Title
Specific α-arrestins negatively regulate Saccharomyces cerevisiae pheromone response by down-modulating the G-protein-coupled receptor Ste2

Permalink
https://escholarship.org/uc/item/5cj6p3gp

Journal
Molecular and Cellular Biology, 34(14)

ISSN
0270-7306

Authors
Alvaro, CG
O'Donnell, AF
Prosser, DC
et al.

Publication Date
2014

DOI
10.1128/MCB.00230-14

Peer reviewed
Specific α-Arrestins Negatively Regulate *Saccharomyces cerevisiae* Pheromone Response by Down-Modulating the G-Protein-Coupled Receptor Ste2

Christopher G. Alvaro, Allyson F. O'Donnell, Derek C. Prosser, Andrew A. Augustine, Aaron Goldman, Jeffrey L. Brodsky, Martha S. Cyert, Beverly Wendland, Jeremy Thorner

Division of Biochemistry, Biophysics and Structural Biology, Department of Molecular and Cell Biology, University of California, Berkeley, California, USA; Department of Cell Biology, School of Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania, USA; Department of Biology, The Johns Hopkins University, Baltimore, Maryland, USA; Department of Biological Sciences, University of Pittsburgh, Pittsburgh, Pennsylvania, USA; Department of Biology, Stanford University, Stanford, California, USA

G-protein-coupled receptors (GPCRs) are integral membrane proteins that initiate responses to extracellular stimuli by mediating ligand-dependent activation of cognate heterotrimeric G proteins. In yeast, occupancy of GPCR Ste2 by peptide pheromone α-factor initiates signaling by releasing a stimulatory Gβγ complex (Ste4-Ste18) from its inhibitory Gαi subunit (Gpa1). Prolonged pathway stimulation is detrimental, and feedback mechanisms have evolved that act at the receptor level to limit the duration of signaling and stimulate recovery from pheromone-induced Gαi arrest, including upregulation of the expression of an α-factor-degrading protease (Bar1), a regulator of G-protein signaling protein (Sst2) that stimulates Gpa1-GTP hydrolysis, and Gpa1 itself. Ste2 is also downregulated by endocytosis, both constitutive and ligand induced. Ste2 internalization requires its phosphorylation and subsequent ubiquitylation by membrane-localized protein kinases (Yck1 and Yck2) and a ubiquitin ligase (Rsp5). Here, we demonstrate that three different members of the α-arrestin family (Ldb19/Art1, Rod1/Art4, and Rog3/Art7) contribute to Ste2 desensitization and internalization, and they do so by discrete mechanisms. We provide genetic and biochemical evidence that Ldb19 and Rod1 recruit Rsp5 to Ste2 via PPXY motifs in their C-terminal regions; in contrast, the arrestin fold domain of the N terminus of Rog3 is sufficient to promote adaptation. Finally, we show that Rod1 function requires calcineurin-dependent dephosphorylation.

For survival, eukaryotic cells sense and respond to changes in external conditions using, in many cases, a G-protein-coupled receptor (GPCR) (1, 2). Most clinically used pharmaceuticals act on GPCRs (3, 4). After an initial response, cells normally adapt by becoming desensitized to the stimulus, whereas chronic GPCR action can lead to inflammation and other pathophysiological states (5, 6). Thus, the mechanisms underlying both GPCR signal transduction and its attenuation have important medical implications.

In *Saccharomyces cerevisiae*, GPCR Ste2 in the plasma membrane (PM) of a MATα haploid cell binds α-factor (a 13-residue peptide) secreted by a MATα haploid cell, and a MATα haploid uses GPCR Ste3 to bind a-factor (a 12-residue prenylated peptide) released by a MATα haploid (7). Engagement of these receptors by these ligands (mating pheromones) initiates a mitogen-activated protein kinase (MAPK) cascade. The activated MAPK evokes transcriptional and morphological responses that arrest cell growth in the G1 phase of the cell cycle and convert the cells to gametes, which conjugate (mate) to form a MATα/MATα diploid (8, 9). Genetic and molecular analysis of this system has established many important concepts in GPCR-initiated signaling and its regulation (10–13).

Hyperactivation or prolonged signaling through the mating pheromone response pathway can cause cell death (14). To avoid this, α-factor also induces feedback mechanisms that downregulate signaling in MATα cells. Paramount among these adaptation processes is upregulation of expression of genes for a variety of negative regulators, such as MSG5, which encodes a phosphoprotein phosphatase that deactivates the terminal MAPK (Fus3) of the pathway (15, 16). However, induced negative regulators also include factors that desensitize the pathway far upstream by acting on α-factor, its receptor, or the associated heterotrimeric G protein, preventing further GPCR-initiated signaling. For example, BAR1 encodes a protease that cleaves α-factor into two inactive fragments (17, 18). SST2 encodes the first regulator of G-protein signaling (RGS) identified (7, 19, 20). Binding of its N-terminal DEP domains to the cytosolic tail of Ste2 (21) delivers Sst2 to the PM. Thus, its C-terminal RGS domain is positioned to stimulate conversion of PM-localized GTP-bound Gpa1 back to its GDP state (22). GDP-Gpa1 then reassociates with and blocks downstream signaling by the Ste4-Ste18 complex (23, 24), which is also PM anchored via S-palmitoylation and S-farnesylation of Ste18 (25, 26). Recoupling and squelching of Gβγ function is further promoted by mass action, because Gpa1 is upregulated in response to pheromone (27), concomitant with an enhanced rate of its N-myristoylation (28), a posttranslational modification essential for Gpa1 PM targeting, coupling to Gβγ, and association with the receptor (23, 29).

Termination of an agonist-initiated GPCR-mediated signal is also thought to involve ligand-induced receptor endocytosis (30, 31).
It has been known for decades that both Ste2 and Ste3 undergo constitutive internalization at a basal rate and that endocytosis is stimulated upon binding of the cognate pheromone (32–36). Pheromone binding causes a conformational change that promotes phosphorylation of the Ste2 and Ste3 C termini (37–39), mediated by PM-anchored casein kinase 1 isofoms (Yck1 and Yck2) (21, 40). Phosphorylation is a prelude to receptor ubiquitylation (39, 41) by a PM-associated HECT domain-containing ubiquitin ligase (E3), Rsp5 (42–44) (the mammalian ortholog is NEDD4L [45]). Ubiquitin attachment targets the receptors for clathrin-mediated internalization (46, 47). The resulting endosomes are delivered to the vacuole (equivalent to a mammalian lysosome) where the receptor is degraded (48, 49).

Subsequent studies have provided more detailed analysis of spatiotemporal aspects of Ste2 (50, 51) and Ste3 (52, 53) internalization and better delineated the phosphorylation and ubiquitylation sites (54, 55). However, how Rsp5 recognizes and is recruited to these GPCRs to catalyze ubiquitylation remained unresolved. Discovery in yeast of a protein family, called the α-arrestins, that serve as adaptors for Rsp5-dependent ubiquitylation and internalization of nutrient permeases provided a clue (56–58). The S. cerevisiae genome encodes 14 currently recognized members of the α-arrestin family (57–60). The name derives from homology of their N-terminal domains to a fold found in mammalian arrestin and β-arrestins (56, 61), first identified and implicated in blocking signaling by rhodopsin (62) and the β-adrenergic receptor (63), respectively.

As described here, we found that three different α-arrestins, Ldb19/Art1, Rod1/Art4, and Rog3/Art7, have overlapping functions and contribute to Ste2 internalization and MAFA cell recovery from pheromone-induced Gαi arrest. Surprisingly, the modes of action of these α-arrestins are distinct. Ldb19 plays a role primarily in basal turnover of Ste2, whereas Rod1 and Rog3 contribute to desensitization of the agonist-occupied receptor. Although paralogous on the basis of primary sequence, Rod1 and Rog3 act by different mechanisms; Rod1 is obligatorily Rsp5 dependent, but Rog3 is not. Moreover, Rod1 (but not Rog3) function during Ste2 downregulation requires calcineurin (CN)-dependent dephosphorylation. Together, our studies extend the function of α-arrestins to another class of polytopic membrane protein and demonstrate discrete actions of α-arrestin family members whose functions previously have been enigmatic.

**MATERIALS AND METHODS**

**Strains and growth conditions.** Yeast strains (Table 1) were grown at 30°C in either rich (YPD) or synthetic complete (SC) medium containing 2% glucose (unless another carbon source is specified) and with appropriate nutrients to maintain selection for plasmids, if present (64). Standard genetic methods were used for strain construction (65).

**Plasmids.** Plasmids (Table 2) were constructed using standard procedures (66). DNA amplification by PCR (66) employed Phusion DNA polymerase (New England Biolabs, Ipswich, MA), and all constructs were verified by sequencing. Site-directed mutagenesis (67) was carried out using the same DNA polymerase and QuikChange methodology (New England Biolabs) according to the manufacturer’s instructions.

**Pheromone-imposed growth arrest.** Response to α-factor was assessed by an agar diffusion (halo) bioassay essentially as described previously (37). In brief, cells (~10^6) were plated on top agar on solid YPD or SC medium as appropriate. On the resulting surface were laid sterile cellulose filter disks, onto which an aliquot (typically, 15 μl) of an aqueous solution (1 mg/ml) of α-factor (GeneScript, Piscataway, NJ) had been sequestically spotted, and the plates were incubated at 30°C for 2 to 4 days. To induce α-arrestin overexpression, strains containing the tripartite S. cerevisiae Gal4-human estrogen receptor-herpes simplex virus transactivator VP16 fusion protein (Gal4-ER-VP16 or GEV) (68, 69) and URA3-marked multicopy (2μm DNA) plasmids expressing from the GALI,10 promoter the α-arrestin of interest (as a fusion to the C terminus of glutathione S-transferase [GST]) were grown to mid-exponential phase. The cultures were then treated with β-estradiol (20 μM final concentration) for 3 h and plated with top agar containing 200 nM β-estradiol. Samples of the same cultures were analyzed by immunoblotting (see below) to confirm α-arrestin expression.

**Mating pathway activation.** As one measure of pathway activation, the level of dually phosphorylated Fus3 was assayed as follows. Strains of interest carrying a bar1Δ mutation were grown in YPD to mid-exponential phase, a sample was removed (zero time point), and immediately thereafter the culture was treated with α-factor (15 nM final concentration). Additional samples of equivalent numbers of cells then were removed at 10, 30, 60, and 90 min, harvested by rapid sedimentation in a microcentrifuge, and immediately frozen in liquid N,2. The cell pellets were thawed on ice, and whole-cell protein extracts were prepared by alkaline lysis followed by collection of total protein by trichloroacetic acid (TCA) precipitation (70, 71). Protein precipitates were solubilized in SDS-urea gel sample buffer (5% SDS, fresh 8 M urea, 1% β-mercaptoethanol, 0.1 M EDTA, 40 mM Tris-HCl [pH 6.8]) with 0.1% bromophenol blue, heated at 37°C for 15 min, resolved in a slab gel by SDS-PAGE (72), and analyzed by immunoblotting.

As an independent measure of pathway activation, induction of an integrated single-copy FUS1prom-enhanced green fluorescent protein (eGFP) reporter (73) was monitored. Strains of interest carrying a bar1Δ mutation and also containing integrated FUS1prom-eGFP were constructed (Table 1). These cells were grown to mid-exponential phase and treated with 15 nM α-factor for 2 h, and the level of GFP expression was quantified by flow cytometry using a Beckman-Coulter FC-500 analyzer. The resulting data were analyzed using Flowjo software (Tree Star, Inc.).

**Immunoblotting.** Equal numbers of cells from mid-exponential-phase cultures were collected by centrifugation and lysed, and total protein was collected and resolved by SDS-PAGE as described above. The proteins in the resulting slab gels were transferred electrophoretically to nitrocellulose sheets (74) using a semidy transfer apparatus (Transblot SD; Bio-Rad, Inc.). After blocking with carrier protein (75), the filters were incubated (generally for several h at room temperature or overnight at 4°C) with the following primary antibodies, as appropriate: rabbit polyclonal anti-GST (Sigma), rabbit polyclonal anti-phospho-extracellular signal-regulated kinase (anti-phospho-ERK; Cell Signaling), goat polyclonal anti-Fus3 (Santa Cruz), mouse monoclonal anti-γH2 (12CA5; Roche), mouse monoclonal antiguibitin (P4D1; Santa Cruz), and, as loading control, rabbit polyclonal anti-Pgk1 (76). The resulting immune complexes were then detected by incubation, as appropriate, with infrared fluorescent protein (or eGFP) reporter (73) was monitored. Strains of interest carrying a bar1Δ mutation and also containing integrated FUS1prom-eGFP were constructed (Table 1). These cells were grown to mid-exponential phase and treated with 15 nM α-factor for 2 h, and the level of GFP expression was quantified by flow cytometry using a Beckman-Coulter FC-500 analyzer. The resulting data were analyzed using Flowjo software (Tree Star, Inc.).
TABLE 1 Yeast strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>BY4741</td>
<td>MATa len1Δ ura3Δ his3Δ1 met15Δ0</td>
<td>Yeast deletion collection</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(Open Biosystems, Inc.)</td>
</tr>
<tr>
<td>9arrΔ (EN60)</td>
<td>ccm21Δ:KANMX cnr2Δ::KANMX budsΔ rog3Δ::NATMX rod1Δ ygr086Δ Δ aly1Δ lbd19Δ ylr392Δ::HIS3 his3Δ leu2Δ ura3Δ</td>
<td>Yeast deletion collection</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(Open Biosystems, Inc.)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>This study</td>
</tr>
<tr>
<td>lbd19Δ</td>
<td>MATa len1Δ ura3Δ his3Δ1 met15Δ0 lbd19Δ::KANMX</td>
<td>Yeast deletion collection</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(Open Biosystems, Inc.)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>This study</td>
</tr>
<tr>
<td>rod1Δ rog3Δ (JT5887)</td>
<td>MATa len1Δ ura3Δ his3Δ1 met15Δ0 rod1Δ::KANMX4 rog3Δ::KANMX4</td>
<td>This study</td>
</tr>
<tr>
<td>aly1Δ aly2Δ (D2-D4A)</td>
<td>MATa len1Δ ura3Δ his3Δ1 met15Δ0 aly1Δ::KANMX4 aly2Δ::KANMX4</td>
<td>This study</td>
</tr>
<tr>
<td>emc21Δ cnr2Δ (JT5751)</td>
<td>MATa len1Δ ura3Δ his3Δ1 met15Δ0 emc21Δ::KANMX4 cnr2Δ::KANMX4</td>
<td>This study</td>
</tr>
<tr>
<td>art5Δ rim8Δ (JT5860)</td>
<td>MATa len1Δ ura3Δ his3Δ1 met15Δ0 art5Δ::KANMX4 rim8Δ::KANMX4</td>
<td>This study</td>
</tr>
<tr>
<td>art10Δ</td>
<td>MATa len1Δ ura3Δ his3Δ1 met15Δ0 art10Δ::KANMX4</td>
<td>Yeast deletion collection</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(Open Biosystems, Inc.)</td>
</tr>
<tr>
<td>rod1Δ rog3Δ lbd19Δ (JT6765)</td>
<td>MATa len1Δ ura3Δ his3Δ1 met15Δ0 rod1Δ::KANMX4 rog3Δ::KANMX4 lbd19Δ::NATMX</td>
<td>This study</td>
</tr>
<tr>
<td>bar1Δ (JT5915)</td>
<td>MATa len1Δ ura3Δ his3Δ1 met15Δ0 bar1Δ::CgLEU2</td>
<td>This study</td>
</tr>
<tr>
<td>lbd19Δ bar1Δ (JT5916)</td>
<td>MATa len1Δ ura3Δ his3Δ1 met15Δ0 lbd19Δ::KANMX4 bar1Δ::CgLEU2</td>
<td>This study</td>
</tr>
<tr>
<td>rod1Δ rog3Δ bar1Δ (JT5917)</td>
<td>MATa len1Δ ura3Δ his3Δ1 met15Δ0 rod1Δ::KANMX4 rog3Δ::KANMX4 bar1Δ::CgLEU2</td>
<td>This study</td>
</tr>
<tr>
<td>sst2Δ (JT6755)</td>
<td>MATa len1Δ ura3Δ his3Δ1 met15Δ0 sst2Δ::SpHIS5</td>
<td>This study</td>
</tr>
<tr>
<td>lbd19Δ sst2Δ (JT6660)</td>
<td>MATa len1Δ ura3Δ his3Δ1 met15Δ0 lbd19Δ::KANMX4 sst2Δ::SpHIS5</td>
<td>This study</td>
</tr>
<tr>
<td>rod1Δ rog3Δ sst2Δ (JT7002)</td>
<td>MATa len1Δ ura3Δ his3Δ1 met15Δ0 rod1Δ::KANMX4 rog3Δ::KANMX4 sst2Δ::SpHIS5</td>
<td>This study</td>
</tr>
<tr>
<td>rod1Δ rog3Δ lbd19Δ sst2Δ (JT6662)</td>
<td>MATa len1Δ ura3Δ his3Δ1 met15Δ0 rod1Δ::KANMX4 rog3Δ::KANMX4 lbd19Δ::NATMX sst2Δ::SpHIS5</td>
<td>This study</td>
</tr>
<tr>
<td>sst2Δ, GEV (JT5919)</td>
<td>MATa len1Δ ura3Δ his3Δ1 met15Δ0 sst2Δ::SpHIS5 len2Δ::GEV::NATMX</td>
<td>This study</td>
</tr>
<tr>
<td>rod1Δ rog3Δ lbd19Δ sst2Δ GEV (JT6716)</td>
<td>MATa len1Δ ura3Δ his3Δ1 met15Δ0 rod1Δ::KANMX4 rog3Δ::KANMX4 lbd19Δ::NATMX sst2Δ::SpHIS5 len2Δ::GEV::NATMX</td>
<td>This study</td>
</tr>
<tr>
<td>STE2-mCherry bar1Δ (JT6677)</td>
<td>MATa len1Δ ura3Δ his3Δ1 met15Δ0 STE2-mCherry::URA3 bar1Δ::CgLEU2</td>
<td>This study</td>
</tr>
<tr>
<td>STE2-mCherry lbd19Δ bar1Δ (JT6678)</td>
<td>MATa len1Δ ura3Δ his3Δ1 met15Δ0 STE2-mCherry::URA3 lbd19Δ::KANMX4 bar1Δ::CgLEU2</td>
<td>This study</td>
</tr>
<tr>
<td>STE2-mCherry rod1Δ rog3Δ lbd19Δ (JT6679)</td>
<td>MATa len1Δ ura3Δ his3Δ1 met15Δ0 STE2-mCherry::URA3 rod1Δ::KANMX4 rog3Δ::KANMX4 lbd19Δ::NATMX</td>
<td>This study</td>
</tr>
<tr>
<td>STE2-mCherry rod1Δ rog3Δ lbd19Δ lbd19Δ (JT6680)</td>
<td>MATa len1Δ ura3Δ his3Δ1 met15Δ0 STE2-mCherry::URA3 rod1Δ::KANMX4 rog3Δ::KANMX4 lbd19Δ::NATMX bar1Δ::CgLEU2</td>
<td>This study</td>
</tr>
<tr>
<td>rod1Δ lbd19Δ STE2-GFP (JT6759)</td>
<td>MATa len1Δ ura3Δ his3Δ1 met15Δ0 STE2-GFP::HPH</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rod1Δ rog3Δ STE2-GFP (JT7660)</td>
<td>MATa len1Δ ura3Δ his3Δ1 met15Δ0 STE2-GFP::HPH</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rod1Δ rog3Δ lbd19Δ STE2-GFP (JT7661)</td>
<td>MATa len1Δ ura3Δ his3Δ1 met15Δ0 STE2-GFP::HPH</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>aly1Δ aly2Δ STE2-GFP (JT7662)</td>
<td>MATa lbd19Δ::KANMX4 rod1Δ::CgLEU2</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>bar1Δ FUS1prom::gEFP (JT6866)</td>
<td>MATa len1Δ ura3Δ his3Δ1 met15Δ0 bar1Δ::URA3 FUS1prom::gEFP::LEU2</td>
<td>This study</td>
</tr>
<tr>
<td>rod1Δ rog3Δ lbd19Δ bar1Δ FUS1prom::gEFP (JT6868)</td>
<td>MATa len1Δ ura3Δ his3Δ1 met15Δ0 rod1Δ::KANMX4 rog3Δ::KANMX4 lbd19Δ::NATMX bar1Δ::CgLEU2</td>
<td>This study</td>
</tr>
<tr>
<td>BJ5449 FUS1prom::gEFP (JT6868)</td>
<td>MATa len1Δ ura3Δ his3Δ1 met15Δ0 lbd19Δ::KANMX4</td>
<td>This study</td>
</tr>
<tr>
<td>BJ5459 GEV (JT6743)</td>
<td>MATa len1Δ ura3Δ his3Δ1 met15Δ0 STE2-GFP::HPH</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cna1Δ cna2Δ (JT5574)</td>
<td>MATa len1Δ ura3Δ his3Δ1 met15Δ0 cna1Δ::KANMX4 cna1Δ::KANMX4</td>
<td>Yeast deletion collection</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(Open Biosystems, Inc.)</td>
</tr>
<tr>
<td>JRY11</td>
<td>MATa ura3Δ-2 Δ ldr1Δ his3Δ200 pep4Δ::HIS3 prb1Δ1.6Δ can1 GAL cir+</td>
<td>Yeast deletion collection</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(Open Biosystems, Inc.)</td>
</tr>
<tr>
<td>cna1Δ cna2Δ GEV (JT6694)</td>
<td>MATa len1Δ ura3Δ his3Δ1 met15Δ0 cna1Δ::KANMX4 cna1Δ::KANMX4</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a To generate strains with the integrated FUS1prom::gEFP reporter, the cassette (73) was amplified by PCR and introduced by DNA-mediated transformation into a bar1Δ derivative of BY4741. The resulting strain (JT6866) was mated to a MATa lbd19Δ rod1Δ rog3Δ bar1Δ mutant. The resulting diploids were sporulated, and MATa lbd19Δ rod1Δ rog3Δ lbd19Δ bar1Δ FUS1prom::gEFP spores were identified after tetrad dissection.

b To generate a GEV-expressing version of the indicated yeast strain, pACT1-GEV (69, 161) was digested with EcoRV and introduced into the cells of interest by DNA-mediated transformation (65), and nourseothricin (NAT)-resistant colonies were selected in which GEV (expressed under the control of an ACT1 promoter) is integrated at the len2Δ locus.

Dura-extended duration subtype (Thermo Scientific, Rockford, IL) detected with Biomax XR film (Eastman Kodak, Rochester, NY).

Protein purification. GST-Rod1 fusions were used to assess the role of the apparent PXIXIT motif (35) (PQKIE36) in this α-arrestin in mediating its association with calciumin as follows. Cells of yeast strain JRY11, which expresses Cna1-GFP, were transformed with pEGK-Grod1WT or pEGK-Grod1AKAKA. The resulting transformants were grown to mid-exponential phase in SC-2% raffinose, and then expression of the α-arrestin was induced by addition of 2% galactose (final concentration). To activate calciumin, cells were treated with 200 mM CaCl2 (final concent-
TABLE 2 Plasmids used in this study

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Genotype</th>
<th>Description/ reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pEGKG</td>
<td>GAL1prom-GST 2 µm URA3</td>
<td>162</td>
</tr>
<tr>
<td>pEGKG-Rod1</td>
<td>GAL1prom-GST 2 µm URA3</td>
<td>163</td>
</tr>
<tr>
<td>pEGKG-Rog3</td>
<td>GAL1prom-GST 2 µm URA3</td>
<td>163</td>
</tr>
<tr>
<td>pEGKG-Ldb19</td>
<td>GAL1prom-GST 2 µm URA3</td>
<td>163</td>
</tr>
<tr>
<td>pEGKG-Art5</td>
<td>GAL1prom-GST 2 µm URA3</td>
<td>163</td>
</tr>
<tr>
<td>pEGKG-Rod1AAKAA ([pJT4954])</td>
<td>GAL1prom-GST 2 µm URA3</td>
<td>This study</td>
</tr>
<tr>
<td>pEGKG-Rod1PASA ([pJT4955])</td>
<td>GAL1prom-GST 2 µm URA3</td>
<td>This study</td>
</tr>
<tr>
<td>pEGKG-Rod1PPXY-less ([pJT4956])</td>
<td>GAL1prom-GST 2 µm URA3</td>
<td>This study</td>
</tr>
<tr>
<td>pEGKG-Rod1KR ([Rod1K235R K245R K264R K267R]) ([pJT4957])</td>
<td>GAL1prom-GST 2 µm URA3</td>
<td>This study</td>
</tr>
<tr>
<td>pEGKG-Rog3PPXY-less ([pJT5045])</td>
<td>GAL1prom-GST 2 µm URA3</td>
<td>This study</td>
</tr>
<tr>
<td>pEGKG-Rog3VASA ([pJT5046])</td>
<td>GAL1prom-GST 2 µm URA3</td>
<td>This study</td>
</tr>
<tr>
<td>pEGKG-Rog3PASA ([pJT5047])</td>
<td>GAL1prom-GST 2 µm URA3</td>
<td>This study</td>
</tr>
<tr>
<td>pEGKG-Rod14KR</td>
<td>GAL1prom-GST 2 µm URA3</td>
<td>This study</td>
</tr>
<tr>
<td>pEGKG-Rod1PPXY-less ([pJT4958])</td>
<td>GAL1prom-GST 2 µm URA3</td>
<td>This study</td>
</tr>
<tr>
<td>pEGKG-Rog3PPXY-less ([pJT4959])</td>
<td>GAL1prom-GST 2 µm URA3</td>
<td>This study</td>
</tr>
<tr>
<td>pEGKG-Rog3VASA ([pJT4960])</td>
<td>GAL1prom-GST 2 µm URA3</td>
<td>This study</td>
</tr>
<tr>
<td>pEGKG-Rog3PASA ([pJT4961])</td>
<td>GAL1prom-GST 2 µm URA3</td>
<td>This study</td>
</tr>
<tr>
<td>pEGKG-Rod1PPXY-less ([pJT4962])</td>
<td>GAL1prom-GST 2 µm URA3</td>
<td>This study</td>
</tr>
<tr>
<td>pEGKG-Rog3PPXY-less ([pJT5060])</td>
<td>GAL1prom-GST 2 µm URA3</td>
<td>This study</td>
</tr>
<tr>
<td>pEGKG-Rod1AQAKAA ([pJT4957])</td>
<td>GAL1prom-GST 2 µm URA3</td>
<td>This study</td>
</tr>
<tr>
<td>pRS313</td>
<td>LDB19prom CEN HIS3</td>
<td>164</td>
</tr>
<tr>
<td>pRS313-Ldb19</td>
<td>LDB19prom CEN HIS3</td>
<td>This study</td>
</tr>
<tr>
<td>pRS313-Ldb19PAPA ([pJT4963])</td>
<td>LDB19prom CEN HIS3</td>
<td>This study</td>
</tr>
<tr>
<td>pRS313-Ldb19PPXY ([pJT4964])</td>
<td>LDB19prom CEN HIS3</td>
<td>This study</td>
</tr>
<tr>
<td>pRS313-Ldb19PPXY-less ([pJT4965])</td>
<td>LDB19prom CEN HIS3</td>
<td>This study</td>
</tr>
<tr>
<td>pRS313-Ldb19PAIA ([pJT4966])</td>
<td>LDB19prom CEN HIS3</td>
<td>This study</td>
</tr>
<tr>
<td>pRS313-Ldb19K255KR ([pJT4967])</td>
<td>LDB19prom CEN HIS3</td>
<td>This study</td>
</tr>
<tr>
<td>pRS313-Ldb19K255KR ([pJT5061])</td>
<td>LDB19prom CEN HIS3</td>
<td>This study</td>
</tr>
<tr>
<td>pGEKW11-GST-Ste2297–431</td>
<td>T7 AMP</td>
<td>163</td>
</tr>
</tbody>
</table>

* Generated by site-directed mutagenesis with synthetic oligonucleotides containing the desired codon alterations and the corresponding codon in pRS313 vectors as the template.

* DNA of the corresponding gene was amplified from genomic DNA by PCR (66) and then cloned into pEGKG.

* Generated by site-directed mutagenesis with synthetic oligonucleotides containing the desired codon alterations and the corresponding LDB19 DNA inserted into pRS313 as the template.

Assessment of Rsp5 copurification with α-arrestins was performed as described previously (78). To assess the state of α-arrestin modification by ubiquitin in vivo, BJ5459 GEV cells carrying a plasmid vector (pEGKG) for the expression of GST-Ldb19 or GST-Ldb19 [K486R] were grown to mid-exponential phase and induced with 20 µM β-estradiol (final concentration) for 3 h. After harvesting by centrifugation, the cells were washed and frozen in liquid N2. Cell pellets were resuspended in 600 µl immunoprecipitation buffer (100 mM NaCl, 0.2% Triton X-100, 15 mM EGTA, 50 mM Tris [pH 7.4]) containing 5 mM N-ethylmaleimide (NEM) and protease inhibitors (1 tablet of cOmplete protease inhibitor cocktail [Roche Applied Science] per 15 ml) and lysed at 4°C by vigorous vortexing with ~1-g glass beads (0.5 mm; BioSpec Products). After clarification, GST-tagged proteins were recovered from equal volumes of these extracts by incubation with GST-agarose beads for 2 h at 4°C. Two washes with immunoprecipitation buffer containing 150 mM NaCl, liquid was removed by aspiration and the beads were resuspended in SDS-PAGE sample buffer, resolved by SDS-PAGE (6% acrylamide gel), and analyzed by immunoblotting.

In vitro ubiquitinylation. The ability of α-arrestins to serve as substrates for Rsp5-mediated ubiquitinylation in vitro was assessed by minor modifications of previous methods (79). Brieﬂy, GST-Rsp5 and a catalytically inactive mutant (GST-Rsp5C777A) were expressed in and puriﬁed from Escherichia coli and the GST tag removed by cleavage with commercial rhinovirus 3C protease (PreScission; GE Healthcare). Uba1 (E1) and

Judgment for 10 min and then incubated for 1 h either with vehicle alone (90% [vol/vol] ethanol and 10% [vol/vol] aqueous Tetra 20) at a designated concentration of 1 µg/ml (added from a 10-µg/ml stock dissolved in ET) prior to galactose induction. After further incubation for 3.5 h, the cells were harvested and lysed by vigorous vortexing with glass beads in radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1 mM dithiothreitol [DTT], 50 mM Tris–HCl [pH 7.4]) containing protease inhibitors (77). After brief centrifugation to remove unbroken cells and debris, the clarified extracts were incubated with glutathione-agarose beads (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom) for 2 h at 4°C and washed three times with 500 µl RIPA buffer. Bound proteins were eluted from the beads in SDS-PAGE sample buffer and resolved by SDS-PAGE, and the presence of Cna1-GFP and GST-Rod1 was analyzed by immunoblotting.

To assess in vivo phosphorylation of Rod1, pEGKG-Rod1 and pEGKG-Rod1AQAKAA were introduced into BY4741 and otherwise isogenic cni1Δ and cna1Δ cna1Δ2 derivatives by DNA-mediated transformation (65).Transformants were grown to mid-exponential phase in SC-2% raffinose. Prior to harvesting, the cultures were treated with 200 mM CaCl2 (final concentration) to activate calcineurin. Protein extracts were prepared by glass bead lysis in RIPA buffer containing both 600 mM NaCl (HS-RIPA) and 1 µg/ml FK506. After clarification by centrifugation, GST-Rod1 and GST-Rod1AQAKAA were collected from the lysates by binding to glutathione-agarose for 2 h at 4°C. The beads were washed twice with 500 µl HS-RIPA, and aliquots of the bead-bound proteins were incubated at 30°C for 45 min either in phosphate buffer alone (1 mM MnCl2, 10 mM NaCl, 2 mM DTT, 0.01% Brij-35, 50 mM HEPES [pH 7.5]) or in the same buffer containing 200 U of lambda phosphatase (New England BioLabs) in either the absence or presence of phosphatase inhibitors (10 mM Na3P2O7, 10 mM NaF, 0.4 mM NaVO3, 0.4 mM Na3VO4, and 0.1 mM glycerol-3-phosphate). Supernatant liquid was removed by aspiration. Bound protein was eluted at 37°C for 15 min in SDS-PAGE sample buffer, resolved by SDS-PAGE (6% acrylamide gel), and analyzed by immunoblotting.

In vitro ubiquitinylation. The ability of α-arrestins to serve as substrates for Rsp5-mediated ubiquitinylation in vitro was assessed by minor modifications of previous methods (79). Brieﬂy, GST-Rsp5 and a catalytically inactive mutant (GST-Rsp5C777A) were expressed in and puriﬁed from Escherichia coli and the GST tag removed by cleavage with commercial rhinovirus 3C protease (PreScission; GE Healthcare). Uba1 (E1) and
UbC1 (E2) were purified from yeast as previously described (80). Plasmid DNA (1 µg) of a vector (pME32) carrying the open reading frames for either ROD1, ROG3, LDB19, or cognate derivatives containing mutated versions of one or more of their P/VPXY motifs was used as the template to generate the corresponding [35S]methionine ([35S]Met)-labeled protein by coupled in vitro transcription-translation using the TNT quick coupled system (Promega, Sunnyvale, CA). The resulting translation mixture was treated with a final concentration of 10 mM NEM for 15 min at room temperature to inactivate the deubiquitinating enzymes and ubiquitin-conjugating enzymes in the rabbit reticulocyte lysate (81). After quenching unreacted NEM with a final concentration of 20 mM DTT, portions (42 µl) of each in vitro translation product were added to a reaction mixture (60 µl final volume) containing the following components at the indicated final concentrations: 600 µM ubiquitin (Sigma, St. Louis, MO), 0.5 mM ATP, 220 nM UbC1, and 3 µM UbC1. An aliquot (10 µl) was removed as the zero time point, and the reaction was initiated immediately thereafter by addition of GST-Rps5 or, as a control, GST-Rps5C777A (100 nM final concentration). Additional aliquots were removed at 5, 15, and 30 min, and each was quenched by immediate mixing with 8X concentrated SDS-PAGE sample buffer followed by incubation at 37°C for 15 min. The resulting products were resolved by SDS-PAGE (4.5% acrylamide gel). After drying the gel, the radioactive species were detected by exposure to a phosphorimager screen for 2 h followed by visualization on a Typhoon FLA 7000 laser scanner (GE Healthcare).

Fluorescence microscopy. Imaging of Ste2-mCherry was performed as described previously (21). Imaging of Ste2-GFP was carried out as described previously (82), using an inverted fluorescence microscope (Axiovert 200; Carl Zeiss GmbH, Jena, Germany) equipped with a charged-couple device (CCD) camera (Sensicam; PCO-Tech, Inc., Romulus, MI), an X-Cite 120 PC fluorescence illumination system (Exfo Ltd., Quebec, Canada), and a 100X (1.4-numerical-aperture) Plan-Apochromat objective. The day before examining the cells, cultures of the strains to be tested were streaked at a low dilution on plates containing minimal medium (yeast nitrogen base), supplemented with appropriate nutrients to select for plasmid maintenance (if needed), and grown overnight at 30°C, selecting small colonies in mid-exponential-phase growth (as judged by the presence of cells in all cell cycle stages in the population). Single colonies were suspended in 2.75 µl of the same medium on the surface of a glass slide under a coverslip immediately before imaging at room temperature using Slidebook software (v5.0.0.32; Olympus America, San Jose, CA) with identical imaging parameters (2 by 2 binning, 500-ms exposure) for all samples. Images were processed using ImageJ (v1.48b) with identical maximum and minimum intensity values applied to all images.

RESULTS

Ldb19, Rod1, and Rog3 function independently from Bar1 and Sst2. Some α-arrestins have been implicated in aspects of vesicle-mediated transport other than endocytosis (86). Therefore, it was possible that lack of Ldb19, Rod1, and/or Rog3 enhances pheromone response simply by impeding the action of known negative regulators of pheromone signaling that require membrane trafficking (such as the secreted α-arrestin protease Bar1/Sat1) or membrane interaction (such as the receptor- and G-protein-associated RGS protein Sst2). However, compared to an otherwise isogenic bar1Δ mutant, an ldb19Δ bar1Δ double mutant, a rod1Δ rog3Δ bar1Δ triple mutant, and an ldb19Δ rod1Δ rog3Δ bar1Δ quadruple mutant exhibited significantly greater pheromone sensitivity at every concentration of α-factor tested (Fig. 1C). Because Bar1 is absent from these cells, the observed additive behavior shows that the increase in pheromone sensitivity has nothing to do with preventing efficient Bar1 secretion or function. Likewise, compared to an otherwise isogenic sst2Δ mutant, an ldb19Δ sst2Δ double mutant, a rod1Δ rog3Δ sst2Δ triple mutant, and an ldb19Δ rod1Δ rog3Δ sst2Δ quadruple mutant exhibited greater pheromone sensitivity at every concentration of α-factor tested (Fig. 1D). Again, this additive effect indicates that the absence of Ldb19, Rod1, and/or Rog3 does not cause increased pheromone sensitivity by interfering with Sst2 action. Because these α-arrestins negatively regulate pheromone response by a mechanism(s) independent from those exerted by either Bar1 or Sst2, we used bar1Δ and sst2Δ cells as sensitized backgrounds in which to further characterize the function of Ldb19, Rod1, and Rog3.

Rod1 and Rog3 promote adaptation. The increased pheromone sensitivity observed when Ldb19 or Rod1 and Rog3 are absent is consistent with a role for these proteins in receptor downregulation and/or signal damping. If so, overexpression of such negative regulators should stimulate recovery from pheromone signaling. The agar diffusion bioassay provides a convenient means to assess desensitization, because adaptation is readily monitored by examining the rate and extent of the resumption of cell growth inside the initial zone of pheromone-imposed G1 arrest. Normally, in the absence of Sst2, once cells are exposed to pheromone, little or no adaptation is observed even after prolonged incubation (19, 21); only occasional papillae arise (see, for
example, Fig. 1E, left), which represent rare cells with a selective advantage (they became pheromone resistant by acquiring a spontaneous mutation) (87). Remarkably, overexpression of either GST-Rod1 or GST-Rog3 in sst2Δ cells caused turbid halos diagnostic of adaptation, recovery from G1 arrest, and a return to cell growth (Fig. 1E). Similar turbid halos have been observed when other negative regulators of pheromone response are overexpressed (88). Although both α-arrestins are produced at an equi-
alent level (Fig. 1F), the adaptation-promoting effect of GST-Rog3 was reproducibly more potent than that of GST-Rod1.

In contrast, overexpression of GST-Ldb19, or untagged Ldb19 (data not shown), in sst2Δ cells did not promote adaptation (Fig. 1E). In multiple trials, GST-Ldb19 expression was always lower than that of GST-Rod1 and GST-Rog3 (Fig. 1F). Therefore, it was formally possible that the level of GST-Ldb19 achieved was insufficient to support adaptation. However, like GST-Ldb19, other GST-tagged α-arrestins that are expressed at a level comparable to or even higher than that of GST-Rod1 and GST-Rog3 (e.g., GST-Art5 and GST-Csr2/Art8) also failed to promote adaptation in sst2Δ cells (data not shown). These observations argue, first, that the adaptation-promoting effects of Rod1 and Rog3 are specific. Second, and tellingly, because α-factor is present continuously in these assays, these observations indicate that Rod1 and Rog3 act on the pheromone receptor in its ligand-occupied conformation, whereas Ldb19 is unable to do so. These data also show that Rod1 and Rog3 act at a different level and/or via a different mechanism than Ldb19. Of course, some other factor may be rate limiting for the Ldb19-promoted adaptation pathway (such as a stimulus that results in more receptor misfolding or unfolding [see Discussion]).

Absence of α-arrestins and mating pathway signaling. Pheromone-imposed G1 arrest is one measure of mating pathway function. We used two other independent methods to confirm that absence of α-arrestins leads to an enhanced pheromone response. First, under conditions where the concentration of α-factor remains essentially constant (cells carried a bar1Δ mutation), we compared the kinetics of activation (via dual phosphorylation) of Fus3 (89), the mating pheromone response-pathway-specific MAPK (12, 90), after exposing an otherwise wild-type strain and an ldb19Δ rod1Δ rog3Δ triple mutant to α-factor (Fig. 2A). As observed in the halo bioassay, there was more efficacious and sustained signaling in cells lacking the three α-arrestins than in the control cells, although the effect was relatively modest. Fus3 itself is known to be a pheromone-induced gene product (27, 89, 91), and the level of Fus3 increased in control cells and cells lacking Ldb19, Rod1, and Rog3. However, the fraction of Fus3 in its activated state was higher and more persistent in the cells lacking the three α-arrestins than in the control cells (Fig. 2B).

Another standard used to measure pheromone response is induction of the pheromone-responsive gene FUS1 (92). We quantified the expression level of an integrated FUS1prom·eGFP reporter gene (73) in bar1Δ cells that were otherwise wild type or carried the ldb19Δ rod1Δ rog3Δ mutations using flow cytometry. It was shown previously that basal signaling in the mating pheromone response pathway arises largely from stochastic spontaneous dissociation of receptor-heterotrimeric G protein complexes (93, 94). An increase in receptor level in the PM shifts the equilibrium toward complex formation and reduces basal signaling (21, 23). For this reason, if the α-arrestin-deficient cells internalize Ste2 less efficiently, then basal signaling should be reduced. Consistent with this prediction, basal expression was significantly lower in cells lacking Ldb19, Rod1, and Rog3 than in control cells (Fig. 2C). Nonetheless, after exposure to α-factor, the level of FUS1 expression achieved in the α-arrestin-deficient cells was nearly equivalent to that in the wild type. Therefore, the induction ratio for the FUS1 reporter was ∼3-fold higher in the ldb19Δ rod1Δ rog3Δ bar1Δ cells than in bar1Δ cells (Fig. 2D). Thus, as judged by three different assays, pheromone signaling is more sustained in cells lacking Ldb19, Rod1, and Rog3 than in control cells, consistent with loss of negative regulation of the pathway.

Efficient Ste2 internalization requires Ldb19, Rod1, and Rog3. In S. cerevisiae, various classes of integral polytopic PM proteins can be endocytosed by clathrin-dependent (95) and clathrin-independent (96) routes. Caveolin-like structures in yeast (“eisosomes”) (97, 98) do not appear to be sites of endocytosis (99, 100) and may even protect cargo from internalization (101). The evidence that various yeast α-arrestins are necessary for efficient internalization of distinct PM-localized nutrient permeases is compelling (57–59, 102). If Ldb19, Rod1, and/or Rog3 contributes to GPCR downregulation by any endocytic route, one would expect to detect some Ste2 accumulation at the PM in cells lacking one or more of these α-arrestins. To examine receptor localization, we first used full-length Ste2 tagged at its C terminus with mCherry (21). We showed before that in otherwise wild-type cells (even in the absence of pheromone), the red fluorescence resides mainly in the vacuole (21), presumably because mCherry of the mCherry chromophore is slow relative to the rate of constitutive endocytosis and/or because the mCherry portion of the fusion persists due to its slow degradation. Indeed, in wild-type cells, the fluorescent signal was confined almost exclusively to the vacuole (Fig. 3A, left), whereas in isogenic ldb19Δ single, rod1Δ rog3Δ double, and ldb19Δ rod1Δ rog3Δ triple mutants, fluorescence was discernible at the PM (Fig. 3A).

To confirm that these conclusions were not dependent on the chromophore used to monitor Ste2 localization and to quantify the results, the same analysis was carried out using cells expressing full-length Ste2 tagged at its C terminus with eGFP. Again, the fluorescent signal in the parental strain was confined largely to the vacuole in virtually every cell, whereas the vast majority of the 9αarr, ldb19Δ, rod1Δ rog3Δ, and especially ldb19Δ rod1Δ rog3Δ cells exhibited readily detectable PM fluorescence (Fig. 3B). In marked contrast, the level of Ste2-GFP fluorescence at the PM was unchanged in mutants lacking other α-arrestins, e.g., aly1Δ aly2Δ (Fig. 3B). These visual impressions were corroborated by averaging the intensity of PM fluorescence determined by taking multiple line scans across large numbers of cells in each field (Fig. 3C).

Together, these results are consistent with Ldb19, Rod1, and Rog3 acting as negative regulators of pheromone-initiated signaling by promoting efficient Ste2 internalization.

The ubiquitin ligase Rsp5 is required for Ldb19 and Rod1 action. Aside from an N-terminal arrestin fold that mediates interaction with specific targets, a defining hallmark of an α-arrestin is that its C-terminal sequence contains multiple copies of PPXY (and/or variants thereof, such as LPXY and VPXY) (Fig. 4A). The PPXY motifs serve as docking sites for binding three tandem WW domains (103) present in the HECT family ubiquitin ligase (E3) Rsp5 (104, 105). In this way, α-arrestins act as adaptors that link PM substrates that lack endogenous PPXY motifs to Rsp5 (99, 100) and may even protect cargo from internalization (101). The evidence that various yeast α-arrestins are necessary for efficient internalization of distinct PM-localized nutrient permeases is compelling (57–59, 102). If Ldb19, Rod1, and/or Rog3 contributes to GPCR downregulation by any endocytic route, one would expect to detect some Ste2 accumulation at the PM in cells lacking one or more of these α-arrestins. To examine receptor localization, we first used full-length Ste2 tagged at its C terminus with mCherry (21). We showed before that in otherwise wild-type cells (even in the absence of pheromone), the red fluorescence resides mainly in the vacuole (21), presumably because mCherry of the mCherry chromophore is slow relative to the rate of constitutive endocytosis and/or because the mCherry portion of the fusion persists due to its slow degradation. Indeed, in wild-type cells, the fluorescent signal was confined almost exclusively to the vacuole (Fig. 3A, left), whereas in isogenic ldb19Δ single, rod1Δ rog3Δ double, and ldb19Δ rod1Δ rog3Δ triple mutants, fluorescence was discernible at the PM (Fig. 3A).

To confirm that these conclusions were not dependent on the chromophore used to monitor Ste2 localization and to quantify the results, the same analysis was carried out using cells expressing full-length Ste2 tagged at its C terminus with eGFP. Again, the fluorescent signal in the parental strain was confined largely to the vacuole in virtually every cell, whereas the vast majority of the 9αarr, ldb19Δ, rod1Δ rog3Δ, and especially ldb19Δ rod1Δ rog3Δ cells exhibited readily detectable PM fluorescence (Fig. 3B). In marked contrast, the level of Ste2-GFP fluorescence at the PM was unchanged in mutants lacking other α-arrestins, e.g., aly1Δ aly2Δ (Fig. 3B). These visual impressions were corroborated by averaging the intensity of PM fluorescence determined by taking multiple line scans across large numbers of cells in each field (Fig. 3C).

Together, these results are consistent with Ldb19, Rod1, and Rog3 acting as negative regulators of pheromone-initiated signaling by promoting efficient Ste2 internalization.

The ubiquitin ligase Rsp5 is required for Ldb19 and Rod1 action. Aside from an N-terminal arrestin fold that mediates interaction with specific targets, a defining hallmark of an α-arrestin is that its C-terminal sequence contains multiple copies of PPXY (and/or variants thereof, such as LPXY and VPXY) (Fig. 4A). The PPXY motifs serve as docking sites for binding three tandem WW domains (103) present in the HECT family ubiquitin ligase (E3) Rsp5 (104, 105). In this way, α-arrestins act as adaptors that link PM substrates that lack endogenous PPXY motifs to Rsp5 (99, 100), which catalyzes substrate ubiquitinylation, thereby marking cargo for endocytosis (57, 58, 102, 108). The primary structure of Ste2 has no PPXY motif or variant thereof in its cytoplasmic tail (21, 41, 55).

To determine whether Rsp5 recruitment is required for negative regulation of pheromone signaling by Ldb19, Rod1, or Rog3, we first mutated the two PPXY motifs in each of these α-arrestins to PAXA and then used two different methods to assess whether
these point mutations successfully abrogated interaction with Rsp5. In the case of Rog3, we also mutated its VPXY motif because an identical motif in H9251-arrestin Rim8/Art9 was shown to mediate Rsp5 association (109). As observed for other H9251-arrestins (57, 78, 108), we found that Rsp5 efficiently copurified with GST-Ldb19, GST-Rod1, and GST-Rog3 (and not with a GST control), whereas the corresponding PPXY-less (or, in the case of Rog3, P/VPXY-less) mutants exhibited dramatic decreases in the amount of Rsp5 recovered: Ldb19, 93% reduction; Rod1, 71% reduction; and Rog3, 83% reduction (Fig. 4B). The residual amount of Rsp5 observed for the mutants does not represent residual interaction but rather nonspecific background, because no further reduction below this threshold was observed when a complete C-terminal truncation [Rog3(Δ400-733)] was examined (Fig. 4B). As an independent and more sensitive indicator of the ablation of Rsp5 interaction by these point mutations, we took advantage of the fact that this E3 efficiently ubiquitinylates endogenous Lys residues in the H9251-arrestins (57, 58, 102, 108). We found that each of the three H9251-arrestins, prepared by coupled in vitro transcription and translation, served as an efficient substrate for Rsp5-dependent ubiquitinylation, as judged by complete conversion of the starting material to a broad spectrum of polyubiquitinylated, slower-mobility species (Fig. 4C). In control reactions incubated with Rsp5C777A, no detectable ubiquitinylated species were observed (data not shown). As expected, the PPXY point mutations abolished Rsp5-dependent modification of each H9251-arrestin almost entirely (Fig. 4C). Thus, as judged by two different criteria, the point mutants we generated in Ldb19, Rod1, and Rog3 clearly compromise their interaction with Rsp5.

We then used a complementation test to determine if Rsp5...
otherwise isogenic promoter at the endogenous Ldb19 and/or Rod1 and Rog3. (A) A copy of Ste2-mCherry (as the sole copy of this receptor) from the native promoter on a low-copy-number CEN plasmid into the ldb19Δ bar1Δ strain reduced the halo size to that observed in the bar1Δ strain, whereas even single PAXA point mutations and the double (PPXY-less) mutant were unable to do so (Fig. 5A and B). Thus, the ability of Ldb19 to down-modulate signaling requires its interaction with Rsp5.

To determine if Rsp5 association is required for either Rod1 or Rog3 function in signal dampening, we exploited their ability to promote recovery and adaptation in ssr2Δ cells exposed to pheromone, as described earlier (Fig. 1E). Overexpression of wild-type ROD1 (as a GST fusion from the GAL1 promoter on a high-copy-number 2μm DNA plasmid) in ssr2Δ cells produced a turbid halo and reduced halo size, whereas even single PAXA point mutations and the double (PPXY-less) mutant were unable to do so (Fig. 5C, top), even though the Rod1 mutants were expressed at a level as high as or higher than that of the wild-type protein (Fig. 5D). Thus, as for Ldb19, the ability of Rod1 to downregulate signaling also requires its interaction with Rsp5.

Strikingly, as judged by the same assay, overexpression in ssr2Δ cells of wild-type Rog3, single PAXA mutants, and the double PPXY-less mutant (Fig. 5C, lower), and even the P/VPXY-less derivative lacking all three of its Rsp5-binding motifs (Fig. 5E) produced turbid halos, even though the P/VPXY mutations eliminated the ability of Rog3 to associate with (Fig. 4B) and be modified (Fig. 4C) by Rsp5. Hence, in contrast to Ldb19 and Rod1, the ability of Rog3 to squelch pheromone signaling does not obligatorily require association with Rsp5. However, it was possible that Rog3 associates with its closest paralog, Rod1, to form a heterodimer (or higher oligomer), and Rsp5 recruited by this partner overcomes the loss of the P/VPXY motifs in Rog3. However, this possibility was eliminated (as well as effects of Rog3 mediated through Ldb19) because overexpressed Rog3 and its PPXY-less derivative still promoted efficient adaptation in cells lacking endogenous Ldb19, Rod1, and Rog3 (Fig. 5E). Thus, negative regulation of pheromone signaling by Rog3 does not require interaction, either direct or indirect, with Rsp5. Indeed, just the arrestin fold domain at the N terminus of Rog3 is sufficient to promote desensitization, because overexpression of a truncation mutant, Rog3Δ400-733), yielded halos just as turbid, if not more so, than those of full-length Rog3. In contrast, Ldb19Δ447-818 and Rod1(402-837) were nonfunctional (data not shown). Therefore, Rog3 can act on the receptor to negatively regulate signaling by a mechanism that is independent of the other two α-arrestins and Rsp5.

Ubiquitylation of Ldb19, Rod1, and Rog3 is dispensable for signal dampening. It has been demonstrated that the cognate α-arrestin itself becomes ubiquitinylated during the process of recruiting Rsp5 to nutrient permeases (57, 58, 78, 102, 108). Moreover, the Lys residues in Ldb19 and Rod1 that are ubiquitinylation is required for Ldb19 function in pheromone signal dampening. As described earlier (Fig. 1A), ldb19Δ bar1Δ cells display a halo of pheromone-induced growth inhibition that is larger than that of isogenic bar1Δ cells (Fig. 5A). Reintroduction of wild-type LDB19 (expressed from its native promoter on a low-copy-number CEN plasmid) into the ldb19Δ bar1Δ strain reduced the halo size to that observed in the bar1Δ strain, whereas even single PAXA point mutations and the double (PPXY-less) mutant were unable to do so (Fig. 5A and B). Thus, the ability of Ldb19 to down-modulate signaling requires its interaction with Rsp5.
As previously mentioned, P/VPXY motifs in Ldb19, Rod1, and Rog3 are required for Rsp5 binding and Rsp5-mediated ubiquitinylation. (A) Schematic depiction of the primary structures of Ldb19, Rod1, and Rog3. Residues (numbers below each bar) comprising the arrestin fold (blue) in Ldb19 according to reference 57 and in Rod1 and Rog3 as predicted by the Phyre2 modeling algorithm (166) are shown, and positions of the consensus Rsp5-binding motifs, PPXY and VPXY, and reported ubiquitinylated Lys residue(s) (black lines) are indicated. (B) Cultures of a GEV derivative of vacuolar protease-deficient strain BJ5459 (160) expressing the indicated α-arrestin or the derived P/VPXY substitution mutant (as a GST fusion from the GAL promoter) were grown to mid-exponential phase. Protein expression was induced with β-estradiol, and the cells were harvested by centrifugation and ruptured by vigorous vortex mixing with glass beads. GST fusions in the resulting extracts (pink dots) were captured by binding to glutathione-agarose beads. After washing, the bound proteins were resolved by SDS-PAGE and analyzed by immunoblotting with the indicated antibodies. MW, molecular weight in thousands; Long Exp., long exposure. (C) The Rsp5-catalyzed and time-dependent ubiquitinylation of an [35S]Met-labeled α-arrestin or its cognate PPXY substitution mutant (pink dots), prepared by coupled in vitro transcription-translation, was performed and analyzed using a phosphorimager as described in Materials and Methods. add., addition; Ub, ubiquitinylation.

Our PPXY mutants of Ldb19, Rod1, and Rog3 should prevent ubiquitinylation of both the α-arrestin and its cargo, because they cannot bind Rsp5. Hence, the failure of PPXY-less versions of Ldb19 (Fig. 5A) and Rod1 (Fig. 5C) to promote signal dampening could be due to defective ubiquitinylation rather than an inability to deliver Rsp5 to the receptor (and/or other targets). To distinguish between these possibilities, we generated K-to-R mutations in the ubiquitinylated sites in Ldb19 (56) and Rod1 (64) to sele-
tively disrupt their ubiquitylation but leave Rsp5 binding intact. First, we generated GST-Ldb19(K486R) (57) and confirmed by pulldown, SDS-PAGE analysis, and immunoblotting that GST-Ldb19 is ubiquitylated in vivo, whereas GST-Ldb19(K486R) is not (Fig. 6A, upper). Strikingly, as judged by the complementation test, reintroduction of either wild-type LDB19 or derivatives containing point mutations in each or both of its PPXY motifs binding was determined as described for Fig. 1A, except that the medium was SC-His. (B) Quantification and statistical analysis of the change in halo diameter, determined as described for panel A, for independent replicate experiments (n = 3). The average halo diameter for control cells was set at 100%, and the halo sizes for each mutant were normalized to that of the control. Error bars indicate ± SEM; *, P < 0.0001. (C) Pheromone sensitivity of cultures of MATa sst2Δ GEV cells (JT5919) overexpressing either Rod1 or Rog3, as indicated, or the derived PPXY point mutants under the control of the GAL promoter on a high-copy-number URA3-marked 2μ DNA plasmid was determined as described in the legend to Fig. 1E. Data from one representative experiment (n = 5) are shown. (D) Proteins from whole-cell extracts of the cells shown in panel C were prepared, resolved by SDS-PAGE, and analyzed by immunoblotting with the indicated antibodies. Data from one representative experiment (n = 5) are shown. MW, molecular weight in thousands. (E) Pheromone sensitivity of cultures of MATa sst2Δ GEV cells (JT5919) or an ldb19Δ rod1Δ rog3Δ sst2Δ GEV derivative (JT6716) overexpressing either Rog3 or the derived cognate P/VPPXY point mutants, under the control of the GAL promoter on a high-copy-number URA3-marked 2μ DNA plasmid, was determined as described for panel C. Data from one representative experiment (n = 3) are shown. (F) Confirmation of protein expression, as described for panel D. Data from one representative experiment (n = 3) are shown.

FIG 5 Ldb19 and Rod1, but not Rog3, require Rsp5 binding to downregulate pheromone signaling. (A) Pheromone sensitivity of a MATa bar1Δ strain (JT5915) and an otherwise isogenic ldb19Δ bar1Δ derivative (JT5916) carrying either empty vector (HIS3-marked CEN plasmid) or the same vector expressing wild-type LDB19 or derivatives containing point mutations in each or both of its PPXY motifs binding was determined as described for Fig. 1A, except that the medium was SC-His. (B) Quantification and statistical analysis of the change in halo diameter, determined as described for panel A, for independent replicate experiments (n = 3). The average halo diameter for control cells was set at 100%, and the halo sizes for each mutant were normalized to that of the control. Error bars indicate ± SEM; *, P < 0.0001. (C) Pheromone sensitivity of cultures of MATa sst2Δ GEV cells (JT5919) overexpressing either Rod1 or Rog3, as indicated, or the derived PPXY point mutants under the control of the GAL promoter on a high-copy-number URA3-marked 2μ DNA plasmid was determined as described in the legend to Fig. 1E. Data from one representative experiment (n = 5) are shown. (D) Proteins from whole-cell extracts of the cells shown in panel C were prepared, resolved by SDS-PAGE, and analyzed by immunoblotting with the indicated antibodies. Data from one representative experiment (n = 5) are shown. MW, molecular weight in thousands. (E) Pheromone sensitivity of cultures of MATa sst2Δ GEV cells (JT5919) or an ldb19Δ rod1Δ rog3Δ sst2Δ GEV derivative (JT6716) overexpressing either Rog3 or the derived cognate P/VPPXY point mutants, under the control of the GAL promoter on a high-copy-number URA3-marked 2μ DNA plasmid, was determined as described for panel C. Data from one representative experiment (n = 3) are shown. (F) Confirmation of protein expression, as described for panel D. Data from one representative experiment (n = 3) are shown.
adaptation assay, overexpression of either wild-type ROG3 or ROG3<sup>K48R</sup> in <i>sst2</i>Δ cells produced turbid halos (Fig. 5D). Thus, the ability of Rog3 to squelch signaling requires neither its Rsp5 interaction (Fig. 5C and E) nor, presumably, its own ubiquitinylation (assuming that, as in Rod1, the 4K-to-R mutations eliminate Rog3 ubiquitinylation).

Ldb19, Rod1, and Rog3 associate with the C-terminal cytosolic tail of Ste2. Extraction of the receptor from membranes requires addition of detergent (21, 110), which might cause misfolding and disrupt protein-protein interactions. Cumulative evidence indicates that one of the most dramatic changes that occurs in the receptor upon pheromone binding <i>in situ</i> is a conformational alteration that makes its C-terminal extension (cytosolic tail) more susceptible to attack by exogenously added trypsin (111), to greatly enhanced Yck1- and Yck2-dependent phosphorylation (21, 37, 38, 41), and to overt Rsp5-dependent ubiquitinylation at multiple sites (41, 55). These findings suggest that Ldb19, Rod1, and/or Rog3 interact with this same region of the receptor once it becomes exposed, facilitating Rsp5-dependent modification. Hence, we examined the ability of these <i>α</i>-arrestins to interact with a purified soluble version of the 135-residue C-terminal extension of Ste2 [GST-Ste2<sup>297-431</sup>] bound to beads. Moreover, because we demonstrated (Fig. 6) that ubiquitinylation of Ldb19, Rod1, and Rog3 is not required for their actions <i>in vivo</i>, it was not necessary to prepare ubiquitin-decorated versions of each molecule to analyze their ability to associate with the Ste2 tail <i>in vitro</i>. Indeed, we found that <sup>35</sup>S-labeled Ldb19, Rod1, and Rog3 reproducibly bound better to the C-terminal tail of Ste2 than to the GST control (Fig. 7A and B). Other <i>α</i>-arrestins (e.g., Aly1/Art6) and Art5 (data not shown) did not show any increase above the level of the control (Fig. 7A and B). Moreover, consistent with the fact that its overexpression promoted recovery of <i>sst2</i>Δ cells,
we found that Rog3(Δ400-733) retained the ability to bind to the Ste2 cytosolic tail (Fig. 7C and D). Thus, Ldb19, Rod1, and Rog3 fulfill all of the requirements of adaptors for the downregulation, modification, and endocytosis of Ste2; they associate specifically with Rsp5, bind preferentially to the C-terminal tail of Ste2, and reduce Ste2 abundance at the cell surface.

**Rod1 must be dephosphorylated by calcineurin to promote signal desensitization.** Endocytosis of PM nutrient transporters is regulated by metabolic conditions. For example, under glucose-replete conditions, the lactate transporter Jen1 is internalized in a Rod1- and Rsp5-dependent manner (102). Upon glucose limitation, however, Snf1 (yeast AMPK) is activated (112) and phosphorylates Rod1 (113), inhibiting its function. This event allows Jen1 to accumulate in the PM (102). Similarly, we noted during the course of our studies that overexpression of Rod1 on galactose medium, another condition under which Snf1 is active (112), failed to promote adaptation of sst2Δ cells (data not shown). These data suggest that Rod1 phosphorylation blocks its association with Ste2. Glc7 (yeast phosphoprotein phosphatase 1) bound to targeting subunit Reg1 has been implicated in the dephosphorylation of Rod1 required for Jen1 internalization (102). For several reasons, we suspected that the role of Rod1 in promoting pheromone signal desensitization is a feedback mechanism that requires dephosphorylation controlled by Ca²⁺ and the Ca²⁺-activated phosphatase CN (yeast phosphoprotein phosphatase 2B). First, elevated Ca²⁺ influx is a consequence of pheromone action (114). Second, we showed previously that optimal CN function is required for efficient adaptation after MATα cells are exposed to α-factor (115). Third, we demonstrated recently that CN-mediated dephosphorylation of another α-arrestin, Aly1, is required to promote endocytosis of the aspartate and glutamate transporter Dip5 (78). Finally, like Aly1, Rod1 was identified in a global screen for CN substrates and found to be efficiently dephosphorylated by CN in vitro (116).

CN is recruited to substrates that contain a conserved docking motif, i.e., PXIXIT and variants thereof (117). Indeed, compared to a GST control, a GFP-tagged derivative of the CN catalytic subunit Cna1 copurified with GST-Rod1 from yeast extracts, whereas an equivalent level of GST-Rod1AQAKAA (in which the sole PXIXIT motif, 545PQIKIE550, was mutated) exhibited a dra-
matic decrease (>90%) in the amount of Cna1-GFP recovered (Fig. 8A). Consistent with this site being required for efficient CN-dependent dephosphorylation in vivo, we found that GST-Rod1<sup>AQAKAA</sup>-derived species were clearly phosphorylated, because they collapsed to a single faster-mobility band upon treatment with λ phosphatase (Fig. 8B, upper). Likewise, GST-Rod1 also migrated as two distinct bands when the cells were treated with the potent CN-specific inhibitor FK506 (Fig. 8B, upper) or in mutants lacking either the Ca<sup>2+</sup>-binding regulatory subunit (Cbn1) of CN (Fig. 8B, middle) or both of its catalytic subunit isoforms (Cna1 and Cna2) (Fig. 8B, lower). These GST-Rod1-derived bands co-migrated with those observed for GST-Rod1<sup>AQAKAA</sup>. These species also collapsed to the same single faster-mobility species after λ phosphatase treatment (Fig. 8B). These findings demonstrate that efficient dephosphorylation of Rod1 in vivo requires CN action.

In agreement with the conclusion that phosphorylation of Rod1 at these CN-sensitive sites blocks its ability to promote desensitization after pheromone response, we observed less turbid halos in the adaptation assay when GST-Rod1<sup>AQAKAA</sup> was overexpressed in ste2Δ cells than with GST-Rod1 (Fig. 8C). Most tellingly, in cells lacking functional CN (either cna1Δ or cna2Δ mutants), overexpression of GST-Rod1 was unable to promote any detectable adaptation (Fig. 8D). In contrast, adaptation promoted by GST-Rog3 remained unaffected (Fig. 8D), even though GST-Rod1 and GST-Rog3 were expressed at an equivalent level in both wild-type cells and the CN-deficient mutants (Fig. 8E). We conclude that dephosphorylation mediated by the Ca<sup>2+</sup>-dependent phosphatase CN is essential for Rod1 to downregulate pheromone signaling.

**DISCUSSION**

It has been presumed that the ligand-induced phosphorylation (37, 38) and Rsp5-dependent ubiquitinylatation of multiple Lys residues (41, 42, 55) in the C-terminal cytosolic tail of Ste2, and the ensuing increased rate of receptor endocytosis (33, 34), contributes to signal dampening and recovery from pheromone response. Consistent with this notion, truncations that eliminate the C-terminal tail of Ste2 prevent receptor internalization and result in increased pheromone sensitivity and marked prolongation of pheromone-imposed G<sub>i</sub> arrest (37, 118). However, we now know that the C-terminal tail of Ste2 is also the primary binding site for the RGS protein Sst2 (121, 122) more efficiently than they are delivered from the Golgi compartment to the PM via its N-terminal lipid-binding C2 domain (119) but interacts with the substrates it modifies via the binding of its three tandem WW domains (103) to PPXY motifs in those targets (57, 120), it was a conundrum as to how Rsp5 recognized Ste2 as a substrate, since this receptor lacks any sequence elements resembling PPXY. The discovery that members of the α-arrestin family of adaptor proteins serve as intermediaries to recruit Rsp5 to nutrient permeases that also lack endogenous PPXY motifs suggested that members of the α-arrestin class of proteins function as the molecular matchmakers for delivering Rsp5 to Ste2.

Indeed, as documented here, three of the 14 known yeast α-arrestins contribute to signal downregulation at the receptor level, as judged by multiple independent criteria. First, lack of either Ldb19 or the paralogous pair Rod1 and Rog3 increases pheromone sensitivity, even in cells that possess all of the other demonstrated mechanisms for recovery from pheromone response. Second, simultaneous absence of Ldb19, Rod1, and Rog3 results in even greater pheromone sensitivity, comparable to that of a cell in which 9 α-arrestins (including Ldb19, Rod1, and Rog3) are absent. Third, among all of the α-arrestins tested, only Rod1, Rog3, and Ldb19 exhibited preferential binding to the site of Rsp5-dependent modification in Ste2, its cytosolic C-terminal tail. Phosphorylation of purified GST-Ste2<sub>tail</sub> with recombinant casein kinase I neither enhanced nor inhibited binding of Ldb19, Rod1, or Rog3 (A. F. O’Donnell, unpublished results), indicating that, rather than creating epitopes for recruitment of these adaptors, phosphorylation simply assists in locking the tail in a solvent-accessible state after it is exposed by the pheromone-induced change in receptor conformation. Fourth, in cells expressing fluorescent derivatives of Ste2, PM accumulation of the receptor occurs in ldb19Δ cells, in rod1Δ rod3Δ cells, and especially in ldb19Δ rod1Δ rod3Δ (and sst2Δ) cells, and it was not observed in control cells or in any other single or multiple α-arrestin deletion mutants.

The bulk of either mCherry- or eGFP-tagged Ste2 resided in the vacuole under all circumstances. This behavior suggests that a large fraction of these chimeras can be recognized as incorrectly folded (and/or improperly glycosylated) and shunted to the vacuole via the Golgi body-to-endosome quality-control pathway (121, 122) more efficiently than they are delivered from the Golgi compartment to the PM. Therefore, the amount of these fluorescently tagged proteins that is properly folded and inserted into the PM may be a minority of the total synthesized. Moreover, the Golgi body-to-endosomal shunt is known to depend on decoration of cargo by ubiquitin (123), making it problematic to analyze the change in modification state of our Ste2 constructs due solely to the presence or absence of Ldb19 and/or Rod1 and Rog3. Nevertheless, the observed increase in the amount of tagged Ste2 at the PM in ldb19Δ, rod1Δ rod3Δ, and ldb19Δ rod1Δ rod3Δ cells is consistent with their role in mediating the Rsp5-dependent endocytosis of the receptor.

Our results also show that the three α-arrestins contribute to Ste2 downregulation in discrete ways (Fig. 9). First, although paralogs Rod1 and Rog3 may have some overlapping role (be-
Rod1-mediated desensitization requires calcineurin-dependent dephosphorylation. (A) A single PXIXIT motif mediates CN-Rod1 interaction. Cultures of strain JRY11, which produces Cna1-GFP from the native CNA1 promoter at the endogenous CNA1 locus on chromosome XII, and also expressing, as indicated, either GST alone, GST-Rod1, or GST-Rod1^{AQAKAA}, were grown to mid-exponential phase, harvested, and lysed, and proteins in the resulting extracts were captured on glutathione-agarose beads, resolved by SDS-PAGE, and analyzed with the indicated antibodies. MW, molecular weight in thousands. (B) Rod1 is phosphorylated at CN-sensitive sites. Cultures of strain BY4741 (WT) or otherwise isogenic cnb1/H9004 (BY4741 cnb1/H9004) and cna1/H9004 cna2/H9004 (JT5574) derivatives, as indicated, expressing either GST-Rod1 or GST-Rod1^{AQAKAA} were grown to mid-exponential phase and stimulated with 200 mM CaCl$_2$ to activate CN in either the absence or presence (+) of the CN inhibitor FK506 (FK). After harvesting and lysis, proteins in the resulting extracts were purified by capture on glutathione-agarose beads (lanes 1 to 4). Samples of the material shown in lanes 1 to 4 then were either left untreated or treated (+) with lambda phosphatase (λ) in either the absence or presence (+) of phosphatase inhibitors (PPase inhibitors), and the resulting products were separated under SDS-PAGE conditions that permit resolution of phospho isoforms and analyzed with anti-GST antibodies. exp., exposure. (C) Lack of CN binding reduces Rod1-mediated adaptation. Pheromone sensitivity of MATa sst2Δ GEV (JT5919) cells expressing either GST-Rod1 or GST-Rod1^{AQAKAA}, as indicated, under the GAL promoter on a high-copy-number URA3-marked 2μm DNA plasmid was determined as described for Fig. 1E. Data from one representative experiment (n = 3) are shown. (D) Absence of CN eliminates Rod1-mediated adaptation but not Rog3-mediated adaptation. Pheromone sensitivity of cultures of MATa sst2Δ GEV (JT5919) cells and isogenic cnb1Δ (JT6694) and cna1Δ cna2Δ (JT6695) derivatives, as indicated, expressing either GST-Rod1 or GST-Rog3 under the GAL promoter on a high-copy-number URA3-marked 2μm DNA plasmid was determined as described for Fig. 1E. Data from one representative experiment (n = 3) are shown. (E) Confirmation of protein expression. Whole-cell extracts of the cells used in panels C and D were prepared, resolved by SDS-PAGE, and analyzed by immunoblotting with the indicated antibodies. Here, phospho isoforms were not separated, because different SDS-PAGE conditions were used. Data from one representative experiment (n = 3) are shown.
cause a detectable phenotype was found only for the rod1Δ rog3Δ double mutant), the loss of Ldb19 and absence of Rod1 and Rog3 had additive effects. Second, like Ldb19, the ability of Rod1 to promote adaptation requires Rsp5 binding, whereas the function of Rog3 in adaptation does not. Third, in the absence of CN-dependent dephosphorylation, Rod1 is unable to promote adaptation, whereas Rog3 is not subject to CN-mediated regulation. The most parsimonious interpretation of our collective findings is that the three different α-arrestins act on different states of the receptor and do so under different conditions (Fig. 9).

Once inserted into the PM, Ste2, like any GPCR, will exist primarily in three conformational states. Even in the absence of pheromone stimulation, Ste2 undergoes spontaneous dissociation from its cognate heterotrimeric G protein. In the absence of the stabilization conferred by receptor-G-protein interaction, Ste2 can misfold. It was shown recently that Ldb19/Art1 is essential for the Rsp5-dependent ubiquitylation and internalization of the lysine permease Lyp1, but only after it had been subjected to heat stress (124). Thus, in the same way, the effects we observed in ldb19Δ cells may be explained if Ldb19 has a similar role in PM quality control in that it mediates Rsp5-dependent ubiquitylation and endocytic removal only of misfolded Ste2. Consistent with this view, Ldb19 bound less avidly than did either Rod1 or Rog3 to the isolated Ste2 tail in vitro, presumably because Ldb19 recognizes an additional determinant only accessible in a misfolded receptor. For example, because native Ste2 functions as a dimer (125–127), perhaps Ldb19 associates with the tail and also a site exposed in a monomer when the dimer dissociates. In any event, a role in removal of misfolded molecules may explain why overexpression of Ldb19 was unable to enhance the rate of recovery in the adaptation assay, which is conducted under conditions where the properly folded state of the receptor is stabilized by ligand binding. In the absence of Ldb19, unfolded receptor is not removed from the cell surface, explaining the increase in Ste2-mCherry and Ste2-GFP observed in ldb19Δ cells. Moreover, since misfolded receptor is not removed from ldb19Δ cells, it has a chance to refold and recouple to the G protein, raising the concentration of receptor competent for signaling in the first place and explaining the modest increase in pheromone sensitivity displayed by ldb19Δ cells.

When MATa cells are exposed to α-factor, Ste2 undergoes a ligand-induced conformational change that activates and dissociates its cognate G protein; however, in contrast to when the G protein stochastically dissociates from the naïve receptor in untreated cells, activated receptor is stabilized by bound pheromone. Because Rog3 potently stimulates desensitization and does not obligatorily require its association with or modification by Rsp5, it may act similarly to classical arrestin or β-arrestin by binding to the C-terminal tail of the receptor and sterically preventing additional rounds of G-protein activation by the pheromone-bound receptor. Later during response to pheromone, Ca2+ influx will stimulate the CN-dependent dephosphorylation of Rod1, making Rod1 competent to mediate Rsp5-dependent ubiquitylation and endocytic removal of the pheromone-bound receptor, a prime example of a stimulus-induced, late-stage, negative-feedback control.

FIG 9 Distinct mechanisms of Ste2 downregulation by the α-arrestins Ldb19, Rod1, and Rog3. The α-factor receptor (Ste2), a polytopic integral membrane protein, exists primarily in three conformational states. In naïve cells, Ste2 undergoes spontaneous stochastic dissociation from its cognate heterotrimeric G protein (not shown for clarity) at a certain rate and thereby becomes destabilized. When it does so, it may misfold. Current evidence suggests that Ldb19/Art1 has a primary role in a PM quality-control pathway that mediates Rsp5-dependent ubiquitylation and endocytic removal of such misfolded PM proteins. In the presence of α-factor, Ste2 undergoes a ligand-induced conformational change that activates and dissociates its cognate G protein; however, in this case, the receptor is stabilized by bound pheromone. Because Rog3-imposed inhibition of pheromone signaling does not obligatorily require its association with or modification by Rsp5, it may act similarly to classical arrestin or β-arrestin by binding to the C-terminal tail of the receptor and sterically preventing additional rounds of G-protein activation by the pheromone-bound receptor.
competes with Gpa1 for receptor binding. Alternatively, Rog3 may promote the clathrin- and Slal-dependent, but ubiquitin-independent, route of endocytosis that has been defined for membrane proteins that contain an exposed NPFX₁₋₃D motif (129–131). Ste2 contains a very similar sequence, 395GPFAD396, in its C-terminal tail. On the other hand, although Rog3 does not need to recruit Rsp5 to execute its role in squelching pheromone signaling, it does possess P/VPXY motifs competent to bind Rsp5. Hence, normally, Rog3 may both interfere with receptor-G-protein recoupling and mediate Rsp5-dependent ubiquitinylation, thereby enhancing the efficiency of Ste2 capture by the components of the endocytic machinery that recognize ubiquitinylated cargo (132). This dual function may explain why Rog3 appears more efficacious than Rod1 in promoting adaptation when overexpressed. Moreover, Ste2 is the first target of Rog3 identified.

Our results indicate that Rod1 is a component of a negative feedback loop that ensures complete receptor clearance after a MATα cell has committed to a productive pheromone response. One hallmark of the later stages of pheromone response is a robust influx of Ca²⁺ (133, 134). This rise in intracellular Ca²⁺ is sufficient to activate the Ca²⁺-dependent phosphatase calcineurin (135). As we have demonstrated here, CN-mediated dephosphorylation makes Rod1 competent to stimulate adaptation after MATα cells are exposed to α-factor (Fig. 9). In contrast, loss of CN activity did not prevent Rog3-promoted adaptation to pheromone, demonstrating that regulation by CN is specific to Rod1. Indeed, the PXIXIT motif in Rod1 is not conserved in Rog3. Along with Aly1 (78), Rod1 is now the second yeast α-arrestin shown to be under CN regulation. Also, in Caenorhabditis elegans, the function of α-arrestin CNP-1/ArrD-17 requires CN-mediated dephosphorylation (136), suggesting that CN control of α-arrestin dephosphorylation is a conserved regulatory mechanism.

Snf1 negatively regulates Rod1 function in response to glucose limitation, thereby preventing endocytosis of the lactate permease Jen1 (102, 113). Similarly, AMPK phosphorylates and promotes degradation of the mammalian α-arrestin family member TXNIP, thereby increasing glucose uptake by preventing TXNIP-mediated downregulation of the glucose transporter GLUT1 (137). Moreover, in glucose control of Rod1 action on Jen1, Reg1-bound Glc7 seems to be responsible for Rod1 dephosphorylation (64) and likely also prevents Snf1-mediated phosphorylation of Rod1 by deactivating Snf1 itself (138). However, we observed a requirement for CN-dependent dephosphorylation for Rod1 action on Ste2 on glucose-rich medium, a condition under which Snf1 is not activated (112). These observations raised two important points. First, Snf1 cannot be the only protein kinase responsible for phosphorylating and inhibiting Rod1. In fact, phosphorylation seems to be a general mechanism for blocking the endocytic action of other α-arrestin family members (78, 108, 139). Second, it is clear, at least in the case of Rod1, that the same α-arrestin is being subjected to differential phospho-regulation as a means to control endocytosis of different targets in response to distinct stimuli.

Previous reports provide evidence that ubiquitinylation of Ldb19 (57) and Rod1 (102) is necessary for their function in internalizing other cargoes. We found, however, that ubiquitinylation of Ldb19, Rod1, and Rog3 themselves was dispensable for negative regulation of Ste2-initiated signaling. Moreover, compared to the wild-type proteins, we observed only modest increases in the steady-state level of nonubiquitinylatable (PPXY-less and K-to-R) variants of these three α-arrestins, suggesting that their Rsp5-mediated modification does not trigger rapid proteasome-mediated degradation. Indeed, Rsp5 is known to install K63-linked polyubiquitin chains on target Lys residues (140), and binding of accessory proteins, such as ESCRT-0 (141) and ESCRT-1 (109), blocks K63-linked chain recognition by proteasomes (141) or K63-linked chain formation on substrates (142). Moreover, paring back of the K63-linked chains by cellular deubiquitinylating enzymes leaves monoubiquitinylated Lys residues (143), which are poorly recognized by the proteasome (144). Although ubiquitinylation of Ldb19, Rod1, and Rog3 is not required for their function, decoration with ubiquitin might prevent Rsp5 binding. If so, ubiquitinylation of these α-arrestins themselves would provide a built-in delay timer that, after an appropriate extent of modification, eventually dissociates Rsp5 and thereby recycles this E3. Alternatively, like phosphorylation, ubiquitinylation may be yet another means to control differentially the interaction of the same α-arrestin with different targets in response to discrete signals.

Presumably, defects in LDB19, and especially in ROD1 and ROG3, were not identified in standard screens for loss-of-function mutations that confer elevated pheromone sensitivity because of their overlapping functions. Indeed, in the case of ROD1 and ROG3, we observed a detectable phenotype only in the rodlΔ rog3Δ double mutant. One reason the effects of these mutations appears modest is that the level of these α-arrestins is quite low (Ldb19, 295 per cell; Rod1, 386 per cell [Rog3 was not reported] [145]) compared to the number of receptors on the surface of a MATα cell, ~8,000 per cell (32, 125, 146). Thus, this difference in stoichiometry may explain why it has been technically difficult to observe colocalization of these molecules with Ste2 under normal cellular conditions (C. Alvaro, unpublished observations). In contrast, colocalization of the much more abundant Sst2 (5,980 molecules per cell [145]) with Ste2 was readily observed (21). Indeed, colocalization of Ldb19 (57) and Rod1 (102) with their respective cargoes has not been demonstrated, even when overexpressed (C. Alvaro, unpublished observations), perhaps indicating the transient nature of α-arrestin-target interaction. Nonetheless, when the quantity of either Rod1 or Rog3 was elevated, the adaptation assay revealed that the ability of these α-arrestins to squelch signaling by the ligand-bound form of the receptor is actually quite potent.

In addition to Ste2, it is formally possible that Ldb19, Rod1, and/or Rog3 mediates the Rsp5-dependent modification of other factors that might lead to downregulation of a pheromone-induced signal. One study indicates that the Gα subunit (Gpa1) of the Ste2-associated heterotrimeric G protein undergoes Rsp5-mediated mono- and polyubiquitinylation, which reportedly downregulates the amount of Gpa1 at the PM by diverting it to the vacuole or to the proteasome, respectively (147). Because Gpa1 holds the Gβγ (Ste4-Ste18) complex in check and prevents signal propagation, loss of any factor that contributes to Rsp5-dependent modification of Gpa1 would stabilize Gpa1, increase Gpa1 abundance at the PM, and make cells less sensitive to pheromone. Hence, Ldb19, Rod1, and Rog3 cannot be involved in the reported Rsp5-dependent modification of Gpa1, because as we have documented here, loss of these α-arrestins makes cells more sensitive to pheromone action. Similarly, it has been noted that Ste4 (Gβ) becomes ubiquitinylated in an Rsp5-dependent manner on Lys340, but the presence or absence of this modification does not affect the rate of turnover of Ste4 and, unlike loss of Ldb19, Rod1,
and Rog3, does not affect the magnitude or duration of pheromone-induced Fus3 activation (148). Hence, Ldb19, Rod1, and Rog3 cannot negatively regulate pheromone responses by being responsible for mediating the reported Rsp5-dependent modification of Ste4.

Taken together, our findings indicate that, in S. cerevisiae, α-arrestins Rod1, Rog3, and Ldb19 are negative regulators of the α-factor receptor (Ste2) in MATa cells. In yeast, there are other GPCRs, including the α-factor receptor (Ste3) in MATα cells (149), an apparent glucose sensor (Gpr1) (150), and an alkaline pH sensor (Rim21) (109). It will be important to determine which α-arrestins regulate these GPCRs and whether, as observed for nutrient permeases (58, 124), there is both specificity and redundancy in which α-arrestins modulate these targets, as we have found for Ste2.

Since the time we first reported our initial observations about the apparent functions of Ldb19, Rod1, and Rog3 in contributing to downregulation of GPCR-initiated signaling in yeast (151), members of the α-arrestin family in animal cells have been implicated in interacting with, modifying, and/or promoting the desensitization and endocytosis of several classes of GPCRs (61, 152–155). However, it has been reported previously that β-arrestins fulfill this role (156, 157); furthermore, the molecular mechanisms by which mammalian α-arrestins may contribute to downregulation of GPCR signaling is currently in dispute (152, 154). Because S. cerevisiae lacks any β-arrestin homolog, our results indicate that α-arrestins alone are capable of promoting GPCR internalization. Thus, our studies in a model organism have helped to resolve an important biological question. Therefore, α-arrestin-mediated downregulation of GPCR-initiated signaling is likely a conserved regulatory mechanism in eukaryotes. In S. cerevisiae, there are currently 14 documented α-arrestin members (60, 108, 158), whereas, to date, only eight (ArrDC1 to ArrDC5, TXNIP, and possibly DSCR3 and RGP1) are recognized in animal cells (159). Moreover, the number of mammalian GPCRs is very large. Hence, it is highly likely that many more mammalian α-arrestins remain to be identified and characterized and their targets delineated.

ACKNOWLEDGMENTS

This work was supported by NIH CBMS predoctoral training grant GM07232 (to C.G.A.), by NIH CBMS predoctoral training grant GM07276 (to A.G.), by NIH R01 research grant GM48729 (to M.S.C.), by NSF grant MCB1024818 (to B.W.), and by NIH R01 research grant GM21841 (to J.T.). For studies at the University of Pittsburgh, work was supported by NIH R01 research grant GM75061 (to J.L.B.) and NIH R01 research grant DA014204 (to Alexander Dohlman HG, Thorner JW). The University of Pittsburgh, work was supported by NIH R01 research grant GM21841 (to J.T.). The University of Pittsburgh, work was supported by NIH R01 research grant GM21841 (to J.T.). Moreover, the number of mammalian GPCRs is very large. Hence, it is highly likely that many more mammalian α-arrestins remain to be identified and characterized and their targets delineated.

REFERENCES


22. Apanovitch DM, Sleip KC, Sigler PB, Dohlman HG. 1998. Sst2 is a


112. Konopka J, Jenness BDD, Hartwell LH. 1987. Multiple interac-


120. Herrador A, Leon S, Hagenauer-Tsapis R, Vincent O. 2013. A mechan-


121. Kee Y, Munoz W, Lyon N, Hrubegset JM. 2006. The ubiquitin-

ating


