 or Fus3 fusion-amplified and cloned into another vector lacking any fu-

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Fig. 3. Disruption of PRR1 or PRR2 does not alter pheromone-dependent transcriptional induction. Gene disruption mutants lacking FUS3 ( fus3Δ), PRR1 ( prr1Δ), or PRR2 ( prr2Δ) were transformed with the FUS1-lacZ reporter plasmid. Cells were treated with α-factor, and β-galactosidase activity was determined as described above. Data shown are typical of three independent experiments performed in triplicate. Error bars, ± S.E.

Fig. 4. Overexpression of PRR1-GST or PRR2-GST leads to diminished transcriptional induction through activation by STE11-4 or STE12. Cells were transformed with a plasmid containing the FUS1 promoter-lacZ reporter, a plasmid that overexpresses either a constitutively active allele STE11-4 (top panel) or wild-type STE12 (bottom panel), and a plasmid containing FUS3-GST, PRR1-GST, PRR2-GST, or no insert (Vector; pYEX 4T-2). Cells were treated with the indicated concentrations of α-factor or water, and β-galactosidase activity was determined as detailed under “Experimental Procedures.” Data shown are typical of three independent experiments performed in triplicate. Error bars, ± S.E.

The ability to suppress both STE11-4 and GAL1-STE12 indicates that Prr2 operates at the level of the transcription factor Ste12. Ste12 activity depends in part on Fus3 binding and phosphorylation. We therefore examined whether Prr2 inhibits Ste12 indirectly, through inhibition of Fus3. It has been shown previously that pheromone activation results in feedback phosphorylation of Ste7. This reaction provides a particularly convenient indicator of Fus3 activity in vivo, because the resulting hyperphosphorylated species of Ste7 exhibits a markedly reduced mobility (~10 kDa) on SDS-polyacryl-

amide gel electrophoresis (35). Thus we used the Ste7 mobility shift assay to determine whether Prr2 or Prr2K390R can inhibit Fus3 activity. As shown in Fig. 7, pheromone induction caused a typical shift in Ste7 mobility (Fig. 7, lane 1 versus lane 6). As
expected, overexpression of either Fus3 or Fus3K42R completely inhibited pheromone-induced Ste7 phosphorylation. In contrast, overexpression of Prr2 or the catalytically inactive derivative Prr2K202R had no effect on Ste7 phosphorylation (Fig. 7, lanes 9 and 10). Overexpression of Prr1 or Prr1K202R similarly had no effect on pheromone-induced feedback phosphorylation of Ste7 (data not shown). These results confirm that inhibition of the pheromone-response pathway by Prr2 (and Prr1) occurs downstream of the MAPK.

FIG. 5. PRR1 but not PRR2 requires GST for inhibition of pheromone-dependent transcriptional induction. Cells were co-transformed with a plasmid containing the FUS1 promoter-loc2 reporter and a plasmid containing FUS3, PRR1, PRR2, or no insert (Vector; pYES2.1 TOP). Cells were treated with the indicated concentrations of α-factor, and β-galactosidase activity was determined as described above. Data shown are typical of three independent experiments performed in triplicate. Error bars, ± S.E.

FIG. 6. PRR2 kinase activity is required for full inhibition of pheromone-dependent transcriptional induction. Cells were co-transformed with the FUS1 promoter-loc2 reporter and a plasmid containing either wild-type or kinase-dead (KR) forms of FUS3, PRR1, PRR2, or no insert (Vector; pYES2.1/V5). Cells were treated with the indicated concentrations of α-factor, and β-galactosidase activity was determined as described above. Data shown are typical of three independent experiments performed in triplicate. Error bars, ± S.E.

FIG. 7. PRR2 does not alter Fus3/Kss1 phosphorylation of Ste7. Immunoblots comparing phosphorylated (P-Ste7; upper arrow) and non-phosphorylated (lower arrow) forms of Ste7 are shown. Strain C699–32 (bar1::HisG ste7::ADE2) expressing Myc-tagged Ste7 (Ste7M) from the CYC1 promoter (pNC318) and one of the following from the GAL1,10 promoter is shown: lane 1, empty vector (pYES2.1/V5); lane 2, Fus3; lane 3, Fus3-KR; lane 4, Prr2; lane 5, Prr2-KR; lane 6, empty vector (pYES2.1/V5); lane 7, Fus3; lane 8, Fus3-KR; lane 9, PRR2; lane 10, PRR2-KR; KR, kinase-dead. Extracts were prepared from each culture either before (right) or after (left) pheromone induction (50 nM α-factor; 90 min). 150 µg of each extract was fractionated by SDS-polyacrylamide gel electrophoresis (10%) and transferred to nitrocellulose filters for immune detection of Ste7M using anti-Myc monoclonal antibody and anti-mouse IgG conjugated to alkaline phosphatase. A wealth of information provided by these efforts will require the development of more systematic approaches to the analysis of gene function. Here we have tested a subset of the Saccharomyces genome, specifically the genes encoding likely protein kinases. Our immediate goal was to identify novel kinase regulators of the pheromone-response pathway. A broader goal was to assess the feasibility of such a systematic screening method for functional genomic analysis in yeast and eventually in mammals.

By screening a protein kinase-GST overexpression library, we identified 12 kinase regulators of the pheromone response. Five of the genes represent previously known components and a regulator of the signaling pathway (STE7, STE11, FUS3, YCK1, and KSS1). The remaining seven have not been shown previously to act in this manner. The two most active regulators, designated PRR1 and PRR2, were characterized further.

Overexpression of either PRR1 or PRR2 is able to diminish signaling, as assessed by two standard bioassays of pheromone sensitivity (Figs. 1 and 2). Gene disruption mutants have no effect on signaling (Fig. 3). Both kinases appear to act late in the pathway, as demonstrated by their ability to diminish signaling by activated forms of STE11 and STE12 (Fig. 4), without affecting the activity of the MAPK (Fig. 7). Inhibition of signaling by PRR1, but not PRR2, is largely dependent upon GST fusion (Fig. 5). Inhibition by PRR2, but not PRR1, is dependent upon its kinase catalytic activity (Fig. 6). Thus our genetic analysis indicates that the pheromone-response regulators identified here act downstream of the MAPK and most likely in conjunction with a terminal-signaling component Ste12.

Future experiments will be aimed at finding the target of the most potent regulator Prr2. One obvious candidate substrate is Ste12. Ste12 is known to be heavily phosphorylated through the action of Fus3 and Kss1 and almost certainly by other kinases (18, 22, 23). Demonstrating that Prr2 phosphorylates Ste12 may be difficult, however. The presence of multiple independent phosphorylation sites has frustrated efforts in the past to identify kinases in addition to the MAPKs that act on Ste12 (23).

Other candidate substrates include a number of DNA-binding proteins that also interact with Ste12, including Mcm1, Matα1, Tec1, Kar4, Dig1, Dig2, and Ste12 itself. Ste12 homodimers are needed for the expression of pheromone-inducible genes in both a- and α-haploid cell types (12). Ste12-Tec1 heterodimers are needed for expression at promoters contain-
ing a filamentous growth response element (13, 15, 51, 52). Ste12-Mcm1 heterodimers are needed for expression of α-cell-specific genes in response to pheromone (13, 14). A ternary complex involving Ste12, Mcm1, and Matα1 is needed for expression of α-cell-specific genes in response to pheromone (e.g., MFα2). Conversely, a ternary complex involving Ste12, Dig1, or Dig2 and either Fus3 or Kss1 (24, 25) can inhibit transcription at pheromone-response elements (48, 50) or filamentous growth response elements (49, 53), respectively. In principle, Prr1/Prr2 could modulate Ste12 activity directly or indirectly through phosphorylation of a Ste12-binding protein.

Prr1, and to a certain extent Prr2, may regulate signaling through a mechanism that does not require kinase activity. We showed that wild-type and kinase-dead forms of Prr1 were equally active in their ability to inhibit the pheromone response. The kinase-dead form of Prr2 was less active than its wild-type counterpart (Fig. 6). In this regard, Prr2 behaves very much like Fus3: a kinase-dead form of Fus3 inhibits pheromone signaling, though less potently than the wild-type version (Fig. 6). Inhibition of signaling by catalytically inactive Fus3 has been attributed, at least in part, to its direct binding and inhibition of Ste12 (48–50). In the absence of a sustained signal, a dephosphorylated form of Fus3 accumulates, blocks Ste12-mediated gene transcription, and permits cell cycle progression. Under pheromone-inducing conditions Ste7 phosphorylates and activates Fus3, and this leads to both removal of inhibition (through dissociation) and activation (through phosphorylation) of Ste12. Thus, Fus3 can act as both an inhibitor and an activator of Ste12, depending on its state of phosphorylation. A similar phenomenon has also been observed for Kss1 (49, 50, 54). Taken together, these results suggest that Prr2 behaves similarly to Fus3, able to function as both an inhibitor and activator of Ste12.

Once the target of Prr2 kinase activity is found, we will attempt to identify upstream activators of Prr2. One likely candidate is the MAPK Fus3. Although the most intensively studied substrates of MAPKs are transcription factors (55), some substrates are themselves protein kinases (16, 66). Although some MAPK kinase substrates act upstream in the same pathway (Ste7, Ste11), it is unlikely that Prr2 acts upstream of Fus3, because Fus3 activity is not altered by PRR2 overexpression (Fig. 7).

There are other reasons to believe that Prr2 acts within the nucleus. We identified three NLS motifs within Prr2 (Fig. 8B). NLSs are utilized for the import of proteins into the nucleus from the cytoplasm through the nuclear pore complex (56). Translation of Prr2 to the nucleus from the cytosol and/or activation of Prr2 within the nucleus may be involved in mediating the inhibition of pheromone signaling. Exploring these areas may lead to new insights into the molecular mechanism of the regulation of nuclear signal transduction.

Another issue is whether Prr1 or Prr2 exists as a dimer, either as a homodimer or as a heterodimer with another regulatory protein. We have shown that the pheromone-response regulation by Prr1 requires the GST moiety. Because the GST moiety exists as a dimer (57), GST could activate Prr1 by imposing homodimerization. Alternatively, GST might sterically exclude binding by another subunit or a regulatory protein. Given the position at which Prr1 and Prr2 act in the pathway (downstream of the MAPK) it seems likely that these proteins do form some sort of dimer. Many MAPK substrates exist as dimers, most notably transcription factors (55). Moreover, many MAPKs appear to undergo dimerization upon activation, which likely facilitates the phosphorylation of dimeric substrates (58). Finally, biochemical (9, 59) and genetic (11, 60) methods indicate that the MAPK scaffold protein Ste5 also self-associates and exists as an oligomer in cell extracts. Ste5 mutants that cannot oligomerize (11) are unable to transmit the pheromone signal (9, 11).

It is not certain why the prr1Δ and prr2Δ mutants lack any signaling phenotype. The absence of an observable phenotype for the prr1Δ mutant might be expected, because only the GST...
fusin protein inhibits the response. However, this explanation does not apply to the prr2Δ mutant. One possibility to consider in this case is that a closely related gene may compensate for the loss of Prr2. For example, loss of STE20 is compensated in part by the closely related gene, CLA4 (14, 61–63). In fact, at least three other kinases appear closely related to Prr2, including NPK1, YDL025c, and YOR267c (Fig. 8A). Analysis of double, triple, and quadruple gene disruption mutants are in progress and may reveal some defect in signaling (if indeed these mutants are viable). Alternatively Prr2 activity might regulate both positive and negative components of the pathway. For example, it has been established that these mutants are viable). Alternatively Prr2 activity might regulate both positive and negative components of the path-

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