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REGULATION OF G PROTEIN–INITIATED SIGNAL TRANSDUCTION IN YEAST: Paradigms and Principles

Henrik G. Dohlman[1] and Jeremy W. Thorner[2]
[1]*Department of Pharmacology and the Boyer Center for Molecular Medicine, Yale University School of Medicine, New Haven, Connecticut 06536-0812, and*
[2]*Department of Molecular and Cell Biology, Division of Biochemistry and Molecular Biology, University of California, Berkeley, California 94720-3202; e-mail: henrik.dohlman@yale.edu; jeremy@socrates.berkeley.edu*

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■ **Abstract** All cells have the capacity to evoke appropriate and measured responses to signal molecules (such as peptide hormones), environmental changes, and other external stimuli. Tremendous progress has been made in identifying the proteins that mediate cellular response to such signals and in elucidating how events at the cell surface are linked to subsequent biochemical changes in the cytoplasm and nucleus. An emerging area of investigation concerns how signaling components are assembled and regulated (both spatially and temporally), so as to control properly the specificity and intensity of a given signaling pathway. A related question under intensive study is how the action of an individual signaling pathway is integrated with (or insulated from) other pathways to constitute larger networks that control overall cell behavior appropriately. This review describes the signal transduction pathway used by budding yeast (*Saccharomyces cerevisiae*) to respond to its peptide mating pheromones. This pathway is comprised by receptors, a heterotrimeric G protein, and a protein kinase cascade all remarkably similar to counterparts in multicellular organisms. The primary focus of this review, however, is recent advances that have been made, using primarily genetic methods, in identifying molecules responsible for regulation of the action of the components of this signaling pathway. Just as many of the constituent proteins of this pathway and their interrelationships were first identified in yeast, the functions of some of these regulators have clearly been conserved in metazoans, and others will likely serve as additional models for molecules that carry out analogous roles in higher organisms.

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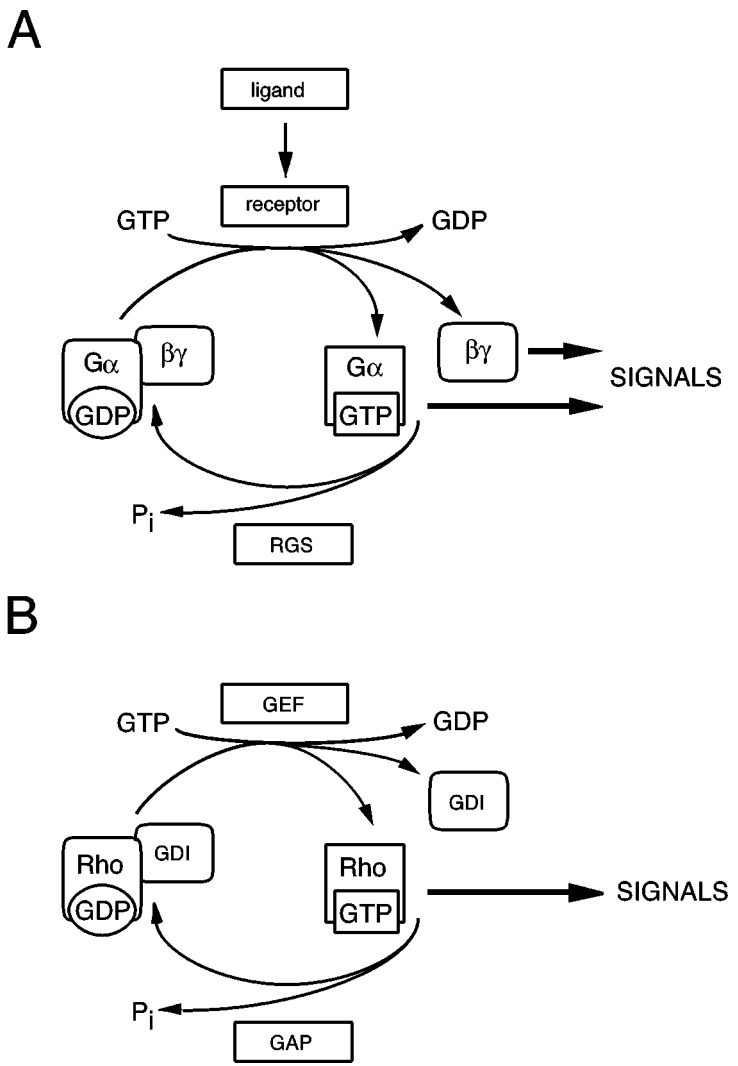
BACKGROUND AND SCOPE

One of the oldest questions in biology is how cells sense and discriminate between various environmental stimuli and then translate these inputs into an appropriate intracellular response. Among the best studied signal transduction cascades are those consisting of (*a*) a cell surface receptor with a seven-transmembrane-segment (heptahelical) structure; (*b*) an associated, heterotrimeric, guanine nucleotide-binding regulatory protein (G protein); and (*c*) an intracellular effector that produces a second messenger (1, 2) (Figure 1*A*). In humans, such G protein–coupled receptors mediate responses to light, flavors, odors, numerous hormones, neurotransmitters, and other signals (3–7). In unicellular eukaryotes, receptors of this type mediate signals that affect such basic processes as cell division, cell-cell fusion (mating), morphogenesis, and chemotaxis (8–13).

An important aspect to understand about signal transduction pathways initiated by G protein–coupled receptors is how cells modulate the intensity of signaling. Indeed, a general feature of all biological stimulus-response systems, and of G protein–coupled receptor pathways in particular, is that prolonged activation

Figure 1 General features of initiation and regulation of signaling by receptors coupled to heterotrimeric G proteins. The cycle of G protein activation and inactivation is shown diagrammatically. (*A*) When GDP is bound, the G protein α subunit (Gα) is associated with the G protein $\beta\gamma$ heterodimer (G$\beta\gamma$) and is inactive. Agonist binding to a receptor promotes guanine nucleotide exchange; Gα releases GDP, binds GTP, and dissociates from G$\beta\gamma$. Dissociated subunits activate target proteins (effectors), which initiates signaling. When GTP is hydrolyzed, subunits reassociate. G$\beta\gamma$ antagonizes receptor action by inhibiting guanine nucleotide exchange. RGS (regulator of G protein signaling) proteins bind to Gα, stimulate GTP hydrolysis, and thereby reverse G protein activation. (*B*) The roles of a receptor, of G$\beta\gamma$, and of an RGS are completely analogous to a guanine nucleotide exchange factor (GEF) (also called a guanine nucleotide dissociation stimulator), a guanine nucleotide dissociation inhibitor (GDI), and a GTPase-activating protein (GAP) that regulate small monomeric Ras-like GTPases, such as Rho.

leads to desensitization (14–16). Desensitization is the property of signaling systems to display a diminished output over time despite the continued presence of a stimulus. In humans, such signal attenuation provides a beneficial adaptation to chronic cell stimulation; in the clinic, however, downregulation of this sort reduces the efficacy of therapeutic drugs (tolerance). In simpler organisms, desensitization allows cells to recover from prolonged but unproductive exposure to growth-arresting signals (such as mating pheromones) and to resume proliferation.



Here we use the terms recovery, adaptation, and desensitization interchangeably. Another important issue is how cells control the specificity of a stimulus-response pathway (17–19). All signal transduction systems must be able to evoke an appropriate response to a particular stimulus. However, different signaling pathways can use the same or homologous components, which can greatly complicate specificity. Cross talk between parallel pathways can be beneficial when it allows a single stimulus to trigger multiple responses in a coordinated manner (20, 21), but it can also be deleterious when it leads to the adventitious activation of the wrong target, as appears to be the case in many human cancers (22, 23).

In this review, we focus attention on proteins that regulate the intensity and specificity of the response of haploid cells of the budding ascomycete, *Saccharomyces cerevisiae* (hereafter yeast), to its peptide mating pheromones. This signal transduction pathway is arguably the best understood multicomponent signaling system in any eukaryotic organism. All the key gene products responsible for propagating the signal—from the cell surface through the cytosol and into the nucleus—have been identified and their biochemical properties characterized. Moreover, the phenotypes of both loss-of-function (temperature-sensitive and null) and gain-of-function (constitutively active) alleles in the genes encoding all these signaling components have been examined and have permitted unambiguous ordering of most of the steps in this pathway. These points are thoroughly explicated, and the rapid pace of advance in this field is dramatically documented, in the many excellent and comprehensive reviews of this pathway that have appeared during the past decade (24–38). Moreover, principles elucidated in yeast have proven to be applicable to more complex organisms, including humans. Indeed, several classes of protein that are key for controlling desensitization and cross talk, which we discuss in detail below, were first discovered in yeast, including the prototype regulator of G protein signaling (RGS), Sst2 (37, 39), and the archetypical mitogen-activated protein kinase (MAPK)-binding scaffold protein, Ste5 (40, 41). Hence, analysis of yeast can reveal aspects of cell regulation that are fundamental to all eukaryotic cells. Here, therefore, we discuss recent advances in the identification and characterization of proteins that regulate yeast mating pheromone response, a G protein–initiated signaling transduction pathway. Our focus is primarily on protein function and protein-protein interactions (rather than on structure). We also address controls exerted at the level of protein localization. Additional details about this aspect of yeast cell biology, pertaining to pheromone response and mating, can be obtained from several recent and insightful reviews (38, 42–44).

OVERVIEW OF G PROTEIN SIGNALING

The basic mechanism of signaling by heterotrimeric G proteins is now well established (Figure 1*A*). On activation, a heptahelical receptor in the plasma membrane catalyzes the exchange of GDP for GTP on its cognate G protein α subunit (Gα), which leads, in turn, to dissociation of Gα from the G protein $\beta\gamma$ heterodimer

($G\beta\gamma$). Either $G\alpha$ or $G\beta\gamma$, or both, are then free to activate downstream effectors. Examples of direct effectors in mammalian cells include targets as diverse as adenylyl cyclase isotypes, phospholipase C isoforms, exchange factors for small GTPases, some calcium and potassium channels, plasma membrane Na^+/H^+ exchangers, the cytosolic tails of cadherins, and certain protein kinases (1, 45, 46). Typically, these effectors produce second messengers or other biochemical changes that lead to stimulation of a protein kinase or a protein kinase cascade (or, as mentioned, are themselves a protein kinase). The resulting changes in protein phosphorylation can affect metabolism, ion flux, gene expression, cell morphology, cell movement, cellular differentiation, and organismal development. Signaling persists until GTP is hydrolyzed to GDP and the $G\alpha$ and $G\beta\gamma$ subunits reassociate, completing the cycle of activation. Thus, the strength of the G protein–initiated signal depends on (*a*) the rate of nucleotide exchange, (*b*) the rate of GTP hydrolysis, and, (*c*) the rate of subunit reassociation.

The above three-component paradigm for G protein–initiated signaling—i.e. receptor, G protein, effector—held firm for more than 20 years, since the discovery of G proteins in the 1970s by Gilman (47) and by Rodbell (48) and their colleagues. The situation changed dramatically, however, with the discovery of the RGS protein family (37, 39, 49–53). One function of RGS proteins is to act as GTPase-activating proteins (GAPs) for a variety of different $G\alpha$ classes and, thereby, to shorten the lifetime of the activated state of a G protein. Genetic and biochemical evidence in a number of systems strongly supports the conclusion that RGS protein action is a major contributor to signal desensitization. Stated differently, RGS proteins act in opposition to the receptor by promoting G protein inactivation. Furthermore, many RGS proteins contain additional modular domains with other known or suspected signaling functions, which suggests that these types of RGS proteins constitute another node that adds to the diversity and complexity with which heterotrimeric G proteins can affect cellular signaling networks (39, 53–55). Similarly, it is now appreciated that, subsequent to activation of their cognate G proteins, receptors can act as scaffolds to recruit to the intracellular face of the plasma membrane of other signaling proteins that elicit additional cellular responses (20). In any event, the existence of RGS proteins has established a new four-component paradigm for G protein–initiated signal transduction pathways that has many parallels to signaling by small GTPases (Figure 1*B*) (39, 56).

OVERVIEW OF THE YEAST MATING PHEROMONE RESPONSE PATHWAY

The molecules (pheromones) that trigger the signaling pathway responsible for mating of the two yeast haploid cell types (*MAT***a** and *MAT*α) are short peptides (Figure 2*A*). *MAT*α cells secrete α-factor (13 residues) (57), and, *MAT***a** cells secrete **a**-factor (12 residues, but its C-terminal Cys carries a S-farnesyl substituent and is carboxymethylated) (58, 59). The α-factor binds to its specific heptahelical

receptor (Ste2)[1] expressed only on *MAT***a** cells; **a**-factor binds to its heptahelical receptor (Ste3) expressed only on *MAT*α cells. Both pheromone receptors are coupled to and activate the same heterotrimeric G protein, consisting of a Gα subunit (Gpa1) and a G$\beta\gamma$ heterodimer (Ste4-Ste18) (8, 60). Cellular responses ultimately elicited include changes in cytoskeletal structure leading to polarized cell growth (43), induction of gene transcription (61–63), changes in nuclear architecture (64), and arrest of cell cycle progression in the G1 phase (31, 65). Polarized cell growth is required to establish the site for cell fusion (plasmogamy) (42, 66). New gene transcription is required to produce, for example, proteins that mediate cell adhesion and cell fusion (66a, 67, 68). Growth arrest is required to synchronize the cell cycles of the two mating partners (69, 70). Nuclear changes are required in preparation for nuclear fusion (karyogamy) and the completion of zygote formation (71). As described in detail below, current evidence suggests that the G protein–initiated signal is transmitted and amplified via multiple effectors that bind to the released G$\beta\gamma$ heterotrimer (Ste4-Ste18). Consequently, the primary (if not exclusive) role of Gα (Gpa1) appears to be to hold G$\beta\gamma$ in check.

One G$\beta\gamma$ effector critical for mating is Cdc24, a guanine nucleotide exchange factor (GEF) for Cdc42 (72) (Figure 2*A*), which shuttles between the nucleus and the cytosol. Cdc42 is a small GTPase (21 kDa) that most closely resembles, but is distinct from, the Rho family of Ras-related small G proteins (300). In all eukaryotes, Cdc42 and Rho proteins are fundamental components of the molecular machinery that controls cell morphogenesis (74–80). In yeast, Cdc42 and Cdc24 are required for the generation of cell polarity and budding in dividing cells, and for the formation of the projection that protrudes from a haploid cell arrested by mating pheromone (81–84). In dividing haploid cells, each new bud forms next to the previous bud site (85, 86). On pheromone stimulation, division stops, but cell growth continues toward the source of pheromone (87, 88). Thus, although bud position is fixed to a predetermined site, projection formation can occur in any direction. Stated differently, an external signal (a pheromone gradient) overrides the internal signal (a previous bud site) and redirects polarized cell growth. Only if pheromone concentrations are uniform does the cell revert to using internal cues and forms a projection next to the previous bud site (89).

The polarized growth that occurs in response to pheromone requires interaction of G$\beta\gamma$ with Cdc24^{GEF},[2] but this binding appears to be bridged by a scaffold protein (90, 91). This scaffold protein is the product of the *FAR1* gene (92). Far1 shuttles between the nucleus and the cytosol. In the absence of pheromone, Far1 with its bound cargo of Cdc24 is sequestered largely in the nucleus because the rate of nuclear import of Far1 exceeds its rate of exit; after exposure to pheromone,

[1] According to standard nomenclature conventions for *S. cerevisiae*, a gene is designated, for example, *STE2*, and its protein product, Ste2. A deletion mutation is designated *ste2*Δ and alleles carrying point (substitution) mutations, *ste2*.

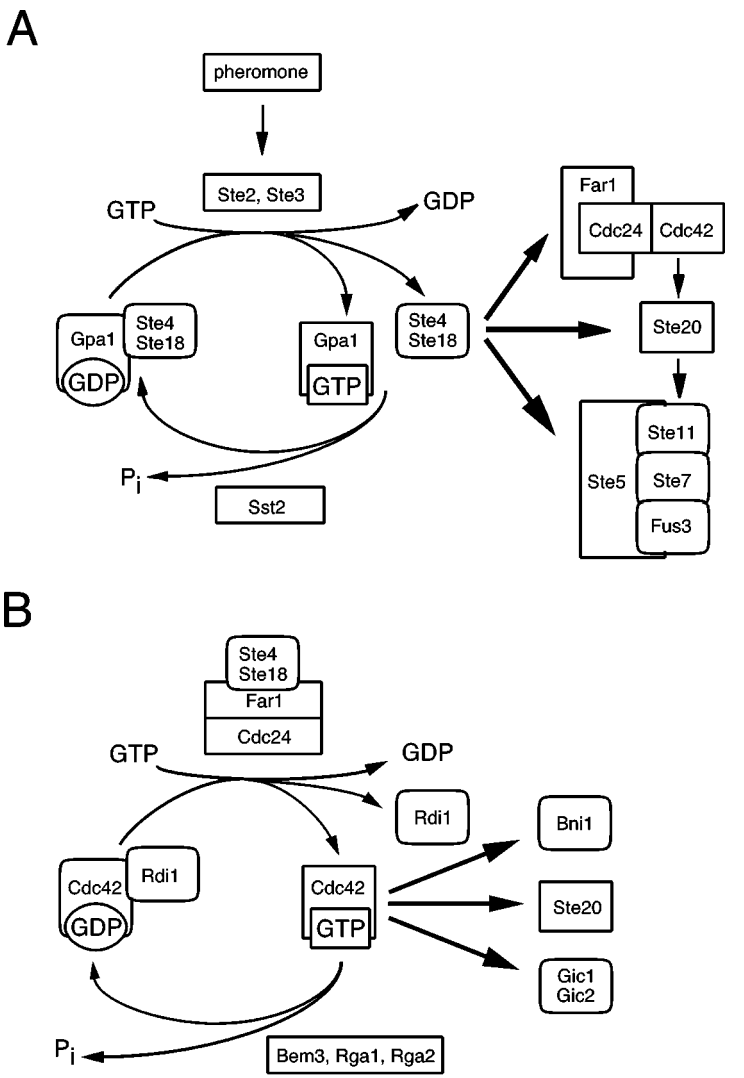
[2] For clarity, a given yeast gene product is sometimes indicated with a generic superscript that depicts its known biochemical function.

however, nuclear export of Far1 is greatly stimulated and Far1 ferries its Cdc24 cargo into the cytosol (93–95). In pheromone-treated cells, the $Cdc24^{GEF}$-Far1-$Ste4^{G\alpha}$-$Ste18^{G\gamma}$ complex localizes to the tip of the mating projection (90, 91), which suggests that it serves as a landmark for orienting the cytoskeleton during polarized cell growth, presumably by mediating efficient and highly localized generation of the GTP-bound state of Cdc42. As discussed further below, there is evidence that, in the nucleus, Far1 also has a function in mediating pheromone-imposed G1 arrest by acting as a direct inhibitor of forms of the cyclin-dependent kinase (CDK), Cdc28, that are required to drive *S. cerevisiae* through the G1 phase of the cell cycle (92, 96). The function of Far1 as a bona fide CDK inhibitor is, however, still somewhat controversial (97). Nonetheless, the regulated localization of Far1 is critical to its functions (98).

Cdc42-GTP has many demonstrated targets that are proteins involved in modulating the state of assembly of actin microfilaments in yeast (99), including the formin homolog, Bni1 (99a), Gic1 and Gic2 (99b–d), and Lsb7 (100), which associates with the yeast homolog (Las17/Bee1) of mammalian Wiskott-Aldrich syndrome protein (WASp) (Figure 2*B*). Like other members of the WASp family, Las17/Bee1 binds the Arp2-Arp3 complex, which is a critical factor for nucleation of actin filaments (100, 101). Moreover, in its GTP-bound state, Cdc42 binds to the N terminus of Ste20, the first p21-activated protein kinase (PAK) to be identified in any eukaryote (102, 103). The action of Ste20 and its nearest homolog, Cla4, also have been implicated in the establishment of cell polarity in yeast (104–106). Cdc42 binds to a high-affinity site (CRIB domain) in Ste20 (107, 108) that is also found in many other known targets of Cdc42 (109). Docking of Cdc42 onto the N terminus of Ste20 accomplishes at least three things. First, as revealed by recent X-ray analysis of crystals of PAK1 (110), a mammalian homolog of Ste20, the unactivated enzyme is a dimer that is stabilized via structural elements that include the CRIB motif. Hence, Cdc42 binding presumably disrupts these dimer contacts, releasing monomeric enzyme. Second, the CRIB motif lies within a larger inhibitory switch element that sterically occludes the active site. Cdc42 binding causes a marked conformational change that unfolds the inhibitory switch region and relieves these steric constraints (111), thereby permitting phosphorylation of the now-exposed activation loop (112, 113), which converts the kinase to its fully active state. Third, because Cdc42 is itself tethered to the plasma membrane (114) via geranylgeranylation and carboxymethylation of its C-terminal Cys (115), association of Cdc42 localizes Ste20 to the plasma membrane (108, 116). Moreover, during pheromone response, activated Ste20 specifically accumulates at the projection tip because, in addition to binding to Cdc42, the C terminus of Ste20 has a specific binding site for $G\beta\gamma$ (117) (Figure 2*A*). The projection tip is where pheromone receptors (118, 119, 119a) and the released $G\beta\gamma$ heterodimer (120; N Dhillon, C Inouye, C Sette, IG Macara & J Thorner, submitted for publication) tend to cluster in pheromone-treated cells.

To initiate the branch of the mating pheromone response pathway that leads to activation of transcription and other events in the nucleus, the substrate of Ste20 is

Ste11 (122, 123), the first mitogen-activated protein kinase (MAPK) kinase kinase (MAPKKK or MEKK) to be identified in any eukaryote (124) (Figure 2*A*). Ste11, in turn, phosphorylates and activates a MAPK kinase (MAPKK or MEK), Ste7 (125), which was also the first such enzyme identified (126). Ste7, likewise, phosphorylates and activates two MAPKs, Kss1 (127, 128) and Fus3 (127, 129), which were, once again, the first such enzymes described in any eukaryote (130, 131). Hence, the three-tiered signal transduction module known as the MAPK cascade,



which is conserved ubiquitously throughout the eukaryotic kingdom, was first identified by genetic and biochemical studies in *S. cerevisiae*. As shown primarily by genetic analysis (132, 133), Fus3 has a dedicated role in the mating pathway, whereas the primary role of Kss1 is in a different developmental response, known as invasive growth (in haploids) and pseudohyphal growth (in diploids) (134–136) (Figure 3). This distinction is not absolute (128, 137–139), however, and there is evidence that components of these MAPK kinase cascades have functions in vegetative growth as well (140, 141).

In haploid cells, a least some significant fraction of the cellular pools of Ste11, Ste7, and Fus3 is bound to a scaffold protein, Ste5 (142–144). Like Far1, Ste5 shuttles between the nucleus and the cytosol (145, 146; N Dhillon, C Inouye, C Sette, IG Macara, & J Thorner, submitted for publication). Also like Far1, Ste5 is sequestered largely in the nucleus in the absence of pheromone stimulation but is exported from the nucleus on pheromone treatment (145–148; N Dhillon, C Inouye, C Sette, IG Macara, & J Thorner, submitted for publication). Export delivers Ste5 to cytosol. Moreover, like Far1 and Ste20, Ste5 also physically interacts with G$\beta\gamma$ (149–151) and accumulates at the projection tip in pheromone-treated cells (Figure 2*A*). This localization juxtaposes Ste5 and Ste20 and, because Ste11 is bound to Ste5, presumably allows more efficient phosphorylation of Ste11 by Ste20. Indeed, there is evidence that interaction of G$\beta\gamma$ with Ste5 induces a conformational change that enhances Ste20-dependent activation of the MAPK cascade (147); hence, in this respect, Ste5 acts as an effector of G$\beta\gamma$ and not merely as a passive scaffold. Because Ste7 and Fus3 are also bound to Ste5 in close apposition to Ste11, activation of Ste11 presumably leads to rapid activation of the entire

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Figure 2 Schematic representation of the components of the *Saccharomyces cerevisiae* mating pheromone signaling pathway. (*A*) Critical roles of the G protein $\beta\gamma$ heterodimer (G$\beta\gamma$) in mating. Release of G$\beta\gamma$ (Ste4-Ste18) from activated pheromone receptors recruits at least three essential regulators to the plasma membrane and tethers them in close juxtaposition: a scaffold protein, Far1 (see also *panel B*), that carries the guanine nucleotide exchange factor (GEF) (Cdc24) for the Cdc42 small GTPase; a Cdc42-activated protein kinase (Ste20) of the p21-activated protein kinase (PAK) family; and a scaffold protein, Ste5, that carries a three-tiered module of protein kinases—a MAPKKK or MEKK (Ste11), a MAPKK or MEK (Ste7), and a MAPK or ERK (Fus3). An RGS (regulator of G protein signaling) protein (Sst2) deactivates the G protein α subunit (Gpa1) by stimulating hydrolysis of bound GTP. (*B*) Critical roles of Cdc42 in mating. Via interaction of free G$\beta\gamma$ (Ste4-Ste18) with the Far1 scaffold protein, the GEF (Cdc24) for Cdc42 is delivered to the site where pheromone receptors have been activated by agonist occupancy and generates at that location the activated (GTP-bound) state of Cdc42. The active (GTP-bound) state of Cdc42 associates with factors (e.g. Bni1, Gic1, Gic2) required for highly polarized growth of the actin cytoskeleton, which leads to generation of the mating projection. Cdc42-GTP also activates the PAK (Ste20). Cdc42 is likely to have other roles important in cell morphology changes required for cell-cell fusion, as yet unidentified. See text for other abbreviations.

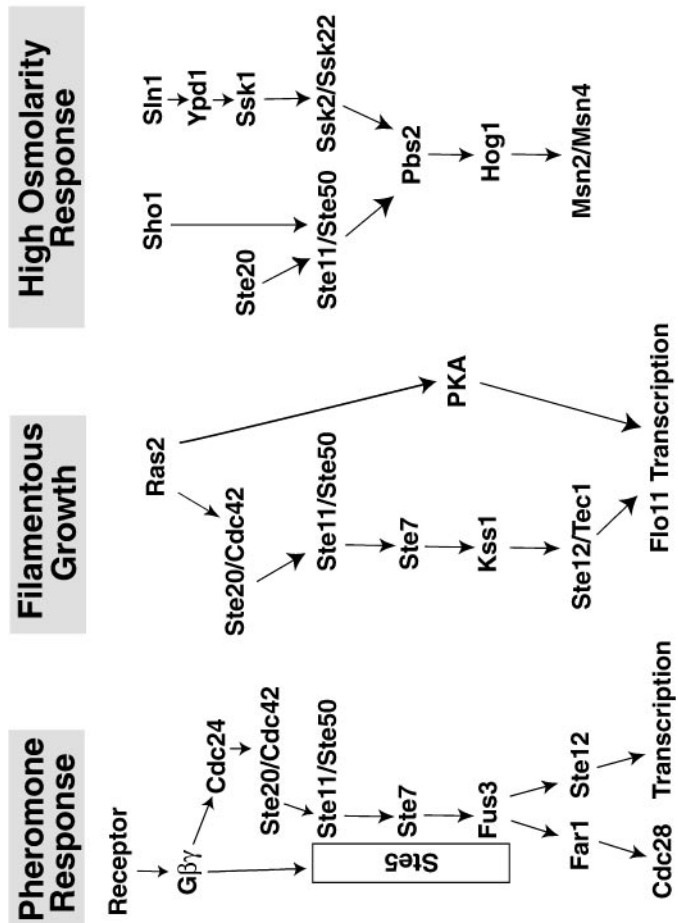


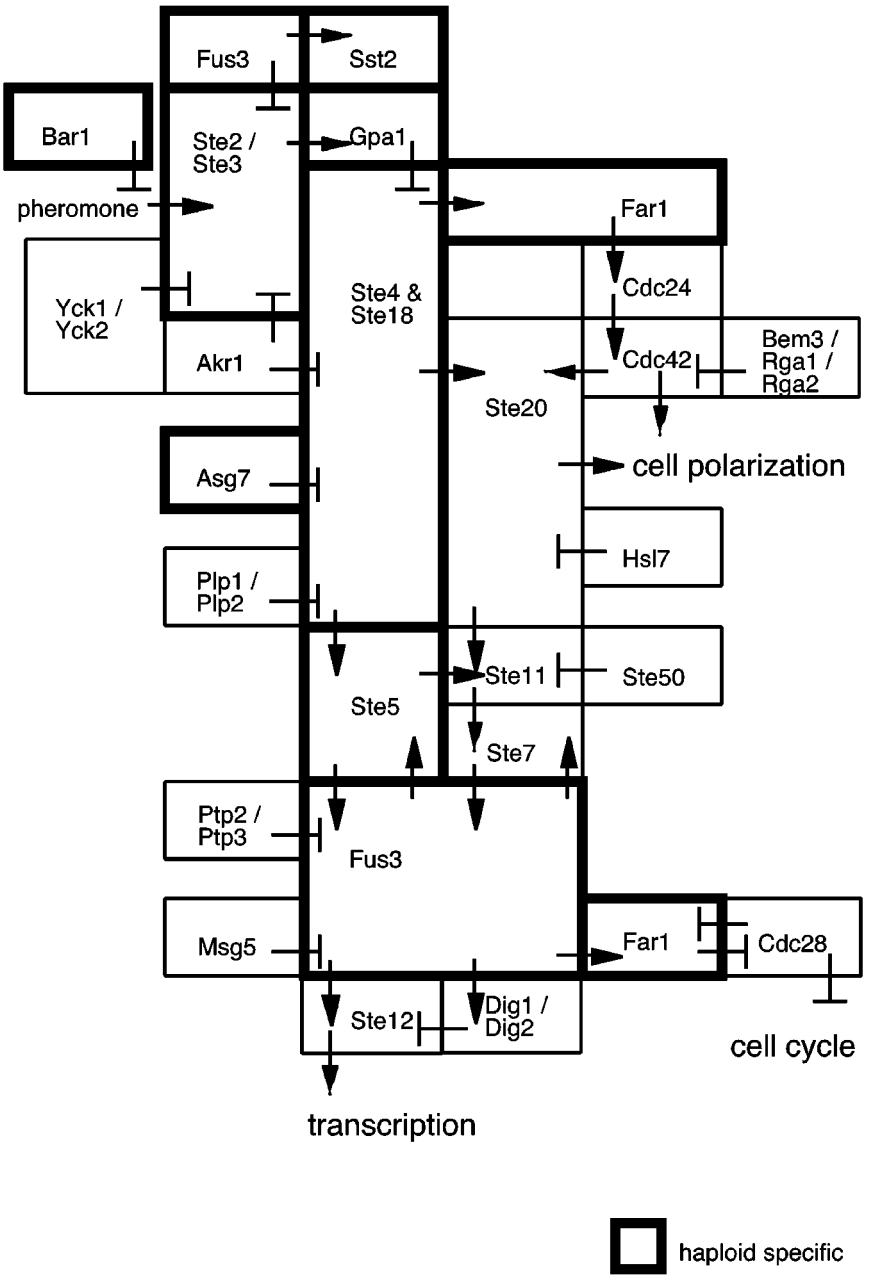
Figure 3 Three mitogen-activated protein kinase (MAPK) signaling pathways in *Saccharomyces cerevisiae* share components in common. Shown schematically are three of the five known yeast signal transduction pathways that activate a MAPK cascade. See text for details and other abbreviations.

MAPK cascade. Cross phosphorylation of the kinases and the efficiency of Fus3 activation may be further enhanced by the fact that Ste5 self-associates to form oligomers (however, whether these oligomers are simple dimers or higher multimers is not resolved) (143, 150–152).

Among the substrates of $Fus3^{MAPK}$ are nuclear proteins, including Far1 (153–155), Ste5 (156, 157), Ste12 (a transcription factor) (153, 158, 159), and Dig1 and Dig2 (inhibitors of Ste12) (160–162). Thus, pheromone-initiated signaling in yeast begins with occupancy of G protein–coupled receptors at the plasma membrane, and it is followed by membrane recruitment and activation of proteins involved in cell morphogenesis, as well as by recruitment and activation of a protein kinase cascade whose action culminates in phosphorylation and activation of nuclear proteins that control cell polarity, transcription, and progression through the cell cycle. All these changes represent a coordinated response to pheromone that permits haploids to differentiate transiently into nonproliferating gamete-like cells that are prepared for cell and nuclear fusion.

LEVELS OF REGULATION

Despite the profound changes yeast cells undergo in preparation for mating, cells that fail to mate eventually become refractory to pheromone action and resume cell division. Thus, yeast cells display the same kind of desensitization and adaptation observed in mammalian cells in response to peptide hormones. As is described in greater detail below, many gene products participate directly in downregulation of the signaling pathway (Figure 4), including a secreted protease (Bar1/Sst1) that destroys α-factor pheromone, enzymes that modify (ubiquitinate, phosphorylate) the pheromone receptors and promote their internalization, GAPs for both $Gpa1^{G\alpha}$ ($Sst2^{RGS}$) and Cdc42 (Bem3, Rga1, Rga2), and phosphatases (Msg5, Ptp2, Ptp3) that deactivate $Fus3^{MAPK}$. Other proteins that clearly contribute to modulating this signaling pathway at various levels have biochemical functions that are not yet as well defined, including Ste50, Mpt5, Akr1, Afr1, Hsl7, and Asg7. In naïve cells, pheromone action initially elicits a signal strong enough to override all the negative regulators. However, prolonged pheromone stimulation leads to transcriptional induction of many of the genes that encode these regulators (e.g. $Gpa1^{G\alpha}$, Sst2, Msg5). As a result, the subsequent accumulation and collective action of these gene products brings about a dramatic dampening of pheromone signaling. Prolonged stimulation also results in posttranscriptional processes that contribute to signal attenuation. For example, feedback phosphorylation by $Fus3^{MAPK}$ may affect the activities and/or stabilities of $Ste7^{MAPKK}$, $Ste11^{MAPKKK}$, $Sst2^{RGS}$, and Ste3 (discussed below). Moreover, if present at a sufficient level, the G1 cyclin (Cln1 and Cln2)-bound forms of $Cdc28^{CDK}$ can phosphorylate $Ste20^{PAK}$ and inhibit its function in the mating pathway (also described below). As a consequence of these processes, cells can recover from exposure to pheromone and resume vegetative growth.


Fus3
Sst2
Bar1
Ste2 /
Ste3
Gpa1
pheromone
Far1
Yck1 /
Yck2
Ste4 &
Ste18
Cdc24
Akr1
Cdc42
Bem3 /
Rga1 /
Rga2
Ste20
cell polarization
Asg7
Hsl7
Plp1 /
Plp2
Ste11
Ste50
Ste5
Ste7
Ptp2 /
Ptp3
Fus3
Msg5
Far1
Cdc28
Ste12
Dig1 /
Dig2
cell cycle
transcription
haploid specific

Mechanisms also exist, first, to prevent inadvertent cross talk between the pheromone response pathway and other signaling pathways that involve distinct MAP kinases and, second, to permit such cross talk at the appropriate time and location during the mating process. Although Fus3^{MAPK} regulates pheromone signaling, three other MAP kinases expressed in haploid yeast cells control invasive growth (Kss1), osmotic stress response (Hog1), and cell wall synthesis (Mpk1) (Figure 3). Upstream components required for mating also participate in these parallel signaling pathways, which lead to different developmental outcomes. For instance, in addition to their roles in the pheromone response pathway, Ste20^{PAK} and Ste11^{MAPKKK} are also involved in both the invasive growth and osmotic response pathways. As mentioned above (and as discussed in greater detail below), one protein that contributes uniquely to signal fidelity by the mating pathway is the Ste5 scaffold protein, which binds selectively to Ste11^{MAPKKK}, Ste7^{MAPKK}, and Fus3^{MAPK}. Clearly, the yeast system provides powerful experimental tools to examine how parallel signaling pathways in the same cell are segregated, as well as coordinately regulated, when appropriate, to permit their sequential engagement.

Regulation of Ligands

One of the simplest ways to modulate receptor activation is to regulate the availability of the initial stimulus. In nerve cells, very rapid regulation of the level of a neurotransmitter is accomplished by controlling (*a*) the extent of synaptic vesicle-mediated neurotransmitter release, (*b*) the efficiency of neurotransmitter uptake by specific transporters, and (*c*) the rate of neurotransmitter destruction by specific enzymes. Most peptide hormones and pheromones are, by design, much longer acting, but very similar mechanisms control their availability. In yeast, **a**-factor and α-factor are released from the cell by two very different routes. The α-factor is produced as a larger prepro-hormone precursor (containing multiple α-factor repeats) that is translocated into the endoplasmic reticulum, processed to its mature form in the Golgi compartment, and released from cells in secretory vesicles (57, 163, 164). In contrast, pro-**a**-factor is produced and processed in the cytosol (165, 166) and exported by Ste6, a dedicated ATP-binding cassette transporter that resides in the plasma membrane (167–169). Ste6 is a homolog of mammalian multiple drug resistance (Mdr) transporters (170) and was the first Mdr-like protein for which a true physiological substrate was found (171, 172). Because secretion becomes highly polarized in pheromone-treated cells (43, 164, 173), the secretory vesicles containing α-factor in *MAT*α cells, and secretory vesicles containing Ste6 in *MAT***a**

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Figure 4 Regulatory factors controlling the yeast mating pheromone response pathway. Depicted in a hard-wired, circuit board–like fashion are the positive (arrowheads) and negative (T bars) regulatory interactions discussed in this review and their targets in this signal transduction pathway. Gene products boxed by thick lines are expressed in a haploid cell–specific manner. See text for additional details.

cells, are delivered to the tip of the mating projection, resulting in a gradient of the cognate pheromone that emanates from the projection tip on each haploid mating partner. Moreover, these gradients become mutually self-reinforcing because transcription of one of the genes that encodes prepro-α-factor (*MFα2*), and both genes encoding pro-**a**-factor (*MFA1* and *MFA2*), as well as *STE6*, is pheromone inducible (26, 63).

As with other secreted peptides, the efficacy and duration of pheromone action are limited by the rate of diffusion and stability. The α-factor is destroyed by Bar1, a secreted pepsin-like protease that cleaves and inactivates α-factor (174, 175). The only physiological function of Bar1 seems to be degradation of α-factor, because the *BAR1* gene is only expressed in **a**-cells, is inducible by α-factor, and has a strict substrate specificity (α-factor is cleaved between Leu6 and Lys7). An activity that inactivates **a**-factor and is expressed in an α cell-specific manner has been reported (176, 177) but not yet substantiated by any subsequent work. On the other hand, the Ste6 transporter (169, 178) required for **a**-factor export and other integral membrane proteins (179) are rapidly turned over constitutively in a ubiquitin-dependent manner, a process akin to that involved in internalization and degradation of the pheromone receptors (see below). Hence, in the absence of continued α-factor–dependent induction of its gene, the steady state level of Ste6 falls precipitously (167), preventing efficient **a**-factor export. Moreover, due to the S-farnesylation and carboxylmethylation of its C-terminal Cys (58), **a**-factor tends to adsorb to hydrophobic surfaces and diffuses poorly (180).

Regulation of Receptors

Once activated, pheromone receptors are subject to various types of regulation. In mammals, hormone desensitization involves, in part, agonist-dependent phosphorylation by a special class of protein kinase, called G protein–coupled receptor kinases (GRKs) (181), and the action of receptor-binding proteins, called arrestins (182). Receptor phosphorylation by a GRK and subsequent arrestin binding both prevent recoupling of a receptor to its cognate G protein (183) and promote receptor removal from the cell surface by stimulating endocytosis (184). In yeast, several investigators have used biochemical approaches to show that loss of pheromone responsiveness also involves ligand-induced processes that downregulate the level of functional receptors at the cell surface. For instance, both the Ste2 and Ste3 pheromone receptors are rapidly phosphorylated at Ser and Thr residues (185–187) and are internalized following agonist activation (188, 189). The pheromone receptors preexist, and are endocytosed, as homooligomers (119, 190).

Receptor phosphorylation contributes to desensitization of the pheromone signal, as shown by mutating potential phosphorylation sites in the C-terminal cytosolic tail of Ste2 (186). Even complete truncation of the Ste2 tail of the receptor does not alter receptor affinity for α-factor (185, 191) or affect receptor oligomerization (119, 190), but it does result in the loss of stimulus-dependent phosphorylation, a marked (10- to 100-fold) increase in pheromone sensitivity, a defect in

ligand-induced endocytosis, a defect in recovery from G1 arrest (inability to resume proliferation after pheromone treatment), and a defect in pheromone-induced morphogenesis (185, 191), as well as less efficient initial coupling of the receptor to the G protein (192). Some shorter C-terminal truncation mutants are also hypersensitive to pheromone but still undergo pheromone-induced endocytosis, which suggests that receptor internalization is only partially responsible for adaptation at the receptor level (185). A five-residue segment near the middle of the C terminal tail of Ste2 is reportedly necessary for endocytosis but is not required for signal transduction (193).

Perhaps surprisingly, G protein–mediated signal transduction is not necessary for Ste2 phosphorylation or internalization. Receptor downregulation still occurs in mutant cells that lack an active G protein and are unable to signal (188, 194). Similarly, a receptor mutant that is defective in initiating a pheromone signal ($Ste2^{L236H}$) is nevertheless capable of undergoing ligand-dependent endocytosis (195). These findings suggest that binding of α-factor to its receptor simply induces a conformational change that increases its accessibility both for phosphorylation and for interaction with the endocytic machinery. Moreover, the protein kinase responsible for receptor phosphorylation must not require the pheromone response pathway for its activation (see below). Biochemical probes, such as changes in susceptibility to protease, indicate that Ste2 does undergo a significant conformational transition when α-factor binds (196).

As mentioned above, Ala substitutions for specific C-terminal Ser and Thr residues in Ste2 result in decreased phosphorylation, loss of ligand-induced endocytosis, and increased sensitivity to α-factor (186). Ste2 is also modified by the attachment of ubiquitin (197), a 76-residue peptide used to tag proteins for degradation (73). Receptor mutations that block phosphorylation also block receptor ubiquitination and internalization (198). A similar phenotype is observed in a strain deficient for casein kinase I (199). In yeast, several casein kinase I isotypes (Yck1, Yck2, Yck3) are associated with the plasma membrane (200), and these enzymes had already been implicated in clathrin-mediated endocytosis on the basis of genetic evidence (201). A point mutation in Ste2 (K337R), removing the likely residue where ubiquitin is attached, allows phosphorylation but eliminates ubiquitination and ligand-induced internalization (193, 198). An *end4* mutant allows Ste2 phosphorylation and ubiquitination but blocks endocytosis (202). End4/Sla2 is a talin-like protein that is associated with cortical actin patches in yeast and is required for efficient endocytosis (203). Taken together, these findings suggest (*a*) that pheromone binding leads to a conformation change in the receptor that permits more efficient phosphorylation by Yck1 (and/or another isoform) already resident at the plasma membrane, (*b*) that phosphorylation is a prerequisite for ubiquitination, and (*c*) that ubiquitination triggers internalization. The fact that these events are only initiated after α-factor stimulation suggests that these processes serve as an adaptation that specifically downregulates the level of Ste2. In this regard, it may be significant that the *YCK3* gene is reportedly pheromone inducible (397).

Most of the events leading to endocytosis of Ste2 also occur for the **a**-factor receptor, Ste3. Ste3 is phosphorylated within its C-terminal domain (204), and phosphorylation leads to ubiquitination, endocytosis, and delivery to the vacuole for degradation (187, 205, 206). As for Ste2, constitutive turnover of Ste3 requires the action of casein kinase I (207). However, in contrast to Ste2, ligand-stimulated phosphorylation and ligand-stimulated turnover of Ste3 require an intact pheromone signaling pathway, including the MAP kinase $Fus3^{MAPK}$ (187). This process appears to require recruitment of Fus3 to the plasma membrane because a membrane-anchored form of Ste5 (145) induces phosphorylation of Ste3 (187). However, to our knowledge, phosphorylation of Ste3 by Fus3 has not been directly demonstrated in vitro. Nonetheless, in response to ligand binding, enhancement of Ste3 phosphorylation and accelerated endocytosis of the receptor seems to occur by a process of feedback regulation that contributes to signal attenuation.

Ubiquitination has long been known to serve as a signal for degradation of cytoplasmic proteins by the proteasome (73). The findings described above for Ste2 were the first to demonstrate that ubiquitination can serve as a signal for the degradation of an integral membrane protein (197). One unique feature of the receptor degradation pathway is that it typically involves monoubiquitination, whereas most cytoplasmic substrates are polyubiquitinated (208). Monoubiquitination appears to be sufficient for endocytosis, because genetic fusion of a single ubiquitin to the receptor C terminus can trigger endocytosis, even in the absence of phosphorylation (209, 210). Another key difference between the ubiquitin-dependent degradation of the pheromone receptors and that of soluble proteins is that the receptors are not degraded by the proteasome but rather are delivered to the vacuole (yeast counterpart of the mammalian lysosome) (211), where the receptors are degraded by resident vacuolar proteases (195). By tracking Ste2 using indirect immunofluorescence (211a), a chimera with the *Aequoria victoria* green fluorescent protein (GFP) (212), or immunoelectron microscopy (212a), it has been shown that, in response to ligand binding, Ste2 is delivered from the cell surface to a peripheral organelle (early endosome), then to late endosomes (also known as the prevacuolar compartment or multivesicular bodies), and finally to the vacuole itself (190, 212b). In agreement with these findings, the disruption of genes required for endosome maturation (*TLG1*, *TLG2*) (213), for fusion of transport vesicles to the vacuole, including *VAM3/PTH1* (214) and *VPS2* (204), and for a phosphatidylinositol 3-phosphate 5-kinase involved in vacuolar morphology (*FAB1*) (212) prevents delivery of endocytosed receptors to the vacuole. Current efforts are aimed at identifying other components of the receptor endocytosis machinery. Clathrin-coated vesicles may be involved, because cells with a temperature-sensitive mutation in clathrin heavy chain ($chc1^{ts}$) exhibit a rapid and reversible defect in receptor internalization (both constitutive and pheromone induced) (215). However, the role of clathrin may be indirect because, at restrictive temperature, the $chc1^{ts}$ mutant displays substantial residual endocytosis (30%–50% of wild type), and once internalized, the receptor is delivered to the vacuole at a normal rate.

In summary, wild-type receptors are delivered to the cell surface via the secretory pathway but are internalized in response to phosphorylation and ubiquitination. On pheromone binding, this process is accelerated, ultimately resulting in attenuated signaling, which contributes to adaptation. Both constitutive and ligand-dependent endocytosis result in delivery of the receptor to the vacuole for degradation.

Another apparent regulator of receptor endocytosis is Akr1, a protein with five predicted membrane-spanning domains, six ankyrin repeats, and a zinc finger-like domain. In the absence of Akr1, constitutive turnover of both Ste2 and Ste3 are blocked and ligand-stimulated uptake of Ste2 is prevented (ligand-stimulated internalization of Ste3 is unaffected) (207, 216). This phenotype resembles that of casein kinase I–deficient mutants. Indeed, Akr1 seems to be necessary to localize both the Yck1 and Yck2 isoforms to the plasma membrane (207). In cells lacking Akr1, Yck1 and Yck2 are mislocalized to the cytoplasm and are presumably less able to phosphorylate their membrane-localized receptor substrates. The mechanism by which Akr1 participates in localizing Yck1 and Yck2 to the plasma membrane is not known, but one possibility is that Akr1 acts as a molecular matchmaker to allow these protein kinases to physically associate with their proper substrate. Consistent with this view, Akr1 binds to the C-terminal domain of Ste3 (187) as well as to free $G\beta\gamma$ (discussed below). Similar to **a**-factor (see above) and $Ste18^{G\gamma}$ (217–219) (see below), Yck1 and Yck2 may be S-prenylated and/or S-palmitoylated (220, 221) at a C-terminal CysCys doublet. Several mammalian GRKs are also C-terminally isoprenylated (222, 223). Perhaps Akr1 acts as a cofactor for introduction of these modifications or as a binding partner for specific recruitment of lipid-modified (prenylated and/or palmitoylated) proteins to the plasma membrane (207).

Another protein, Afr1, was thought to act at the level of the pheromone receptors for four reasons (224): (*a*) The *AFR1* gene was originally isolated on the basis of its ability, when overexpressed, to inhibit pheromone signaling by cells expressing a normal receptor, but not by cells expressing Ste2 lacking its C-terminal cytosolic tail; (*b*) *AFR1* overexpression was unable to block constitutive signaling in cells that lack $Gpa1^{G\alpha}$; (*c*) the Afr1 sequence has weak homology to mammalian arrestins; and (*d*) the *AFR1* gene is highly pheromone inducible, which suggested that it might participate in a negative feedback loop to control receptor signaling. However, an *afr1*Δ (null) mutation does not enhance the sensitivity of cells significantly and influences the efficiency of pheromone signaling and mating independently from other factors that affect receptor desensitization and receptor endocytosis (225). Indeed, the function of Afr1 seems much more important for polarized cell growth and formation of the mating projection (226, 227). Consistent with a role in apical growth of the mating projection, Afr1 does not seem to interact with the pheromone receptors directly, but rather physically associates with other proteins (228), including a component (Cdc12) of the septin filaments (229) and a Cdc42-interacting protein, Iqg1 (230), both of which are normally found at the

bud neck and are important for the isotropic growth needed for morphogenesis of a round bud (231). Perhaps Afr1 interdicts the normal function of these proteins and, hence, allows for more facile assembly of the factors that are required for the anisotropic growth that leads to mating projection formation.

Regulation of Heterotrimeric G Protein Subunits

As the intermediaries between cell surface receptors and intracellular effectors, G proteins transduce initial pheromone binding into a downstream signal. Hence, factors that control the competence of G protein components to couple to upstream activators and factors that control the lifetime of the activated state of G proteins are especially well positioned to modulate both the intensity and the duration of signaling. G protein activity depends on the relative rates of GTP binding (accelerated by receptors, discussed above) and GTP hydrolysis (stimulated by RGS proteins, discussed below). In addition, there is growing evidence for regulation of G protein and RGS protein function, stability, and localization, in part through posttranslational modifications. Several types of modifications have been described for the different subunits of the pheromone receptor–coupled G protein.

GPA1$^{G\alpha}$ Gpa1$^{G\alpha}$ is polyubiquitinated, and this modification leads to rapid proteasome-dependent degradation of the protein (232). This route of degradation involves the N-end rule ubiquitination pathway (233). Because overexpression of Gpa1$^{G\alpha}$ is known to reduce pheromone sensitivity (234, 235), changes in its rate of turnover could alter the ratio of Gα to G$\beta\gamma$ and, hence, affect signal intensity (236). Although the sequence element conferring this instability has been localized (237), an in vivo test of the hypothesis that the rate of Gpa1 turnover affects the intensity of signaling will require that the site of ubiquitination be identified and mutationally replaced. Ubiquitination has not yet been demonstrated for any other G protein subunit in any other organism.

All Gα subunits that have been examined are N-myristoylated and/or S-palmitoylated (238, 239). Gpa1 has both of these modifications. N-myristoylation, catalyzed by N-myristoyltransferase (240), involves cotranslational formation of a stable amide linkage between the carboxyl group of a C_{14} saturated fatty acid (from its activated donor, myristoyl-CoA) and the N-terminal amino group of a Gly residue (Gly2) that is exposed after removal of the initiator Met residue by the action of Met-specific aminopeptidase (241). Palmitoylation (also referred to as thioacylation) involves formation (possibly nonenzymatic) of an unstable thioester bond between the carboxyl group of a C_{16} saturated fatty acid (from its activated donor, palmitoyl-CoA) and the thiol group of the side chain of an internal (but N-terminally located) Cys residue (typically Cys3) (238, 239). Both modifications have been intensively studied because they can have profound effects on Gα activity and localization. In some cases, these modifications appear to be regulated by extracellular signals, for example S-palmitoylation of mammalian $G_{s\alpha}$ and $G_{q\alpha}$ (238) and N-myristoylation of Gpa1$^{G\alpha}$ (242).

Gpa1 is normally not myristoylated at full stoichiometry; on pheromone stimulation, the efficiency with which newly synthesized Gpa1 undergoes this modification is markedly enhanced (242). Mutations in the N-myristoyltransferase gene (*NMT1*) (243), or those that eliminate the target residue for myristoylation (*gpa1*^{G2A}) (244), mimic the phenotype of *gpa1*Δ (null) mutations, resulting in sustained release of G$\beta\gamma$ and constitutive signaling. Thus, N-myristoylation appears to be essential for Gpa1 function; therefore, the observed pheromone-dependent change in the stoichiometry of myristoylated vs unmyristoylated protein presumably affects G protein signaling. Nonmyristoylated Gpa1 can still form a high-affinity complex with G$\beta\gamma$ (Ste4-Ste18) in vitro but fails to associate with the plasma membrane in vivo. In cells expressing the *gpa1*^{G2A} mutant, a pool of G$\beta\gamma$ remains at the plasma membrane, which is presumably responsible for the constitutive signaling phenotype (244, 245). In support of the conclusion that N-myristoylation is important for efficient delivery of Gpa1-Ste4-Ste18 heterotrimers to the plasma membrane, fusion of Gpa1$^{G\alpha}$ to the C terminus of Ste2 allows coupling to G$\beta\gamma$ and the formation of signaling-competent receptor–G protein complexes, even though the Gpa1$^{G\alpha}$ moiety cannot be N-myristoylated (246). Yeast Gpa1 is S-palmitoylated at Cys3, and absence of this modification has similar (though less severe) consequences for localization of Gpa1 and the efficiency of signaling (219, 247). Conversely, in the absence of G$\beta\gamma$, Gpa1 still associates with the plasma membrane efficiently, although it cannot productively couple to pheromone receptors (244, 248). These findings indicate that fatty acylation is needed primarily for proper membrane targeting of G$\alpha\beta\gamma$, rather than for subunit-subunit association and assembly of the heterotrimer (246).

STE4$^{G\alpha}$ On pheromone stimulation, Ste4$^{G\beta}$ is dynamically phosphorylated at several sites (249). A small internal deletion (*ste4*Δ*310-346*) prevents pheromone-stimulated phosphorylation of the protein and results in a modest (sixfold) increase in pheromone sensitivity (manifested as a defect in recovery from α-factor–induced G1 growth arrest), which suggested that Gβ phosphorylation is a response that contributes to attenuation of the pheromone signal (249). One possibility is that phosphorylation reinforces G$\beta\gamma$ binding to the Ste5 scaffold protein, since the pool of Ste4$^{G\beta}$ found associated with Ste5 in coimmunoprecipitation experiments is predominantly in the phosphorylated state (143, 151). However, a recent study indicates that the defect in adaptation exhibited by Ste4(Δ310-346) is due to disruption of its ability to interact efficiently with, and be sequestered by, Gpa1$^{G\alpha}$ (250), and even point mutations in Ste4$^{G\beta}$ that also reduce its affinity for Gpa1 display a similar apparent defect in recovery from pheromone-induced growth arrest (251). Like Ste4(Δ310-346), two different Gβ substitution mutants, Ste4(T320A S335A) and Ste4(T322A S335A), remain unphosphorylated on pheromone stimulation; however, these two mutants, unlike Ste4(Δ310-346), have no discernible effect on either initial signaling or subsequent adaptation. These findings indicate that contrary to the original suggestion, pheromone-induced phosphorylation of Ste4$^{G\beta}$ does not contribute to desensitization. Although several Gα subtypes

are phosphorylated in mammalian cells and in the slime mold *Dictyostelium discoideum* (252, 253), Ste4 is, to our knowledge, the only Gβ whose phosphorylation has been demonstrated in any organism. Perhaps various cell types have evolved so as to phosphorylate the subunit that is primarily responsible for effector activation. However, what agonist-stimulated phosphorylation contributes to Ste4 function in *S. cerevisiae* remains an open question.

STE18$^{G\gamma}$ Like **a**-factor, Cdc42, and perhaps Yck1, all Gγ subunits that have been examined are isoprenylated. Introduction of this modification involves linkage of a C_{15} (farnesyl) or C_{20} (geranylgeranyl) isoprenoid chain via a thioether bond to the sulfhydryl group of a Cys located four residues penultimate to the C terminus (CAAX box motif). This attachment reaction is catalyzed by heterodimeric prenyltransferases specific for farnesylation or geranylgeranylation (254). The prenylated protein then undergoes endoproteolytic truncation of the last three amino acids, a cleavage catalyzed by a special class of proteases, called CAAX converting enzymes (255). Finally, the newly exposed carboxyl group is converted to the methyl ester by a dedicated S-adenosylmethionine–dependent prenylcysteine carboxyl methyltransferase (256, 257). Whereas most Gγ proteins are geranylgeranylated, Ste18 is farnesylated (at Cys 107) (258) and also S-palmitoylated (at Cys 106) (218, 219). Replacement of Cys 107 (with Ala or Ser) results in a sterile phenotype (217, 259), whereas replacement of Cys 106 significantly reduces, but does not eliminate, Ste18 function. Unmodified Ste18 is still targeted to the plasma membrane (presumably via its association, as the G$\beta\gamma$ dimer, with Gpa1$^{G\alpha}$), but it is readily dissociated from membranes following G protein activation (218, 219). Thus, it appears that prenylation and palmitoylation are dispensable for G$\beta\gamma$ association, for heterotrimer assembly, and for receptor-dependent G protein activation but are required for the released G$\beta\gamma$ to remain stably tethered to the plasma membrane.

SST2^{RGS}, Regulator of GPA1$^{G\alpha}$ Sst2^{RGS} plays a predominant role in signal desensitization. The *SST2* gene was first identified in a search for factors that act as negative regulators of pheromone signaling by screening for mutants that showed greatly increased sensitivity to the growth-arresting affects of pheromone (260, 261). It was demonstrated that *sst2* mutants are deficient primarily in the ability to recover and resume growth after their exposure to pheromone, which suggested that they were defective in a function critical for adaptation. Cells carrying strong *sst2* loss-of-function mutations (262) or an *sst2*Δ (null) mutation (130, 263) respond to doses of pheromone at least two orders of magnitude lower than do wild-type cells and are completely unable to recover from pheromone-imposed cell cycle arrest. When the *SST2* gene was cloned (264), its deduced amino acid sequence failed to provide any information about its mechanism of action or its target. Moreover, at the time, it was not yet known that heterotrimeric G proteins existed in yeast, and the relevance of pheromone signaling to hormone signaling in mammalian cells was not broadly appreciated. Hence, the discovery

of $Sst2^{RGS}$ did not capture much attention at the time. However, by 1992, it was already explicitly proposed that the role of Sst2 was to act as a GAP on Gpa1 (26).

Subsequent work has confirmed that $Gpa1^{G\alpha}$-specific GAP activity is the primary function of Sst2. Initially, a dominant "sterile" allele of *SST2* was used to deduce its intracellular target. One such dominant gain-of-function mutant, *SST2*(*P20L*), could block response to pheromone but could not prevent activation of the mating pathway downstream of the receptor, which was achieved, for example, by overexpression of normal $Ste4^{G\beta}$, by expression of a constitutively active Gβ mutant ($Ste4^{Hpl}$), or by disruption of the *GPA1* gene (265). Moreover, like *sst2*Δ mutants, cells lacking *gpa1*Δ never recover from pheromone-induced growth, which indicated that, in the absence of $Gpa1^{G\alpha}$, Sst2 could not exert its adaptation-promoting effect (263). These arguments and other genetic evidence implicated $Gpa1^{G\alpha}$ as the direct target of $Sst2^{RGS}$. It was subsequently shown that $Sst2^{RGS}$ and $Gpa1^{G\alpha}$ colocalize at the plasma membrane and copurify as a complex from yeast (263). It was then directly demonstrated that purified Sst2 stimulates the conversion of purified GTP-bound Gpa1 to the GDP-bound state by stimulating nucleotide hydrolysis (266).

Since the identification of Sst2, a family of homologous proteins has been discovered in more complex organisms (37, 39, 49–53). Several RGS family members from metazoans have been purified in recombinant form and shown to be potent GAPs for certain classes of Gα subunits (267–269). Moreover, the structural basis for the observed enhancement of the rate of GTP hydrolysis (RGS binding stabilizes the transition state form of the Gα subunit) has been elucidated at atomic resolution (270) and confirmed by site-directed mutagenesis in both yeast (271) and mammalian cells (271–273, 273a). These findings demonstrate that Sst2 and other RGS proteins promote desensitization by stimulating the GTPase activity of their target Gα subunit, thereby shortening the lifetime of the active (GTP-bound) species, accelerating reassociation with G$\beta\gamma$, and, as a result, attenuating cellular response to a signal.

Many questions about the function of the 698-residue Sst2 protein remain, however. First, expression of its C-terminal RGS domain (residues 406–698) alone is not sufficient to promote adaptation (263), which suggests that other regions of the protein are important for its function. Second, and in this same regard, just amino-proximal to the RGS domain in Sst2 and its closest homologs from other fungi, including FlbA from *Aspergillus nidulans* (274) and Rgs1 from *Schizosaccharomyces pombe* (275), there is a segment (residues 279–358 in Sst2) with detectable homology to so-called DEP domains. DEP domains are conserved sequence elements of ~80 residues, first found in three proteins: (*a*) a *Drosophila melanogaster* adapter protein (Dishevelled) and its mammalian homologs (Dvl), which are components of the Wingless (Wnt) signaling pathway downstream of the receptor (Frizzled); (*b*) another RGS protein, from the nematode *Caenorhabditis elegans* (EGL-10); and (*c*) an actin-binding cytoskeletal protein (pleckstrin) (276). Although DEP domains have been implicated in recruitment to the plasma membrane (277), their precise role or binding partners are not known. Nonetheless,

if the DEP domain in Sst2 is required for its localization to the plasma membrane, then the isolated RGS domain alone might be unable to efficiently encounter its substrate, $Gpa1^{G\alpha}$ [tethered at the membrane by its N-myristoylation and S-palmitoylation (see above)], explaining its lack of efficacy. Third, even short truncations (e.g. 55 residues) of the N terminus of Sst2 also ablate its function (263). Sst2 and its fungal homologs (FlbA and Rgs1) share a high degree of sequence similarity in this region (residues 1–300 in Sst2), and as was noted previously, this segment of Sst2 possesses weak homology to a portion of the region of mammalian $p120^{Ras\text{-}GAP}$ that is both necessary and sufficient for its GAP activity (263). Moreover, this region of Sst2 contains a second potential DEP domain (residues 50–135). The function of the N-terminal region of Sst2 also remains to be explored. In this regard, however, it is noteworthy, first, that the dominant gain-of-function alleles reside in this region and, second, that Sst2 appears to undergo endoproteolytic cleavage (mainly between Ile413 and Ser414) in vivo to yield separate N- and C-terminal domains (278). When coexpressed, the N- and C-terminal halves display at least partial function (as judged by the degree of amelioration of the pheromone-hypersensitive phenotype of *sst2*Δ cells), whereas the C-terminal (RGS) half alone, although stably expressed, does not (278).

A related question about $Sst2^{RGS}$ (and for that matter about other RGS proteins) is how it is itself regulated. *SST2* is a pheromone-inducible gene, and *SST2* mRNA level (264) and Sst2 protein level (263) both increase markedly on prolonged receptor stimulation. This behavior suggests that pheromone-induced accumulation of Sst2 provides a built-in feedback mechanism for limiting signaling to a restricted time window. $Sst2^{RGS}$ also undergoes posttranslational phosphorylation (279). Phosphorylation of one site (Ser539) occurs only in response to pheromone stimulation, requires a functional MAPK cascade, and lies in a canonical MAPK consensus sequence, PxSP. Ser539 is located just proximal to a 120-residue insert that interrupts the RGS domain of Sst2 and that resembles the PEST regions found in unstable proteins (280). Notably, phosphorylation at Ser539 appears to slow the overall rate of $Sst2^{RGS}$ turnover (279). Perhaps the insert serves as a proteolytic signal that can be modulated by pheromone-dependent phosphorylation. This kind of control seems physiologically reasonable in the sense that in the absence of pheromone (when Sst2 is not needed), degradation could occur unimpeded, whereas after pheromone induction, a reduction in the rate of Sst2 breakdown should enhance the efficiency of its pheromone-induced accumulation and further promote its ability to inactivate Gpa1.

A putative Sst2-binding protein, Mpt5/Uth4, was identified in a two-hybrid screen and was reported to interact physically with Sst2, as well as with Fus3, Kss1, and Cdc28 (281). An *mpt5*Δ mutation has pleiotropic effects, which include temperature-sensitive growth and a modest increase in pheromone sensitivity. However, more recent work indicates that Mpt5 is an RNA-binding protein of the *Drosophila pumillo* repeat family (282). Mpt5 (also known as Uth4) has a role in chromatin silencing, especially of the ribosomal RNA genes, by enhancing the amount of the Sir3 and Sir4 proteins in the nucleolus versus the amount of

these proteins at telomeres (283). Moreover, the *MPT5* gene was also isolated as a dosage suppressor of a null mutation in a general transcription factor, Pop2/Caf1 (284), that is part of a larger protein ensemble (CCR4-NOT complex) that interacts with the TATAA box-binding protein, TBP (285). These observations suggest that Mpt5 is not a specific regulator of Sst2 at all but rather may be required for efficient packaging or nucleocytoplasmic transport of mRNA-containing ribonucleoprotein particles, or some other function that affects yeast gene expression globally.

Regulators of STE4$^{G\beta}$-STE18$^{G\gamma}$ Because free G$\beta\gamma$ (Ste4-Ste18) is an essential trigger for initiating various aspects of the pheromone response pathway (Figure 2), it seems reasonable that, like Gpa1$^{G\alpha}$, it should be a target for regulation.

In mammals, cells contain a G$\beta\gamma$-binding protein, first discovered in the retina, dubbed phosducin (286–288). Unphosphorylated phosducin binds to G$\beta\gamma$ in vitro, but phosphorylation reduces this affinity, which potentially provides a mechanism in vivo for reversibly sequestering G$\beta\gamma$ and presumably inhibiting its interaction with effectors and/or its ability to recouple to its cognate Gα. The structural basis for this switch has been determined (289). The *S. cerevisiae* genome encodes two apparent phosducin homologs, Plp1 and Plp2 (290). GST fusions to Plp1 and Plp2 can fish G$\beta\gamma$ (Ste4-Ste18) out of cell extracts, and binding is enhanced by pheromone stimulation of the cells prior to preparing extracts and by addition of GTPγS to the extracts, which are conditions that favor dissociation of G$\beta\gamma$ from Gα. Cells overexpressing either *PLP1* or *PLP2* exhibit a substantial (70%–80%) decrease in expression of a pheromone-inducible reporter gene (*FUS1-lacZ*), yet there is no effect on pheromone-imposed growth arrest. These data indicate that Plp1 and Plp2 can regulate early G$\beta\gamma$-dependent signaling events selectively. However, a *plp1*Δ mutant is viable and, unlike an *sst2*Δ mutant, exhibits only a very modest increase in pheromone-mediated gene induction. Moreover, unlike the *SST2* gene, neither *PLP1* nor *PLP2* is pheromone inducible. Furthermore, a *plp2*Δ mutation is lethal, and cell viability is not restored by a mutation (e.g. *ste7*Δ) that should disrupt the ability of the pheromone pathway to induce G1 arrest, indicating, first, that lack of growth is not due to constitutive signaling and, second, that Plp2 must have another essential function in the cell (290). Whether agonist-induced phosphorylation of Ste4 prevents G$\beta\gamma$ binding to Plpl and/or Plp2, or whether Plp1 and/or Plp2 is regulated by phosphorylation (like mammalian phosducin), have not yet been explored.

Another purported G$\beta\gamma$-binding protein is Syg1. A truncated mutant form of Syg1, designated SYG1-1, was isolated in a screen for dosage suppressors of a *gpa1*Δ mutant (291). When overexpressed, the truncated Syg1 protein also suppresses normal pheromone signaling, as well as the constitutive signaling caused by overproduction of Ste4$^{G\beta}$. However, normal Syg1, when overexpressed, is a weak suppressor of *gpa1*Δ, and a *syg1*Δ mutation has little or no effect on pheromone response or mating. The Syg1 protein has eight predicted membrane-spanning domains and, based on sequence homology, is clearly a member of a group of plasma membrane transport proteins known as divalent anion:Na^+ symporters

(DASS family; more commonly called the phosphate permease family) (292, 293). Hence, the ability of this polytopic membrane protein to interact with G$\beta\gamma$ was presumably unmasked by its truncation, and hence, normal Syg1 almost certainly does not play any physiologically relevant role as a regulator of G$\beta\gamma$ (Ste4-Ste18).

Another apparent G$\beta\gamma$ regulator is Akr1 (294, 295), which we encountered above in its role in contributing to phosphorylation of pheromone receptors by conveying potentially prenylated protein kinases (Yck1 and other casein kinase I isoforms) to the plasma membrane. Like Syg1, Akr1 contains multiple predicted membrane-spanning domains and is a membrane-localized protein. Like SYG1-1, overexpressed Akr1 suppresses the growth arrest phenotype of *gpa1*Δ cells or of cells overproducing G$\beta\gamma$. In contrast, overexpressed Akr1 cannot block activation of the pathway downstream of G$\beta\gamma$, for example pathway stimulation caused by an activated allele of Ste20^{PAK}. As judged by two-hybrid analysis, Akr1 can interact with free G$\beta\gamma$, but not with the G$\alpha\beta\gamma$ heterotrimer (294, 295). Mutations in *AKR1* display synthetic lethality with a weak *gpa1* allele and increase expression of a pheromone-inducible gene (*FUS1*), which is blocked by mutations in downstream components (e.g. *ste7*Δ) of the pathway. These findings suggest that Akr1 normally (and not just when overexpressed) contributes to constraining signaling. However, both haploid and diploid cells (which are nonresponsive to mating pheromone) that lack Akr1 grow slowly and display deformed buds or projections, which suggests that Akr1 has a function that is necessary for the proper control of cell shape and is separate from any role in the pheromone response pathway. Indeed, the morphological abnormalities of *akr1*Δ cells are not rescued by mutations in the pheromone response pathway (294, 295). Although the function of Akr1 has not been elucidated, one possible role (which was proposed above) is that Akr1 serves as the long-sought receptor necessary for the recognition of prenylated proteins for their delivery and insertion into the plasma membrane. It should be recalled that in addition to Gγ (Ste18), which is farnesylated, at least one key regulator of cell polarity and cell shape, Cdc42, is geranylgeranylated (115). Thus, the ability of overexpressed Akr1 to squelch signaling could merely reflect its ability to bind and sequester G$\beta\gamma$ simply because the γ subunit is prenylated.

Finally, a potential G$\beta\gamma$ regulator has been identified from analysis of a regulatory phenomenon, called receptor inhibition (296). The observation is that pheromone signaling is blocked when the **a**-factor receptor (Ste3) is expressed inappropriately in **a**-cells (297). Function of the *ASG7* gene, which is expressed in an **a**-cell–specific manner and is also highly induced by α-factor, is required for the receptor inhibition phenomenon (120). Asg7 apparently acts by redirecting Ste3 and G$\beta\gamma$ (Ste4-Ste18) from the plasma membrane to an internal compartment where they cannot contribute to signaling. Such action may be appropriate during cell fusion, when Ste3 (from the α-cell) and Asg7 (from the **a**-cell) are at a least transiently expressed in the same diploid zygote, and signaling must be stopped to permit resumption of cell cycling. In agreement with this suggestion, mating of *asg7*Δ mutants produces diploid zygotes that continue to form a mating projection and are slow to reinitiate vegetative growth (120, 298). The mechanism

by which Asg7 promotes redistribution of G$\beta\gamma$ from the plasma membrane to the still ill-defined internal location is not yet understood.

Regulation of the Protein Kinase Cascade

STE20 (p21-Activated Protein Kinase) As discussed earlier and summarized here, the primary positive regulator of $Ste20^{PAK}$ is the small GTPase, Cdc42 (82). As mentioned above, Cdc42 has well characterized roles in cell morphogenesis, in particular actin rearrangements required for the polarized cell growth that is necessary for formation of the bud in vegetatively growing cells and for extension of the mating projection in pheromone-treated cells (299, 300). The activity of Cdc42, both temporally and spatially, depends, in turn, on the activity and subcellular localization of its GEF, Cdc24, and its GAPs (Bem3, Rga1, Rga2) (Figure 2*B*). In naïve cells, most of the cellular content of Cdc24 is bound to the scaffold protein, Far1, which is itself located predominantly in the nucleus, although there is always a small pool of both Far1 and Cdc24 located at the emerging edge of the bud (91, 94, 301). Far1, however, continuously shuttles in and out of the nucleus and is a G$\beta\gamma$-binding protein (90, 91, 93, 95). Hence, on occupancy of pheromone receptors and the release of significant amounts of free G$\beta\gamma$, the amount of Far1 and its cargo of Cdc24 that are tethered to the plasma membrane at the incipient projection tip increases dramatically (94, 95, 301). The deposition of Far1 and Cdc24, in turn, establishes a landmark for localized activation of Cdc42. Indeed, certain mutations in Cdc42 (99), or mutations in $Cdc24^{GEF}$ that block its binding to Far1 (or vice versa) and, hence, to G$\beta\gamma$ (Ste4-Ste18) (84, 90), prevent oriented growth of the projection toward a source of pheromone.

This chemotropic growth does not require $Ste20^{PAK}$, Ste5, or any of the three component protein kinases of the MAPK module (302), which suggests, first, that localized G$\beta\gamma$-dependent recruitment of $Cdc24^{GEF}$ (via Far1) and activation of Cdc42 are sufficient to dictate the cytoskeletal changes necessary for projection formation and, second, that $Ste20^{PAK}$, Ste5, and the MAPK cascade are responsible primarily for eliciting other aspects of pheromone response, such as transcriptional regulation and cell cycle arrest. Consistent with the view that activation of Cdc42 is crucial for all aspects of the pheromone response pathway are the observations that certain loss-of-function alleles of $Cdc24^{GEF}$ and Cdc42 block both projection formation and transcriptional response (81, 82, 303) and, conversely, that cells that overexpress Cdc42 or that contain a GTPase-deficient form of Cdc42 (81, 82), or that lack $Rga1^{Cdc42\text{-}GAP}$ (304), exhibit a marked increase in pheromone-dependent gene transcription.

Cdc42 regulates transcription and cell division through $Ste20^{PAK}$. In vitro, Cdc42-GTP binds to and stimulates Ste20 (81, 82). In response to pheromone, Cdc42 is able to potently activate Ste20 and presumably does so in a highly localized manner because, like Cdc42 itself, Ste20 becomes concentrated at the projection tip by virtue of the fact that the C terminus of Ste20 has a high-affinity binding site for G$\beta\gamma$ (117). In other words, Ste20 is itself a G$\beta\gamma$ effector,

and its ability to interact with both Cdc42 and $G_{\beta\gamma}$ makes mutually reinforcing contributions to its locations at the projection tip. Thus, $G\beta\gamma$ recruitment of both Cdc24 (via Far1) and Ste20 bring this target enzyme together with its activator (Cdc42) at high local concentration. Analysis of Ste20 deletion mutants lacking the Cdc42-binding site (CRIB domain) indicated that Cdc42 interaction was not necessary for pheromone-induced gene expression (107, 108); however, these results were misleading because, based on both genetic criteria (116) and structural information (110), such Ste20 deletions, in effect, relieve the inhibitory constraints of the N-terminal regulatory domain of the enzyme (the structural basis for Cdc42-dependent activation of Ste20, discussed earlier). Although Cdc42 and $G\beta\gamma$, by virtue of their prenylated C termini, anchor Ste20 at the projection tip and, by virtue of their direct interaction, alter the conformation of Ste20, these changes are likely to be necessary, but not sufficient, to fully activate the kinase activity of Ste20. This supposition is based on the fact that in addition to binding of Cdc42, catalytic competence of mammalian PAKs is achieved only after phosphorylation of a residue in the activation loop of the kinase domain (112). At least one mammalian enzyme able to carry out this phosphorylation is PDK1 (113). Yeast cells possess two gene products (Pkh1 and Pkh2) that are functional homologs of PDK1 (305). However, it is not known whether either of these protein kinases is a physiologically relevant activator of Ste20. Also, the phosphatase(s) responsible for deactivation of Ste20 is not known. In any event, once activated, Ste20 is able to phosphorylate downstream targets, which include Myo3 (306), a myosin-I–type molecule that can recruit the Arp2-Arp3 complex and nucleate actin polymerization (307), which may contribute to the efficiency of projection formation. Another target of Ste20 vital for the pheromone response pathway is $Ste11^{MAPKKK}$ (122, 123), the first protein kinase of the three-tiered MAPK module.

Another protein that interacts with Ste20 and is a potential positive regulator of its localization (116) and/or activity is Bem1 (308, 309). Bem1 contains three domains found in other adapter proteins: two N-terminal SH3 (src-homology-3) domains (310) and a C-terminal phox homology (or PX) domain (311). Bem1 reportedly interacts with both Ste20 and Ste5, and *bem1* mutants display reduced pheromone-induced responses (312, 313). Moreover, *bem1* mutants that are compromised for both signaling and pheromone-induced polarized morphogenesis interact with Ste5 (and with actin) but do not interact with Ste20, which suggests that Bem1 may modulate the efficiency with which Ste20 gains access to Ste11 bound on Ste5 (116) and perhaps other targets (e.g. Myo3 contains a Pro-rich region that might represent an SH3 domain-binding site).

It has been reported that a putative protein-arginine methyltransferase, Hsl7 (314, 315), acts as a negative regulator of Ste20, perhaps by competing for Cdc42 binding (316). However, this conclusion is at odds with recent evidence that the primary function of Hsl7 is as a negative regulator of another protein kinase, the *S. cerevisiae* Wee1 homolog, Swe1 (317). Hsl7-dependent inactivation of Swe1 is an essential reaction in a morphogenetic checkpoint that links

septin assembly to activation of the B-type cyclin (Clb)-bound forms of Cdc28 (318–320).

Ste20 is subject to phosphorylation by the G1-type cyclin (Cln1 and Cln2)-bound forms of Cdc28 (321–323). If this phosphorylation negatively regulates Ste20, as seems to be the case, this finding may explain the mechanism by which overexpression of either Cln1 or Cln2 is able to squelch pheromone signaling (324). This kind of control mechanism makes physiological sense. If the cell has made the commitment to progress through G1 by synthesizing sufficient levels of Cln1- and Cln2-bound Cdc28, it would simultaneously provide a mechanism to override a signal (pheromone) that would threaten to provoke arrest in G1 and inappropriate morphological changes. In this regard, genetic evidence indicates that Pog1 (a candidate transcriptional activator) promotes recovery through up-regulation of the *CLN2* gene and that the resulting Cln2 protein promotes recovery primarily by enhancing Cdc28-dependent phosphorylation of Ste20 (325).

STE11 (Mitogen-Activated Protein Kinase Kinase Kinase) Ste11 has a long N-terminal regulatory domain and a C-terminal kinase domain. Biogenesis and stability of Ste11 require the molecular chaperone Hsp90 (326) and its cofactor chaperone, Cdc37 (327). It has been known for nearly 20 years (328) that stable expression and membrane association of various protein kinases in animal cells (e.g. Src family members) also require Hsp90 (for a recent example, see 329). So, in one sense, Hsp90 and Cdc37 act as positive regulators of Ste11. However, sequestration with chaperones may act to restrict the number of Ste11 molecules in the cellular pool that are available for signaling and, hence, have a negative regulatory function as well.

At least three proteins that interact with the N terminus of Ste11 should compete with the chaperones for association with Ste11. The first of these proteins is Ste50 (330). Ste50 is required for optimal pheromone response and mating (331), but it is also required in the other MAPK pathways in which Ste11 is involved (Figure 3), including the invasive growth pathway (332) and the high osmolarity stress reponse pathway (333, 334). Ste50 is a relatively small protein (39 kDa) and contains at its N terminus a sterile alpha motif (SAM), which is found in numerous other signal transduction proteins (335, 336), including close relatives (p63 and p73) of the mammalian tumor suppressor protein p53 (337). SAMs are protein-protein interaction domains. In a limited set of other proteins for which structural information is available (338–341), SAM domains mediate homodimerization. The N terminus of Ste11 also has an obvious SAM domain, and hence, the ground state of Ste11 may be a homodimer, although to our knowledge this possibility has not been directly examined. However, it is clear that Ste50 interacts stably with Ste11 and does so via heterotypic association of their respective SAM domains (332, 342). Hence, just as Cdc42 binding may disrupt $Ste20^{PAK}$ dimers, binding of Ste50 to Ste11 may dissociate Ste11 dimers. Another effect of Ste50 binding may be to cause a conformational change in the otherwise inhibitory

N-terminal domain of Ste11 (343) that makes the kinase domain more accessible for activation by Ste20.

Indeed, both in vivo and in vitro, Ste20 phosphorylates Ste11 (122). Therefore, Ste20 and Ste11 represent an enzyme-substrate pair; hence, Ste20 represents the second-known Ste11-interacting protein. Ste20 phosphorylates Ser302 (and/or Ser306) and Thr307, which are residues conserved at the equivalent positions in the N-terminal regulatory domains of MAPKKs (MEKKs) from other organisms (123). Mutation of these sites to nonphosphorylatable Ala residues abolished Ste11 function, whereas mutation to Asp (to mimic the negative charge introduced by phosphorylation) constitutively activated Ste11 in vivo in a Ste20-independent manner. Moreover, the N-terminal regulatory domain of Ste11 interacts with its own C-terminal catalytic domain, as judged by both two-hybrid analysis (342) and direct biochemical experiments (123). In addition, overexpression of a small amino-terminal fragment of Ste11 was able to inhibit signaling in response to pheromones. These results suggest that Ste20-dependent phosphorylation of Ste11 by Ste20 alleviates an inhibitory effect of the N-terminal regulatory domain of Ste11, which may represent a general mechanism for positive regulation of MEKKs by PAKs. However, whether Ste11 also requires phosphorylation of a residue in the activation loop of the kinase domain for activity and, if so, what the nature is of the protein kinase that introduces this activating phosphorylation are still open questions. There is some evidence that Ste11 is capable of autophosphorylation (342); however, to our knowledge, it has not been demonstrated that this self-phosphorylation is sufficient (or required) for activation of the enzyme. Moreover, the phosphatase(s) responsible for deactivation of Ste11 is not known.

The third protein that interacts with Ste11 is Ste5 (40). Cells carrying *ste5* mutations are completely sterile, but such mutations have no other obvious effects on growth or the ability of cells to respond to other signals (344). Ste5 is a large protein (103 kDa) capable of binding each of the three constituent protein kinases of the MAPK module for mating ($Ste11^{MAPKKK}$, $Ste7^{MAPKK}$, and $Fus3^{MAPK}$), as first shown by two-hybrid analysis (142, 345, 346) and confirmed by biochemical methods (142, 143, 144, 147). As demonstrated by two-hybrid analysis, the N terminus of Ste11 is required for its interaction with Ste5. Hence, like interaction of Ste50 or phosphorylation by Ste20, association of Ste11 with Ste5 should block the autoinhibitory interaction between the N terminus of Ste11 and its C-terminal kinase domain. It is not known whether docking of Ste11 on Ste5 and binding of Ste50 are mutually exclusive. However, in this regard, it is noteworthy that *ste50*Δ mutations cause a relatively modest reduction in pheromone response and mating (compared with *ste5*Δ mutants, which are completely sterile) (303, 331), whereas *ste50*Δ mutations block the invasive growth response almost totally (332).

There is some evidence that Ste11 can be negatively regulated via phosphorylation by the Cln2-bound form of Cdc28 (347). However, as discussed above, more recent work from at least two different laboratories indicates that this kind of control is more likely exerted at the level of Ste20 (321, 322).

STE7 (Mitogen-Activated Protein Kinase Kinase) The only known target of $Ste11^{MAPKKK}$ in the mating pathway is $Ste7^{MAPKK}$, a dual-specificity protein kinase (also known as a MEK). Ste11 phosphorylates and activates Ste7 (125, 348) by phosphorylating two residues in the activation loop of Ste7 (Ser359 and Thr363) (349). The positions of these two phosphorylation sites are highly conserved in all members of the MEK family. Mutation of these residues to Ala completely abolishes the biological function of Ste7 (349). Conversely, mutation of these residues to Asp in mammalian MEKs (350) or to Glu in Ste7 (351) converts the enzyme to a constitutively active form (but less well than does authentic phosphorylation). The phosphatase(s) responsible for deactivation of Ste7 is not known.

The only known function of Ste7 is to activate the MAPKs Fus3 and Kss1 by phosphorylating both a Tyr and Thr residue in the activation loops of these enzymes (127–129, 153, 348, 352). In addition to this enzyme-substrate relationship between Ste7 and the MAPKs (the kinase domain of Ste7 resides at its C terminus), Ste7 is also a highly specific and high-affinity MAPK binding protein (352). A short motif (-K/R-R/K-X_{2-6}-L/I-X-L/I-) at the N terminus of Ste7, dubbed the MAPK docking site, is highly conserved in MEKs (353) and is responsible for the specific association of Fus3 with Ste7 and of mammalian ERKs with their cognate MEKs (354). It is now appreciated that the MAPK docking motif first identified in Ste7 is conserved not only in MEKs but also in other signaling proteins and confers upon them the ability to bind MAPKs with high affinity (355–357).

The other known protein that interacts with Ste7 is Ste5, as shown by both two-hybrid analysis (345, 346) (142) and biochemical methods (143, 144, 147). Ste7 interacts with Ste5 via its C-terminal kinase domain, which presumably leaves its N-terminal end free to participate in the MAPK docking interaction with Fus3. The interaction of Ste7 with Ste5 is required for efficient signal transmission. Mutations in Ste5 that reduce its apparent affinity for Ste7 about 30-fold reduce mating proficiency by three orders of magnitude, as judged by quantitative mating assays (143). In contrast, mutations that eliminate the MAPK docking site in Ste7 reduce mating only modestly (352). Likewise, mutations in the Ste7-binding site of Ste5 that reduce its apparent affinity for Ste7 less than 10-fold have a barely discernible effect on mating proficiency (143). However, if the two types of mutations are combined in the same cell, signal propagation is almost totally eliminated and mating proficiency is drastically reduced (354). This result suggests that the MAPK docking interaction and the scaffold function of Ste5 make mutually reinforcing contributions to the efficiency of signaling in this MAPK cascade. The interaction of Ste7 with Ste5 also seems to contribute to the fidelity of signal transmission. For example, an activated Ste7 allele, Ste7(S368P), stimulates the mating pathway weakly, but not the MAPK of the cell integrity pathway, Mpk1/Slt2 (which is normally phosphorylated by two dedicated MEKs, Mkk1 and Mkk2); however, in the absence of Ste5, Ste7(S368P) stimulates Mpk1/Slt2 sufficiently well to bypass the need for its normal upstream activators (358). However, this bypass might be due to differential activation of Kss1 (over Fus3) by Ste7 (S368P) because Kss1 action contributes to cell wall integrity regulation (140).

FUS3 (Mitogen-Activated Protein Kinase) Fus3 (and Kss1) are Ser/Thr-specific protein kinases. Fus3 has multiple targets in both the cytosol and the nucleus. As discussed in previous sections, Fus3-dependent phosphorylation leads to stabilization of Sst2 (279) and to destabilization of Ste3 (187). Fus3 also phosphorylates Ste11 (348), Ste7 (343, 351, 352, 359), Ste5 (157), Far1 (153, 155), the transcription factor Ste12 (153), and the Ste12 repressors, Dig1 and Dig2 (160, 161). There are likely to be additional, as yet unidentified, targets (360). Recent advances in the use of mass spectrometry for proteomics should make it feasible to perform a global analysis on *S. cerevisiae* cells to determine all proteins that are phosphorylated in a pheromone- and Fus3-specific manner (361).

Fus3 also associates with Ste5, as demonstrated by two-hybrid analysis (142, 345, 346) and confirmed by biochemical methods (143, 144, 147). Kss1 can also associate with Ste5 (142, 345, 346), and in the absence of Fus3, Kss1 can replace Fus3 as the MAPK for the pheromone response pathway because *fus3*Δ *KSS1* cells are mating competent, whereas *fus3*Δ *kss1*Δ cells are not (128, 137, 138). However, in the presence of Fus3, Kss1 normally has a more modest role in propagation of the mating signal (132, 133, 139). In fact, in coimmunoprecipitation experiments from extracts of normal cells, Fus3 is found stably associated with Ste5 (147), whereas the amount of Kss1 bound is undetectable (C Sette & J Thorner, unpublished results).

Once Fus3 is activated by Ste7-dependent phosphorylation, a number of factors help to limit the active lifetime of the MAPK. One particularly important mechanism involves dephosphorylation of either Tyr or Thr, which inactivates Fus3. Two phosphotyrosine-specific phosphoprotein phosphatases, Ptp3 (mainly) and Ptp2 (to a much lesser extent), clearly regulate Fus3 in this manner (362). Mutations in Ptp3 or Fus3 that abolish their interaction lead to unregulated kinase activity and delay recovery after pheromone stimulation (363). In addition, a dual-specificity phosphatase, Msg5, also contributes to turning off Fus3 activity. The *MSG5* gene is highly pheromone inducible, and cells carrying a *msg5*Δ mutation display increased pheromone sensitivity and increased steady state Fus3 phosphorylation both before and after pheromone treatment (362, 364), which suggests that Msg5 action contributes to adaptation and recovery. Fus3 was the first example of a MAPK downregulated via the coordinate actions of both phosphotyrosine-specific and dual-specificity phosphatases (362). Msg5 also downregulates other *S. cerevisiae* MAPKs, including Kss1 (365), Slt2/Mpk1 (366, 367), but evidently not Hog1 (366). Likewise, the Ptp3 and Ptp2 phosphatases have differential efficacy in dephosphorylating the various MAPKs. For example, in contrast to Fus3, Ptp2 (mainly) and Ptp3 (somewhat) are responsible for dephosphorylating Hog1 (368) and Mpk1/Slt2 (369).

Regulation of Scaffold Protein Localization and Stability

FAR1 An important substrate of Fus3 (and, more weakly, Kss1) is Far1 (153, 154). Far1 is degraded in a ubiquitin- and proteasome-dependent manner, and

Fus3-dependent phosphorylation leads to its stabilization (370). It was originally thought that this phosphorylation-dependent stabilization was an important aspect of one of the apparent functions of Far1 in the nucleus, which is to serve as a direct inhibitor of the Cln1-, Cln2-, and Cln3-bound forms of $Cdc28^{CDK}$ (96, 154, 155, 371). However, the mechanism of inhibition of Cln-bound Cdc28 by Far1 is still a matter of some controversy (97), and it even appears that the mating pathway can also lead to inhibition of a B-type cyclin (Clb5)-bound form of Cdc28 (65, 372). Moreover, it is now appreciated that Fus3-dependent phosphorylation stabilizes Far1, because this modification greatly stimulates the nuclear export of Far1 via a specific export receptor (Msn5/Ste21) (93) and because Far1 is only subject to degradation in the nucleus (373). The Msn5/Ste21 exportin is also required for the stimulus-induced exit of many other nuclear proteins regulated by phosphorylation (374–376).

Ubiquitination of Far1 is mediated by a Skp1-Cdc53/cullin-1-F-box protein (SCF) complex, which also contains (*a*) a ubiquitin conjugating enzyme, Cdc34; (*b*) another subunit, Hrt1, which acts as a protein:ubiquitin ligase (or E3) to transfer ubiquitin to its ultimate target; and (*c*) an F-box–containing subunit, Cdc4, that acts as selectivity factor to dictate substrate specificity (373, 377). Because Cdc4 is normally confined to the nucleus, SCF^{Cdc4} can only target Far1 for degradation in the nucleus; consequently, once exported from the nucleus, Far1 is stable (373). It has recently been appreciated that two specific types of Zn^{2+}-binding motif, called RING and RING-H2 domains, previously thought to be exclusively involved in protein-protein interactions, are diagnostic of a class of protein:ubiquitin ligases distinct from the HECT (homologous to the E6 C terminus) class of E3s (378–380). The Hrt1 subunit of SCF^{Cdc4} is such a RING-H2 domain–containing E3. Curiously, the N terminus of Far1 also possesses a canonical RING-H2 domain (residues 202–251). Other RING and RING-H2 E3s carry out self-ubiquitination, as well as ubiquitination of their targets (378–380). Hence, it is somewhat puzzling that ubiquitination and turnover of Far1 involves SCF^{Cdc4} rather than an autocatalytic process. Perhaps the RING-H2 of Far1 acts to modify some of its binding partners (e.g. $Cdc24^{GEF}$) to regulate their rate of degradation.

Phosphorylation of many targets appears to be a prerequisite to their recognition and modification by SCF complexes (378, 381, 382). Far1 is no exception. In fact, consistent with degradation confined to the nucleus, Far1 is also a substrate of $Cdc28^{CDK}$, which is located almost exclusively in the nucleus (383), and Cdc28-dependent phosphorylation leads to ubiquitin-mediated Far1 degradation (370, 384). Presumably, this type of feedback phosphorylation of Far1 by Cdc28 would help to eventually overcome Far1-imposed inhibition of Cdc28, allowing resumption of cell cycle progression. Although this situation may seem somewhat paradoxical, it has also been observed that mammalian CDK inhibitors are often substrates for the kinases they inhibit (385, 386).

As mentioned earlier, the cellular role of Far1 has expanded with the discovery that it ferries $Cdc24^{GEF}$ out of the nucleus and can bind $G\beta\gamma$ (Ste4-Ste18) (90, 91). Cells expressing Far1 mutants unable to bind $G\beta\gamma$ are defective in

orienting their growth toward a mating partner (90). When most $Cdc24^{GEF}$ is sequestered in the nucleus with Far1, growth is prevented presumably by the lack of sufficient activation of the Cdc42 GTPase. Copious release of $Cdc24^{GEF}$ into the cytosol can be triggered either by entry into the cell cycle (via activation of Cln-Cdc28 complexes) or by mating pheromone (via activation of $Fus3^{MAPK}$) (94, 95). In the former case, Cdc28-dependent phosphorylation of Far1 leads to its degradation by the mechanism described immediately above; in the absence of its nuclear tether, Cdc24 is free to exit the nucleus (until sufficient Far1 is resynthesized). In the latter case, Fus3-dependent phosphorylation of Far1 stimulates Msn5/Ste21-mediated nuclear export of Far1 (and its bound cargo of $Cdc24^{GEF}$) (93). As expected for any process that requires nucleocytoplasmic transport, nuclear export of Far1 requires the essential *S. cerevisiae* homolog of the Ran GTPase, Gsp1 (387, 388). Cells lacking Msn5/Ste21 fail to export Far1 in response to pheromone and exhibit a partial mating defect, although they are still able to undergo growth arrest (93), as expected if Far1 functions in the nucleus as a CDK inhibitor. Conversely, Msn5/Ste21 overexpression drives Far1 export even in the absence of pheromone. However, cells lacking Far1 are able to form mating projections, albeit misoriented, which suggests that another Msn5/Ste21 target that helps to direct highly polarized cell growth must be exported during mating (93). Indeed, pheromone-stimulated export of Ste5 also requires Msn5/Ste21 (146) (see below).

STE5 Another effector of $G\beta\gamma$ (149–151) and substrate of Fus3 (157) is the Ste5 scaffold protein. Biochemical (151, 152) and genetic (143, 150, 152) methods indicate that Ste5 also self-associates and exists as an oligomer in cell extracts. Ste5 mutants that cannot bind to $G\beta\gamma$ act as though they are unable to oligomerize (150) and are unable to transmit the pheromone signal from $Ste20^{PAK}$ to $Ste11^{MAPKKK}$ (150, 151). However, Ste5 oligomerization does not appear to be regulated by pheromone or by any known Ste5 binding partner (147, 151). Although multimerization of Ste5 may be necessary for signaling, it is not sufficient (151). A forced dimer of Ste5 (Ste5-GST) will complement a *ste5*Δ strain, but not a *ste4*Δ *ste5*Δ strain (150), whereas a Ste5-GST mutant altered in its $G\beta\gamma$-binding domain complements both *ste5*Δ and *ste4*Δ Ste5cells (150), which suggests that $G\beta\gamma$ interaction with preexisting Ste5 multimers alleviates some negative constraint in Ste5 that promotes signaling (147). In this regard, mutational and biochemical analysis suggests that binding of $G\beta\gamma$ may induce a conformational change in Ste5 that promotes association of its N- and C-terminal domains, enhancing Ste20-dependent phosphorylation of $Ste11^{MAPKKK}$ and subsequent activation of $Ste7^{MAPKK}$ and $Fus3^{MAPK}$ (147).

Pheromone treatment and Fus3-dependent phosphorylation stimulate the nuclear export of Ste5 (145, 146). Cytosolic Ste5 becomes rapidly tethered to the tip of the mating projection (145, 146) in a manner that depends on free $G\beta\gamma$ (147; N Dhillon, C Inouye, C Sette, IG Macara, & J Thorner, submitted for publication). If Ste5 lacks its $G\beta\gamma$ binding domain, or if $G\beta\gamma$ is not expressed, Ste5 cannot signal (150, 151) and remains in the nucleus (145–147; N Dhillon, C Inouye,

C Sette, IG Macara, & J Thorner, submitted for publication), at least when Ste5 is overexpressed. However, if Ste5 is fused to a membrane-targeting domain, it can signal in the absence of G$\beta\gamma$ (145), which indicates that in addition to inducing a conformational change (as described above), another primary role of the G$\beta\gamma$-Ste5 interaction is to tether Ste5 to the plasma membrane. It has been reported that a Ste5 deletion mutant that lacks a putative, highly basic N-terminal nuclear localization signal remains in the cytoplasm but cannot signal (146). On this basis, it was suggested that translocation of Ste5 to the cytosol was not sufficient for signaling and, therefore, that cycling of Ste5 through the nucleus was required for it to become competent for membrane recruitment. However, the deletion used destroys sequences in Ste5 that are necessary for its N- and C-terminal domains to interact (147); hence, the deletion mutant may simply be nonfunctional for that reason. Moreover, there is compelling genetic and cytological evidence that reimport of Ste5 is a significant contributing factor to downregulation of the signaling pathway (148), which is consistent with the view that once in the cytosol, Ste5 is active for signaling.

Another means by which the Ste5 scaffold protein contributes to signal propagation is by holding the various components of the signaling pathway at high local concentration and presumably optimizing their relative orientation in a way that enhances their sequential interaction, thereby maximizing the efficiency of signaling (151, 343). Since the discovery of Ste5, analogous scaffold proteins for MAPK cascade components have been found in mammalian cells (41, 389, 390). Consistent with this model, point mutations in Ste5 that eliminate only its ability to bind Ste7 or Ste11, respectively, drastically reduce mating proficiency (143). Likewise, mutations in either Ste5 (143, 151) or Ste20^{PAK} (117) that alter only their ability to bind G$\beta\gamma$ result in sterility. The surface residues on the Gβ subunit (Ste4) of the G$\beta\gamma$ heterodimer, which is a donut-shaped toroid (391, 392), that mediate its interaction with Ste20 and Ste5, respectively, are different, but overlapping (117, 393). Thus, mutual G$\beta\gamma$-mediated membrane recruitment of Ste20 and Ste5 achieves assembly of, and cooperative interactions between, multiple components that would otherwise reside in different subcellular compartments at different times. It is not clear, however, whether a single G$\beta\gamma$ can bind both Ste5 and Ste20 simultaneously, or whether Ste5 and Ste20 compete for the available pool of free G$\beta\gamma$.

Yet another way that Ste5 may promote signaling is by facilitating activation of Ste11, as mentioned earlier in the discussion of Ste11. Like other members of the MAPKKK family, Ste11 has a large N-terminal domain that clearly plays a negative autoinhibitory role, because deletion of or mutations in this region leads to a constitutively active kinase (343, 359). The N-terminal domain of Ste11 mediates its interaction with Ste5 (142, 345, 346). Hence, Ste5-Ste11 association may assist in relieving autoinhibition of the C-terminal kinase domain, promoting Ste20-dependent phosphorylation of Ste11, Ste11 autophosphorylation, and subsequent Ste11-dependent phosphorylation of Ste7^{MAPKK} (342, 343, 345, 359). It was observed previously that Ste5 seemed to bind preferentially to an inactive hypophosphorylated form of Ste7 (142), which suggests that phosphorylation and

activation of Ste7 may lead to its dissociation from Ste5. However, it is clear that active and hyperphosphorylated forms of Ste7 are fully capable of binding to Ste5 (147). In any event, it appears that the most active pool of cellular $Fus3^{MAPK}$ exists in a complex of 350–500 kDa that contains, at least, Ste11 and Ste7, as well as Ste5 (142, 144, 157). Because of the high-affinity MAPK docking site on Ste7 (mentioned above in the discussion of Ste7), stable Ste7-Fus3 complexes can be readily isolated from cell extracts, are not detectably diminished in extracts from *ste5*Δ (or *ste11*Δ) cells, and are not disrupted by the activated state of either enzyme (352). These latter observations suggest that if phosphorylation and activation cause ejection of Ste7 and Fus3 from Ste5, this MAPKK and its target MAPK may dissociate as a heterodimeric pair. Because Ste5 is a target for phosphorylation by both Ste20 (C Sette & J Thorner, unpublished results) and Fus3 (157), it is possible that modification of Ste5 itself, in addition to controlling its nucleocytoplasmic transport, may also modulate its affinity for one or more of its binding partners or its own stability.

Finally, it seems likely that Ste5 has a function in the branch of the pheromone response pathway that leads to changes in morphology, in addition to its generally accepted functions in the branch that leads to activation of the MAPK cascade. Although G$\beta\gamma$ is responsible for recruiting Far1 and $Cdc24^{GEF}$ to the tip of the mating projection, dominant constitutively active alleles of *STE5* can induce projection formation, even in cells that totally lack G$\beta\gamma$ (147). Also, membrane-targeted Ste5, even lacking its G$\beta\gamma$-binding domain, can induce projection (145). Thus, the fact that Ste5 may have a semiredundant role with Far1 in somehow serving as a landmark for establishing a site for polarized growth of the actin cytoskeleton. In fact, many other aspects of the behavior of Ste5 also mirror those described above for Far1. In pulse-chase analyses, Ste5 turnover is almost totally abrogated by the presence of a low-molecular-weight proteasome inhibitor (A Conery, C Sette & J Thorner, unpublished results), which suggests that, like Far1, Ste5 is degraded via a ubiquitin-dependent mechanism. Also, like Far1, the rate of Ste5 degradation is reduced after treatment of cells with pheromone (A Conery, C Sette & J Thorner, unpublished results), which suggests that, once exported into the cytosol, Ste5 is more stable than when it resides in the nucleus. Like Far1, Ste5 has a RING-H2 domain in its N-terminal domain (residues 177–229), which suggests that Ste5 might have E3 activity for either self-ubiquitination or ubiquitination of one or more of its passenger proteins. In any event, the fact that both Far1 and Ste5 are primarily nuclear proteins in the absence of pheromone stimulation prevents these proteins from participating in signaling. This sequestration mechanism creates a situation that ensures that basal signaling is minimal and that adventitious activation is difficult. Thus, both Far1 and Ste5 clearly play much more active roles in signaling than simply acting as scaffold proteins.

Regulation of Transcription

One of the best-documented substrates of $Fus3^{MAPK}$ is the nuclear-localized transcriptional transactivator Ste12 (153, 158, 159). $Kss1^{MAPK}$ is also able to

phosphorylate Ste12 (133, 160, 394). Ste12 is required for expression of pheromone-responsive genes. All the pheromone-inducible and Ste12-dependent genes in the *S. cerevisiae* genome have been identified by the use of DNA microarray technologies (62, 63, 395) and other methods (396, 397). At least a dozen such genes are open-reading frames whose functions have yet to be explored. The DNA-binding domain of Ste12 is a distant relative of the homeodomain (398) and recognizes an eight-base pair motif, called the pheromone response element (PRE), that is tandemly repeated (typically two to four copies) in the promoter regions of pheromone-inducible genes (399–402). Pheromone-inducible genes expressed in both haploid cell types (e.g. *FUS1*) are regulated by Ste12 homooligomers (403), whereas **a**-cell–specific genes responsive to pheromone (e.g. *STE2*) are regulated by Ste12 associated in heterooligomers with Mcm1, the prototype member of the MADS-box (yeast Mcm1, plant Agamous, plant Deficiens, mammalian Serum-Response-Factor) family of DNA-binding proteins (404, 405). For α-cell–specific genes responsive to pheromone (e.g. *MFα2*), regulation requires a ternary complex involving Ste12, Mcm1, and an α-cell–specific homeodomain-containing protein, Matα1 (404, 406).

Induction of a subset of Ste12-dependent and pheromone-inducible genes that are required late in mating for karyogamy, the last step of zygote formation, requires yet another transcription factor, Kar4 (406a). *KAR4* is itself a Ste12-dependent and pheromone-inducible gene. Ste12-dependent genes whose full induction requires cooperation with Kar4 include *KAR3*, which encodes a kinesin specifically associated with the extranuclear microtubule bundle that is required for nuclear congression, and *CIK1*, which encodes a Kar3-associated light chain. Kar4 exists in two forms that are derived from translation of alternative transcripts that contain different AUG initiation codons (406b). The transcript encoding the shorter protein is the one that is pheromone-induced; when expressed at an equivalent level, the longer form is less efficacious in supporting mating. In pulse-chase experiments, the induced (short) form of Kar4 was stable during mating but rapidly turned over in vegetative cells, whereas the constitutively-expressed (long) form is degraded at the same rate in either condition. Overexpression of either form is toxic to vegetative cells. Presumably this elaborate regulation (involving transcriptional induction, alternative translational initiation, and differential protein turnover) reflects the requirement for a high level of the short form of Kar4 late in the mating process and for a much lower level of either form during other growth states.

It is evident from the spectrum of pheromone-induced genes that transcriptional regulation has both positive and negative consequences for signaling and cell cycle control. Many pheromone-induced genes are themselves required for signal propagation, such as the genes encoding the **a**-factor and α-factor precursors, the Ste2 and Ste3 receptors, Fus3, Far1, and the genes for proteins required for agglutination, cell-cell recognition, and cell-cell fusion. Hence, pheromone induction would seem to be a self-reinforcing process. However, as mentioned in each relevant section above, many of the negative regulators of the mating response pathway are

also highly induced by pheromone, including the genes encoding $Gpa1^{G\alpha}$ (acts on $G\beta\gamma$), $Sst2^{RGS}$ (acts on Gpa1), Asg7 (acts on Ste3 and $G\beta\gamma$), Msg5 (acts on Fus3), and Dig2 (acts on Ste12) (see below). Clearly, the kinetics with which each of these factors is induced and accumulates in functional form will determine how long signaling is sustained and will dictate the onset of processes that lead to desensitization and resumption of cell cycling. In any event, it is clear that the transcriptional induction of such an array of negative regulators constitutes a carefully orchestrated program of feedback inhibition that provides the means to allow a haploid cell to resume growth should mating with its partner not be consummated.

Two other substrates of $Fus3^{MAPK}$ critical for transcriptional regulation are Dig1/Rst1 and Dig2/Rst2. These gene products were originally discovered as Kss1 (and Fus3)-interacting proteins that act as negative regulators of the invasive/filamentous growth pathway (160). Like pheromone-responsive genes, genes necessary for invasive growth, such as *FLO11* (407, 408), require Ste12 for their expression. At promoters containing such a filamentous growth response element (FRE), Ste12 acts in conjunction with Tec1, a prototype member of the ATTS family (mold AbaA, yeast Tec1, mammalian TEF-1, Drosophila Scalloped) of DNA-binding proteins (409, 410). However, Dig1 and Dig2 also negatively regulate Ste12 function at PREs (161, 411). Dig1 and Dig2 act by binding directly to and inhibiting Ste12 (160, 161), but they do so by forming ternary complexes that also involve Kss1 (at FREs) (394, 411) and possibly Fus3 (at PREs) (133, 360).

It has been reported that Dig1 binds to sequences within an internal segment (residues 262–594) of the 688-residue Ste12 protein, whereas Dig2 binds instead to the N-terminal DNA-binding domain of Ste12 (162). However, these findings are somewhat at odds with an earlier study, which found that the minimum element of Ste12 necessary for pheromone induction (i.e. the lifting of MAPK- and Dig-mediated repression) was residues 301–355, and that both Dig1 and Dig2 were able to interact with this segment, as judged by the two-hybrid method (412). In any event, seven different Ste12-containing clones isolated as interacting with Kss1 in a genome-wide two-hybrid screen (160) share a common region of Ste12 (residues 298–482). Thus, at least Dig1 and Kss1 interact with the same general region of Ste12, consistent with the need for the juxtaposed contact surfaces that are presumably required for ternary complex formation.

Such ternary complexes are thought to exist because, as documented in the references cited above, the MAPKs bind to Ste12, the Digs bind to Ste12, and the MAPKs bind to the Digs. Evidence that prior to their activation, certain mammalian MAPKs reside in the nucleus prebound to transcription factors has been obtained for Erk5 (413) and Jnk (414, 415). Consistent with the view that the yeast MAPKs act as direct repressors in their unactivated state, a *fus3*Δ *kss1*Δ double deletion or a *dig1*Δ *dig2*Δ double deletion is each sufficient to constitutively derepress expression at FREs, but only a *dig1*Δ *dig2*Δ double deletion causes constitutive derepression at PREs. These findings suggest that at FREs, loss of the MAPKs from the ternary complex is sufficient to cause dissociation of the Digs and to permit Ste12-Tec1 to activate transcription, whereas at PREs dissociation of the

Digs from Ste12-Ste12 (and Ste12-Mcm1) complexes requires MAPK-dependent phosphorylation of Ste12 and/or the Digs (or both) (411). Both Fus3 and Kss1 are able to phosphorylate Ste12 and the Dig proteins in vitro (153, 160, 161).

Normally, during pheromone response, what event triggers dissociation of Kss1 and/or Fus3 from the repressive ternary complexes and sets transcriptional induction in motion? A clue is provided by the fact that nonphosphorylatable alleles of Kss1 (and Fus3) permanently repress Ste12 (133, 360, 394). This finding suggests that in the absence of an upstream activating signal, the MAPKs act to block gene transcription. However, on phosphorylation by $Ste7^{MAPKK}$, modification of the MAPKs on their activation loop causes a conformational change sufficient to dissociate the enzyme from Ste12 (394). As long as Kss1 can be phosphorylated on its activation loop by Ste7, even *kss1* alleles that are catalytically incompetent (owing to mutation of conserved active site residues) will be released, permitting derepression at FREs (132, 394, 411). For derepression at PREs, however, in addition to the dissociation of Kss1 caused by its Ste7-dependent phosphorylation, the catalytic activity of Fus3 is required, presumably to phosphorylate the Digs to release them from Ste12 (411). Apparently, Ste12-Ste12 homodimers have a higher affinity for the Dig proteins than do Ste12-Tec1 heterodimers. Thus, the MAPKs Kss1 and Fus3 can act as both repressors and activators of the transcription factor, Ste12, depending on their state of activation. Such a dual role can also be ascribed to other signaling proteins in the mating response pathway. For example, depending on the guanine nucleotide bound to it, Gα (Gpa1) can either bind and inhibit G$\beta\gamma$ (Ste4-Ste18) or release it to permit interaction with its downstream effectors.

CONTROL OF SIGNAL FIDELITY

As the content of this article demonstrates, even in the simplest organisms, cell signaling is a highly complex process. Mechanisms must exist to control the specificity, as well as the intensity, of a transmembrane signal. The issue of specificity is perhaps the most difficult question to address experimentally because many of the spatial and temporal mechanisms that contribute to accuracy and fidelity cannot be reconstituted easily and analyzed in vitro.

One obvious way to impose specificity is through the assembly of components of a signaling cascade into a supramolecular complex, through mutual binding to a common scaffolding protein. As discussed in detail above, the first protein shown to have such a function is Ste5. It is noteworthy that Ste5 does not bind the homologous kinases of the other MAPK cascades present in yeast cells, which ensures that signal transmission is channeled appropriately. Even though Ste5 can bind Kss1, it does not normally do so when Fus3 is present in the cell, either because most of the Kss1 is sequestered in complexes with Ste12 and not free to associate with Ste5 or because, even at equivalent concentrations of each MAPK, the binding site on Ste5 has higher affinity for Fus3. In addition to selecting the right

kinases, binding to Ste5 may also position the enzymes for optimal interaction and to exclude their interaction with other components. For example, Ste11^{MAPKKK} is required for the pheromone response pathway (via activation of Ste7^{MAPKK} and Fus3^{MAPK}), for the invasive/filamentous growth pathway (via activation of Ste7^{MAPKK} and Kss1^{MAPK}), and for one branch of the high osmolarity response pathway (via activation of Pbs2^{MAPKK} and Hog1^{MAPK}) (Figure 3). In haploid cells, where Ste11 is presumably constrained by binding to Ste5 and to Pbs2 (see below), pheromone stimulation of Ste11 is unable to initiate invasive growth efficiently. In fact, even under optimum conditions, the invasive growth response of haploids is weak. In contrast, in diploids, where Ste5 (and Fus3) are not expressed, the cells are able to display a robust filamentous growth response and to form luxuriant pseudohyphae (416, 417), at least in part because, in the absence of Ste5 (and Fus3), more Ste11 (and Ste7) are available for activation of Kss1.

Viewed from the perspective of Ste5, a number of proteins in other MAPK pathways serve somewhat analogous functions. One example is in the pathway necessary for response to increased external osmolarity (35, 36). An essential component of this pathway is the *SHO1* gene product, which is necessary for activation of Hog1^{MAPK} (Figure 3). Sho1 possesses four transmembrane domains, and a cytoplasmic tail containing an SH3 domain that binds to a Pro-rich tract in the N-terminal regulatory domain of Pbs2^{MAPKK} (418). Sho1 does not appear to be the osmosensor per se. Rather, its primary function seems to be the recruitment of Pbs2^{MAPKK} to the plasma membrane at regions of polarized cell growth (419, 420). Fusion of just the SH3 domain of Sho1 to another integral membrane protein (Ste2), or to a peripheral membrane targeting sequence (N-terminal myristoylation site of Gpa1$^{G\alpha}$), is sufficient for Sho1 activity (419). Pbs2, in turn, has high-affinity binding sites for both its upstream activator, Ste11^{MAPKKK}, and its downstream target, Hog1. Hence, Sho1 serves as a membrane anchor, just as G$\beta\gamma$ serves as a membrane anchor for Ste5; Pbs2 itself acts as a scaffold protein, like Ste5, to recruit the other two component protein kinases of its MAPK module into a complex (421). Just as in the mating pathway, the purpose of tethering Pbs2 at the membrane is to deliver its Ste11 cargo for phosphorylation and activation by Ste20 (which is located at the membrane in an activated state by virtue of its association with Cdc42) (419, 420).

As discussed in detail above, Ste20^{PAK} is the most upstream protein kinase in the mating pathway. It serves as an efficient initiator because it is activated by GTP-Cdc42 (sustained by Far1-mediated delivery of additional Cdc24^{GEF}) and because, by virtue of the mutual binding of Ste20 and Ste5 to G$\beta\gamma$, Ste20 is localized in close proximity to its substrate, Ste11^{MAPKKK}. Ste11 is, in turn, positioned to activate only the Ste7^{MAPKK} and Fus3^{MAPK} bound to Ste5. However, as mentioned with regard to the high osmolarity stress response pathway, Ste20 is not an enzyme dedicated to the mating pathway. In addition to the pheromone response and osmotic stress response pathways, Ste20^{PAK} also functions as an upstream activator of Ste11^{MAPKKK} in the invasive growth pathway (Figure 3). The yeast 14-3-3 homologs, Bmh1 and Bmh2, reportedly interact physically with Ste20 and positively affect its function, but only in the invasive growth pathway (422).

Moreover, Ste20 regulates various aspects of cell adhesion (67), bud formation (104, 423), and myosin (Myo3) function (306). Finally, deletion of *STE20* does not produce complete sterility (102, 103). Perhaps another PAK family member, Cla4, which is normally required for cell division, can partially substitute. Some degree of functional redundancy between these enzymes is suggested by the fact that both a *ste20*Δ single mutant and a *cla4*Δ single mutant are viable, whereas a *ste20*Δ *cla4*Δ double mutant is inviable (104, 423). An activated allele of *STE20* (which lacks its entire N-terminal regulatory domain) is toxic, apparently due to disruption of actin organization (108), and this effect is not alleviated by *ste4*Δ, *ste5*Δ, *ste11*Δ, *ste7*Δ, or *ste12*Δ mutations (103), whereas induction of a *FUS1-lacZ* reporter was blocked by *ste* mutations (321). Overexpression of *CDC42*, or an activated (GTPase-deficient) allele of *CDC42*, is also toxic, even in a variety of *ste* mutants (424). These observations reaffirm the conclusion that Ste20 and its activator, Cdc42, have dichotomous roles in regulation of the cytoskeleton versus activation of the Ste11-Ste7-Fus3 protein kinase cascade.

There are situations where parallel MAPK signaling pathways can intersect. $Kss1^{MAPK}$ was originally thought to be part of the pheromone response pathway because deletion of both $Kss1^{MAPK}$ and $Fus3^{MAPK}$ is required to fully block mating. However, $Kss1^{MAPK}$ alone confers only weak pheromone-dependent growth arrest (128, 138). It appears likely that these proteins normally act separately, but one can function in the absence of the other (132, 133, 137). Pheromone stimulation (425), or a hyperactive Ste7 allele (358), can trigger activation of the MAPK that controls cell wall synthesis (Mpk1), but it does so much more efficiently in the absence of Ste5. Similarly, Pbs2 and $Hog1^{MAPK}$ can under certain conditions negatively regulate the mating response pathway (304, 333, 426). Given the profound changes that occur on stimulation of either the mating or the high osmolarity pathway, it make sense that activation of one pathway should regulate the activity of the other, as a means of coordinating the activities of the different pathways.

Another mechanism for ensuring certain aspects of signal fidelity is spatial segregation of proteins within different subcellular compartments. As discussed in detail above, both Far1 and Ste5 are localized within different subcellular compartments, depending on the presence or absence of an appropriate stimulus. In naïve cells, Ste5 and Far1 are sequestered mainly in the nucleus; but in response to a pheromone signal, they are translocated to the cytosol, where they can encounter and engage $G\beta\gamma$, and thereby dock to the plasma membrane. Presumably, nuclear localization provides a simple means of storing proteins so that they can be mobilized rapidly en masse (rather than waiting for their accumulation by de novo synthesis) when they are needed for purposes such as signal transduction (Ste5) or cell polarization (Far1/Cdc24).

In addition to spatial regulation, there are a number of mechanisms that provide temporal regulation, which can contribute to signaling efficiency and fidelity. The mRNA levels for many pheromone-regulated genes are elevated in G1 (324, 427, 428), the period in which basal activity of $Ste7^{MAPKK}$ and $Fus3^{MAPK}$ is highest (347). Cell cycle regulation of $Fus3^{MAPK}$ is mediated, in part, by Far1 (384) and by the G1 cyclins, Cln1 and Cln2 (324, 347), all of which are most

abundant in late G1. Such cell cycle–dependent changes may confer maximum pheromone sensitivity in the time window of the cell cycle when mating can proceed best. In contrast, other MAPK signaling cascades, such as the osmotic stress ($Hog1^{MAPK}$) and invasive growth ($Kss1^{MAPK}$) pathways, are presumably less dependent on the need to delay progression through an individual cell cycle and may be activated on a much slower timescale. In these cases, pathway activation is likely to be a graded response that occurs over the course of time as metabolic waste products are released into the medium and external osmolarity increases (Hog1), or as nitrogen sources in the immediate vicinity are depleted, demanding that the cells forage through the growth medium to maintain an adequate nitrogen supply (Kss1).

Finally, all signaling events ultimately result in altered patterns of gene expression (429). With regard to genes under Ste12 control, it is clear that combinatorial interactions of Ste12 with other DNA-binding proteins (e.g. Mcm1, Matα1, Tec1, Kar4) and differential interactions of the resulting complexes with regulatory factors (e.g. Dig1, Dig2, Kss1, Fus3) may confer a considerable degree of the specificity required to dictate how distinct extracellular stimuli (e.g. pheromone versus nitrogen limitation) can utilize components of the same MAPK cascade, yet elicit two different transcriptional programs. In addition, parallel stimuli can modify an output. For example, there is good evidence that both carbon source limitation (141) and the cAMP-dependent protein kinases (430, 431) act in concert with nitrogen-limitation triggered activation of Ste20 (and, ultimately, Kss1) to turn on genes for invasive growth (e.g. *FLO11*) optimally (408, 432). In this regard, there is a surprising degree of overlap between the global transcription profiles specified by mutants expressing either Kss1 alone or Fus3 alone, despite their nonoverlapping functions in a wild-type cell (62, 63). Other mechanisms may help impose differential responses in a normal cell. For example, competition between Fus3 and Kss1 for activators, such as Ste7 or Ste5, may preclude activation of Kss1 and prevent the induction of FREs by pheromone. Also, because both Dig2 and Fus3 are more potent negative regulators of invasive growth than of mating, the induction of these genes in response to pheromone may further reinforce the blockade to induction of FREs during pheromone response. In addition, perhaps posttranscriptional regulation plays a significant role in specifying differential responses. For example, Fus3 is preferentially dephosphorylated by Ptp3 (compared with either Ptp2 or Msg5) (362); perhaps Kss1 is especially susceptible to dephosphorylation by the pheromone-inducible Msg5 enzyme, providing an additional reason for the lack of sustained Kss1 activation in cells responding to pheromone (127, 128).

CONCLUSIONS AND PERSPECTIVE

In this article, we have summarized in detail the current state of understanding of yeast mating pheromone response as a model for a signal transduction pathway that is initiated via the action of both a heterotrimeric G protein and a small

GTPase. Our primary mission was to clarify, where possible, the complexity of this network and to focus in particular on the specific roles of gene products and processes that regulate the activity, subcellular location, and stability of the components that actually mediate signaling. It is clear that enormous progress has been made, especially in the past decade or so. Indeed, it has been exhilarating to be engaged ourselves in this endeavor. However, many unanswered questions remain to be addressed, and we have attempted to highlight many of these issues in the text where they were most pertinent. As a general point, it is clear that to fully understand both the temporal and spatial aspects of the assembly and regulation of the various multiprotein complexes that participate in the signaling events we have described (and their downregulation), much more detailed information needs to be obtained about the biochemical features of the purified proteins, the physicochemical properties of the protein-protein interactions in which they engage, and the detailed three-dimensional structures of these molecules. We anticipate that continued experimental study of this pathway will allow it to endure as a paradigm for workers interested in basic signal transduction mechanisms and will generate insights that, as in the past, will reveal additional general principles applicable to more complex organisms.

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