N-myristoylation is required for function of the pheromone-responsive G_{α} protein of yeast: conditional activation of the pheromone response by a temperature-sensitive N-myristoyl transferase

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In a screen designed to identify novel mutations in the mating response pathway of *Saccharomyces cerevisiae*, we isolated conditional alleles of *NMT1*, the gene encoding N-myristoyl transferase. Genetic data indicate that Nmt1 deficiency results in the activation of the pheromone response at the level of Gpa1, the α subunit of the pheromone-responsive G protein. We show that Gpa1 is myristoylated by Nmt1, and without this normally stable modification, Gpa1 is unable to inhibit pheromone signaling. This loss of Gpa1 function is probably not the result of improper subcellular localization. Unlike the mammalian $G_{\alpha i}$ proteins α_i and α_o , nonmyristoylated Gpa1 is able to associate with membranes. In addition to Gpa1, our data indicate that Nmt1 myristoylates other proteins essential to vegetative growth.

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In the life cycle of the budding yeast *Saccharomyces cerevisiae* two haploid cells of opposite mating type can conjugate to form a diploid cell. The mating response, which involves the induction of mating-specific genes, arrest of the mating partners in the G_1 phase of the cell cycle, and a morphological change called projection formation, is triggered by the exchange of peptide mating pheromones (for review, see Cross et al. 1988). *MATa* cells constitutively secrete a-factor and express a receptor that binds α -factor (Jenness et al. 1983; Burkholder and Hartwell 1985; Blumer et al. 1988). Conversely, *MAT α* cells secrete α -factor and bind a-factor (Hagen et al. 1986). The mechanisms by which yeast cells receive the signal to conjugate and transmit it across the plasma membrane are analogous to the G protein-mediated signaling systems found in mammalian cells. The receptors for a- and α -factor, encoded by the *STE3* and *STE2* genes, respectively, are predicted to be topologically similar to members of the β -adrenergic/rhodopsin family of receptors, which are characterized by seven transmembrane domains (Burkholder and Hartwell 1985; Nakayama et al. 1985; Hagen et al. 1986; Blumer et al. 1988). Like

their mammalian counterparts, the yeast pheromone receptors are coupled to a heterotrimeric G protein. The α , β and γ subunits of this G protein are the products of the *GPA1*, *STE4*, and *STE18* genes, respectively (Dietzel and Kurjan 1987; Miyajima et al. 1987; Nakafuku et al. 1987; Jahng et al. 1988; Whiteway et al. 1989). Receptors activated by pheromone are thought to catalyze the exchange of GDP for GTP on the G_{α} subunit (Gpa1), which in turn causes a conformational shift in G_{α} , and the dissociation of the $G_{\beta\gamma}$ dimer (for review, see Stryer and Bourne 1986; Gilman 1987). In contrast to most mammalian systems, it is the $G_{\beta\gamma}$ dimer and not the G_{α} subunit that is the active signal transducer, stimulating the downstream events preparatory to conjugation (Blinder et al. 1989; Cole et al. 1990; Nomoto et al. 1990; Whiteway et al. 1990). The pheromone-responsive G_{α} subunit does, however, appear to generate a signal that counteracts the one created by the release of $G_{\beta\gamma}$ (Miyajima et al. 1989; Cole et al. 1990; Stone and Reed 1990). Thus, the G_{α} subunit plays two roles: In its GDP-bound form, the G_{α} subunit negatively regulates $G_{\beta\gamma}$; in its activated, or GTP-bound form, it is thought to stimulate adaptation. Both the mating and the adaptive signals are most likely terminated when G_{α} , by virtue of its intrinsic GTPase

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activity, hydrolyzes GTP to GDP, reverts to its inactive conformation, and binds $G_{\beta\gamma}$.

Although the effectors and second messengers that are coupled to some vertebrate G proteins (e.g., G_s , G_i , and transducin) are known (for review, see Neer and Clapham 1988), downstream signaling mechanisms in the mating pheromone response have yet to be elucidated. To identify novel elements in the pheromone response pathway, we attempted to isolate conditional mutations that would allow mating in the absence of pheromone receptors (Jahng et al. 1988). We reasoned that mutational activation of the pheromone response downstream of the receptor might restore mating competency to cells lacking a functional copy of *STE2*. It was necessary to look for conditional mutations, as constitutive activation of the pheromone response would result in permanent G_1 arrest, and hence, lethality. From a set of 60 independently isolated temperature-sensitive *STE2* bypass mutations, that is, mutations that allowed *MATa* cells lacking the α -factor receptor to mate at restrictive temperature, three loci were identified. Mutations at one locus, initially designated *cdc70* (for cell division cycle), were found to be allelic with *GPA1*, the gene that encodes G_α . It is not surprising that we recovered mutations in *GPA1*, as temperature-sensitive inactivation of G_α allows the release of $G_{\beta\gamma}$, and stimulation of the mating response (Dietzel and Kurjan 1987; Miyajima et al. 1987; Jahng et al. 1988). Recessive mutations at the two additional loci, *cdc72* and *cdc73*, confer temperature-sensitive phenotypes similar to loss of *Gpa1* function: arrest in G_1 and projection formation. It was presumed that these mutations also activate the pheromone response pathway in the absence of pheromone. We report here that *cdc72* is allelic with *NMT1*, the gene identified previously that encodes *N*-myristoyl transferase (NMT) (Duronio et al. 1989). NMT attaches the 14-carbon saturated fatty acid myristic acid to the amino-terminal glycine residue of target proteins by amide linkage (for review, see Towler et al. 1988). We show that *Gpa1*, like some other G_α proteins α_i and α_o , however, the membrane localization of *Gpa1* does not depend on its acylation. Rather, failure to myristoylate *Gpa1* may result in the activation of the pheromone response pathway by reducing the affinity of *Gpa1* for $G_{\beta\gamma}$. Although *Nmt1* is essential in all yeast cell types, the conditional *cdc72* mutation has allowed us to demonstrate that the most sensitive target of the enzyme in mating competent cells is the pheromone-responsive G protein.

Results

CDC72 is identical to NMT1, which encodes an NMT

The *CDC72* gene was cloned from a plasmid library of *S. cerevisiae* genomic fragments by complementation of the temperature-sensitive allele *cdc72-2*. A preliminary analysis of the *CDC72*-containing segment has been reported elsewhere (Reed et al. 1988). The smallest sub-

clone capable of rescuing the *cdc72-2* mutation was sequenced, and an open reading frame (ORF) with a coding capacity of 455 amino acids was revealed. Insertional disruption of this ORF eliminated its ability to rescue the *cdc72-2* marker. Standard genetic methods were used to confirm that this sequence corresponds to the *CDC72* gene. Unlike many of the genes whose products are known to be involved in the mating pheromone signal transduction pathway, *CDC72* is expressed in diploid as well as haploid cells, and its transcription is not induced by pheromone. When we compared the predicted primary structure of the *CDC72* ORF to a data base of known and predicted polypeptide sequences, we found a perfect match to the product of the *NMT1* gene (data not shown). *NMT1* encodes an *N*-myristoyl transferase, an enzyme that attaches myristic acid (C-14 : O) via amide linkage to the amino-terminal glycine residues of target proteins (Duronio et al. 1989).

cdc72 mutations activate the mating pheromone response in haploid cells

cdc72 mutations were originally isolated as temperature-sensitive suppressors of the mating defect exhibited by cells lacking a mating pheromone receptor. The following analysis describes one of two alleles identified, *cdc72-2*, although *cdc72-1* has similar properties. Haploid cells carrying the *cdc72-2* mutation and growing at the permissive temperature (23°C) are enlarged relative to wild-type cells, and some form the projections (shmoo) typical of cells responding to mating pheromone (Fig. 1). When raised to the restrictive temperature (36°C), *cdc72-2* haploids arrest as large unbudded cells, diagnostic of the G_1 phase of the cell cycle but continue to shmoo. Therefore, one explanation for the ability of

Figure 1. Haploid *cdc72* mutant cells have a morphology characteristic of wild-type cells exposed to mating pheromone. The two left panels depict wild-type cells before and after pheromone treatment. The middle panels show a congenic *cdc72-2* mutant growing at 23°C (top) and at the restrictive temperature for the *cdc72-2* mutation, 36°C (bottom). The two right panels demonstrate that the morphological changes associated with the mutant are still present in cells lacking mating pheromone receptor (*cdc72 ste2*), but not in cells deleted for the G_β subunit (*cdc72 ste4*).

cdc72-2 to suppress the mating deficiency of receptorless cells is that the mutation constitutively activates the pheromone response.

To determine whether the pheromone response is activated by the *cdc72-2* mutation, we examined the expression of the pheromone-responsive gene *FUS1*. The *FUS1* gene is required for cell fusion during mating, and its transcript is induced at least 50-fold by the mating pheromone α -factor (McCaffrey et al. 1987; Trueheart et al. 1987). If the *cdc72-2* mutation activates the pheromone response pathway, *FUS1* expression should be elevated in *cdc72-2* cells. For convenience, we utilized a *FUS1::lacZ* hybrid gene to examine the expression of *FUS1* (Trueheart et al. 1987). Congenic wild-type and *cdc72* mutant strains were transformed with the reporter construct, and β -galactosidase levels were measured in cells grown at permissive and restrictive temperatures. As indicated in Table 1, the *FUS1* promoter exhibited a very low basal activity in wild-type cells regardless of growth temperature, but was highly induced (>180-fold) by treatment with α -factor. In contrast, the basal *FUS1::lacZ* activity of *cdc72-2* mutant cells grown at 23°C was 100-fold greater than in control cells and was only minimally elevated above this level by raising the

temperature or by treatment with α -factor. This result is consistent with the aberrant morphology exhibited by mutant cells growing at 23°C. Taken together, these results suggest that the pheromone response pathway is partially active at the permissive temperature and fully active at the restrictive temperature in mutant cells. The induction of cell cycle arrest is apparently less sensitive to partial pathway activation than is the induction of mating-specific genes and projection formation.

CDC72 is required for vegetative growth as well as regulation of the mating pheromone response

By several criteria then—cell cycle arrest in G₁, cell size and morphology, expression of a mating-specific gene, and restoration of mating competency to cells lacking a pheromone receptor—the *cdc72-2* mutation appears to activate the pheromone response pathway. However, loss of Nmt1 activity also leads to a growth defect in *MATa/α* diploid cells, which do not express critical elements of the mating response pathway, are insensitive to pheromone, and do not mate. The phenotypes conferred by *cdc72-2* in both mating-competent and mating-incompetent cells were investigated more fully, as shown in Figure 2. A *MATa/a* strain homozygous for the *cdc72-2* mutation was used to control for the effects of ploidy. Diploid *MATa/a* cells, like haploid cells, express all the elements of the pheromone response pathway and are competent to mate. In Figure 2A, we can see that diploid cells homozygous for the *cdc72-2* mutation arrest during the first cell cycle after being shifted to the restrictive temperature (38°C), regardless of the information at the mating-type locus (*MAT*). The demonstration that *MATa/α cdc72/cdc72* diploid cells fail to proliferate at the restrictive temperature argues that *cdc72-2* is necessary for the vegetative growth of mating-incompetent cells, as well as being essential for the proper regulation of the pheromone response in haploids. It appears, however, that the regulation of the pheromone response is more sensitive to the loss of Nmt1 activity than is the affected function(s) in *MATa/α* cells: the restrictive temperature of the mating-competent *cdc72-2* mutant cells is significantly lower than that of the mating-incompetent mutant strain. As shown in Figure 2B, *cdc72/cdc72 MATa/α* cells are able to proliferate at 36°C, whereas congenic *MATa/a* cells cease dividing at this temperature. In contrast, both the wild-type *MATa/α* and *MATa/a* control strains grow at 38°C, as does a *MATa/a* strain heterozygous for *cdc72-2*. Neither the *MATa/α* nor the *MATa/a* homozygous mutant strains are able to grow at this temperature.

When the terminal arrest morphologies of the *MATa/α* and the *MATa/a* homozygous mutant cells are examined, striking differences can be seen (Fig. 2C). Whereas *MATa/α* diploid cells arrest heterogeneously throughout the cell cycle as both budded and unbudded cells, *MATa/a* diploid cells appear identical to the haploid *cdc72-2* mutants depicted in Figure 1: They arrest as large unbudded G₁ cells and form projections characteristic of cells responding to mating pheromone. The less

Table 1. Epistasis analysis of the *cdc72-2* mutation: *FUS1* expression and mating efficiency

Strain	β -Galactosidase units (°C)		α -Factor (2 μ g/ml)
	23	37	
<i>CDC⁺ STE⁺</i>	0.3	0.3	54.5
<i>CDC⁺ ste2Δ</i>	0.3	0.7	0.5
<i>CDC⁺ ste4Δ</i>	<0.1	<0.1	<0.1
<i>CDC⁺ ste18Δ</i>	<0.1	<0.1	<0.1
<i>cdc72 STE⁺</i>	37.9	43.1	46.0
<i>cdc72 ste2Δ</i>	24.6	30.0	28.2
<i>cdc72 ste4Δ</i>	<0.1	<0.1	<0.1
<i>cdc72 ste18Δ</i>	<0.1	<0.1	<0.1
<i>cdc72</i> (YEP13)	27.6	33.1	37.9
<i>cdc72</i> [YEP[GPA1]]	4.9	5.4	13.4

Strain	Mating efficiency (%)	
	23°C	37°C
<i>CDC⁺ STE⁺</i>	100	100
<i>CDC⁺ ste2Δ</i>	<0.0005	<0.0005
<i>CDC⁺ ste4Δ</i>	<0.0005	<0.0005
<i>CDC⁺ ste18Δ</i>	<0.0005	<0.0005
<i>cdc72 STE⁺</i>	80.53	41.80
<i>cdc72 ste2Δ</i>	1.05	0.27
<i>cdc72 ste4Δ</i>	<0.0005	<0.0005
<i>cdc72 ste18Δ</i>	<0.0005	<0.0005

(Top) β -Galactosidase activity is expressed in Miller units, determined as described by Slater and Craig (1987). The *cdc72* [YEP13[Gpa1]] strain carries *GPA1* on the high copy number YEP13 plasmid. (Bottom) Mating efficiency was determined as described in Materials and methods and normalized to the proportion of zygotes produced in the wild-type cross (first line).

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Figure 2. *CDC72/NMT1* is essential for vegetative growth, but a mating-specific function is the most sensitive target of *N*-myristoyl transferase in mating competent cells. The responses of *cdc72-2* mutant strains to various conditions were assayed. To control for ploidy, a *MATa/a* strain was used to assess the effects of the *cdc72-2* mutation on mating competent cells [see text]. (A) Growth curves for mating competent (*MATa/a*) and mating-incompetent (*MATa/α*) strains. Cells growing in rich medium (YEPD) at 23°C were shifted to 38°C and monitored for growth. Both *MATa/a* and *MATa/α* diploids homozygous for the *cdc72-2* mutation cease dividing within one cell cycle, whereas non-mutant cells continue to proliferate. (B) Mating-competent cells are more sensitive to partial loss of *CDC72* function than mating incompetent cells. Congenic strains of the specified genotype were patched onto rich (YEPD) agar plates and incubated at the indicated temperatures for 2 days. Mating-competent cells show a reduced restrictive temperature (36°C) relative to mating-incompetent cells (38°C). (C) Terminal arrest morphology of *cdc72* mutants. Mating-incompetent cells arrest at random positions in the cell cycle with both budded and unbudded cells in the population. Congenic mating-competent cells arrest in the G_1 phase of the cell cycle as large unbudded cells with a morphology characteristic of cells responding to mating pheromone.

severe growth defect exhibited by *MATa/α cdc72/cdc72* cells indicates that there are other Nmt1 targets critical to vegetative growth, but they are less sensitive to the immediate loss of Nmt1 activity than the regulator of the pheromone response.

The cdc72 mutation activates the pheromone response pathway at the level of G $_{\alpha}$ (G $_{\alpha}$)

The finding that *CDC72* encodes an NMT suggested that myristoylation of a protein or proteins involved in the mating response is critical for the regulation of the pheromone signal transduction pathway. To determine the point at which this activation occurs relative to reference points at which previously identified gene products are known to function, we performed an epistasis analysis. It has been inferred from a preponderance of genetic evidence that G_{β} and G_{γ} are required for pheromone signaling and that they function at a point downstream of G_{α} (Blinder et al. 1989; Cole et al. 1990; Nomoto et al. 1990; Whiteway et al. 1990). The pheromone responsive G_{α} , in turn, is downstream of the receptor and acts to negatively regulate $G_{\beta\gamma}$ (Dietzel and Kurjan 1987; Miyajima et al. 1987; Jahng et al. 1988). Congenic double mutant strains were therefore constructed in which the

cdc72-2 allele was paired with one of three null alleles: *ste2Δ* (α factor receptor), *ste4Δ* (G_{β}) or *ste18Δ* (G_{γ}). The double mutants were then scored for cell growth and morphology, *FUS1* expression, and mating efficiency.

The results of our epistasis analysis can be summarized as follows. Disruption mutations in *STE4* or *STE18* normalized the cell size and shape of *cdc72-2* mutants (Fig. 1) and blocked the ability of the *cdc72-2* mutation both to induce *FUS1* expression and to stimulate mating behavior (Table 1). A loss of *STE2* function, on the other hand, had no effect on these phenotypes. We conclude that loss of *NMT1* activates the pheromone response pathway downstream of the receptor and upstream of $G_{\beta\gamma}$, that is, at the level of G_{α} (Gpa1). Our observation that the overexpression of Gpa1 partially normalizes *FUS1* expression in a *cdc72* background (Table 1), and also partially suppresses the cell growth and morphology defects in haploid cells (data not shown), is consistent with the notion that Gpa1 itself is central to the *cdc72* phenotype in mating-competent cells. Mutational inactivation of Gpa1 causes induction of the pheromone response, analogous to the pathway activation conferred by the *cdc72-2* mutation. An obvious explanation for these genetic data is that Gpa1 is the most critical target of Nmt1 in cells that express the pheromone response

pathway. Our presumption is that the myristoylation of Gpa1 is necessary for its negative regulatory function (e.g., sequestration of $G_{\beta\gamma}$) and that the defect in the mutant enzyme encoded by *cdc72-2* can be partially suppressed by increasing the amount of available substrate. Elevating the level of Gpa1 most likely increases the total amount of myristoylated Gpa1 and thus partially restores the inhibitory effect of G_{α} on pathway activity.

Gpa1 is myristoylated by Nmt1 and this is an essential modification

A subset of mammalian G_{α} proteins— G_{α_i} , G_{α_o} , and G_{α_z} , but not G_{α_s} —are myristoylated (Jones et al. 1990; Mumby et al. 1990). Our genetic data strongly suggest that Gpa1 is also modified in this way. Moreover, the predicted primary structure for *GPA1* reveals both the mandatory glycine at residue 2 and the serine preferred by Nmt1 at position 6 (Nakafuku et al. 1987; Towler et al. 1987). To determine whether Gpa1 is a substrate for amino-terminal myristoylation, we metabolically labeled yeast cultures with either [3 H]myristic acid or [35 S]methionine and immunoprecipitated Gpa1 from whole-cell extracts using affinity-purified antibodies. The results from one such experiment are shown in Figure 3. Four strains were grown in medium containing [35 S]methionine: a *MATa/α* diploid strain (in which Gpa1 is not produced), a *CDC72* wild-type strain, a *cdc72-2* mutant strain grown at permissive temperature, and a wild-type strain

Figure 3. Gpa1 is a substrate for Nmt1. (Lanes 1–4) Four congenic yeast strains were metabolically labeled with [35 S]methionine: *MATa/α CDC72/CDC72* diploids, which do not express Gpa1; *MATa cdc72-2* mutant haploids, *MATa CDC72* wild-type haploids, and the wild-type haploid strain transformed with a mutated allele of *GPA1* whose product cannot be myristoylated. (Lanes 5–6) A congenic pair of wild-type yeast strains, one of which was transformed with a hybrid gene consisting of the *GPA1*-coding region fused to the *GAL1* promoter, were grown in galactose-containing medium, and metabolically labeled with [3 H]myristate. Under these conditions, the cells carrying the *GAL1p::GPA1* vector (lane 6) overexpress Gpa1 by at least 20-fold relative to the wild-type cells (lane 5). Proteins were extracted from total cellular lysates, immunoprecipitated with affinity-purified anti-Gpa1 polyclonal antibodies, and separated on an SDS-PAGE gel. To adjust for the difference between the 3 H- and 35 S-generated signals, lanes 5 and 6 were exposed 2.5-fold longer than lanes 1–4. The arrow marks the myristoylated form of Gpa1; the asterisk indicates the nonmyristoylated species. Molecular mass is indicated in kilodaltons (left).

transformed with a mutated copy of *GPA1* in which residue 2 has been changed from glycine to alanine. Because Nmt1 requires a glycine at residue 2, the mutated Gpa1 (*gpa1^{ala2}*) should not be a substrate for Nmt1 even if the wild-type product is myristoylated. In the wild-type (*CDC72⁺*) extract, the Gpa1 antibodies specifically precipitated a [35 S]methionine-labeled protein of ~54 kD, as well as a less abundant, slower migrating species of ~56 kD. We conclude that these two bands represent different forms of Gpa1 because they run near its predicted molecular mass (54.1 kD), are not present in *MATa/α* diploid cells (Fig. 3, lane 1), and are detectable more readily when Gpa1 is overproduced (data not shown). Although the same two forms of Gpa1 are present in *cdc72-2* cells grown at permissive temperature (lane 2), as well as in the *gpa1^{ala2}/GPA1* strain (lane 4), the ratio of the two bands is distinct in each of the three immunoprecipitates. The slower-migrating species of Gpa1 is relatively more abundant in *cdc72-2* cells than in wild-type cells, and most abundant in the *gpa1^{ala2}/GPA1* cells. These observations suggest that the faster-migrating species of Gpa1 is myristoylated, whereas the species with higher apparent molecular mass lacks this modification. The results shown in Figure 3, lanes 5 and 6, support this inference. A single radioactive protein was immunoprecipitated from an extract of cells overproducing Gpa1 and labeled with [3 H]myristic acid. It comigrates with the [35 S]methionine-labeled species of lower apparent molecular mass. This protein is not detectable in extracts of [3 H]myristic acid-labeled *cdc72-2* cells overproducing Gpa1 (data not shown) or in extracts from wild-type cells (lane 5). Apparently, the amount of [3 H]-labeled Gpa1 in wild-type cells is insufficient to detect in our immunoprecipitation assay due to the low specific activity of the label. We conclude that Gpa1 is amino-terminally myristoylated by Nmt1 and that this modification increases its electrophoretic mobility. The myristoylated forms of α_i and α_o have also been reported to run faster than the nonmyristoylated species in SDS-PAGE gels (Jones et al. 1990; Linder et al. 1991).

The conditional activation of the pheromone response pathway in *cdc72* temperature-sensitive mutants implies that the myristoylation of Gpa1 is an essential modification. This was confirmed in the following experiment. Diploid cells heterozygous for a *gpa1* null allele (*MATa/α GPA1/gpa1::URA3*) were transformed with a centromeric plasmid containing the *gpa1^{ala2}* allele and *TRP1* as a selectable marker, sporulated, and dissected. The meiotic segregants were then scored for uracil and tryptophan prototrophy, as well as for growth and cellular morphology. Because a loss of Gpa1 function is lethal, diploids heterozygous for the *gpa1::URA3* allele yield two viable and two inviable spores. All of the viable spores are *ura⁻*. Complementation of the *gpa1* null allele should allow the recovery of some *URA⁺ TRP⁺* colonies. Of 11 tetrads, however, numerous *URA⁻ TRP⁺* segregants, but no *URA⁺ TRP⁺* segregants were recovered. Viability segregated 2 : 2 in each case. Microcolonies, presumed to be *gpa1::URA3/gpa1^{ala2}* or *gpa1::URA3*, were arrested as enlarged, unbudded cells

morphologically similar to cells responding to pheromone. Thus, inability to myristoylate Gpa1 results in recessive lethality, apparently as a result of constitutive activation of the pheromone response pathway. Neither the growth nor the morphology of cells that express both the wild-type and mutant alleles of *GPA1* was affected.

Nonmyristoylated Gpa1 is localized to membranes

In their inactive state, G_{α} proteins are positioned on the cytoplasmic face of the plasma membrane, where they interact with membrane-bound receptors and effectors, as well as with $G_{\beta\gamma}$ dimers (Stryer and Bourne 1986; Gilman 1987). The nature of this membrane association is unclear, because G proteins lack obvious hydrophobic domains. Recently, two groups have shown that the membrane localization of α_i and α_o depends on amino-terminal myristoylation (Jones et al. 1990; Mumby et al. 1990). Changing the second amino acid of these proteins from glycine to alanine, which blocks myristoylation, causes them to accumulate in the cytoplasm. If membrane localization of Gpa1 also depends on its myristoylation, then the absence of the myristoyl moiety could account for the activation of the pheromone response pathway. An improperly localized G_{α} might be unable to sequester $G_{\beta\gamma}$, which acts as the positive signal transducer in yeast. To examine this possibility, we prepared crude membrane and cytosolic fractions from wild-type, *cdc72-2*, and diploid cells grown at permissive and restrictive temperature. Proteins were separated by electrophoresis, electroblotted to nitrocellulose, and incubated with an affinity-purified anti-Gpa1 antibody. The results of this experiment are shown in Figure 4. We used the difference in electrophoretic mobility between the

Figure 4. Inactivating Nmt1 does not cause Gpa1 to accumulate in the cytoplasm. Three congenic strains of yeast—a *MATa/α* diploid, which does not express Gpa1; a *CDC72* wild-type haploid; and a *cdc72-2* mutant haploid—were grown to mid-logarithmic phase at 24°C, whereupon half of each culture was shifted to 38°C and grown for an additional 2.5 hr (about one generation). Crude membrane and cytosolic fractions were normalized by cell equivalents, electrophoresed, blotted to nitrocellulose, and probed with an affinity-purified anti-Gpa1 peptide antibody. Immunoreactive proteins were visualized as described in Materials and methods. The arrow marks the myristoylated form of Gpa1; the asterisk indicates the nonmyristoylated species. Molecular mass is indicated in kilodaltons (left).

myristoylated and nonmyristoylated forms of Gpa1 as a means of distinguishing the two species. As expected, there was no indication of *GPA1* expression in the diploid cells, and only myristoylated, membrane-bound Gpa1 was detectable in wild-type cells. The mutant cells, on the other hand, accumulated both forms of Gpa1 when grown at the permissive temperature. Myristoylated Gpa1 was found exclusively in the membrane fraction, whereas the nonmyristoylated form was partitioned largely, but not entirely, to membranes. Two points should be emphasized: First, there was very little myristoylated Gpa1 left in the mutant cells after they had been maintained at restrictive temperature for a full generation, consistent with their first cycle arrest; second, most of the nonmyristoylated Gpa1 was associated with membranes. Thus, the activation of the pheromone response in cells unable to myristoylate Gpa1 is probably not the result of its solubilization.

We cannot determine from these results whether the nonmyristoylated Gpa1 found in the membranous fraction of mutant cells was localized as such, or whether the protein was targeted to membranes in its myristoylated form and subsequently demyristoylated. Myristoylation may be necessary to target Gpa1 to the plasma membrane, but not for its continued membrane association. To address this question, we again employed the *gpa1^{ala2}* allele. If the myristoyl moiety is required to localize Gpa1 to the membrane, the product of the *gpa1^{ala2}* allele should remain soluble. Diploid, wild-type, and wild-type cells transformed with the *gpa1^{ala2}* centromeric vector were labeled with [³⁵S]methionine, and Gpa1 was immunoprecipitated from crude membrane and cytosolic fractions. We also immunoprecipitated the enzyme alcohol dehydrogenase from aliquots of these same extracts to demonstrate the proper partitioning of a known cytoplasmic protein (Fig. 5B). As expected, the wild-type cells produced predominantly myristoylated and membrane-bound Gpa1. The wild-type protein was not detectable in the cytosol (Fig. 5A, lane 5). This result is consistent with previous data (Figs. 3 and 4). The behavior of Gpa1 in wild-type cells carrying the *gpa1^{ala2}* vector appeared to be similar to that of Gpa1 in *cdc72* mutant cells (cf. Fig. 5, lanes 3 and 6, with Fig. 4, lanes 5 and 11); a large amount of nonmyristoylated Gpa1 was produced and was found mostly in the membrane fraction. The majority of this nonmyristoylated Gpa1 must be encoded by the *gpa1^{ala2}* allele and not by the genomic copy of *GPA1*, as its expression greatly surpassed that in wild-type cells. Thus, nonmyristoylated Gpa1 is localized to membranes.

Amino-terminal myristoylation of Gpa1 is a stable modification

When *cdc72-2* mutant cells are shifted to restrictive temperature, most of the myristoylated Gpa1 disappears over a period of 2.5 hr, or approximately one generation time (Fig. 4). This may be the result of turnover of the *GPA1* gene product, or it may simply reflect dilution of myristoylated Gpa1 in cells that are synthesizing only

Figure 5. Nonmyristoylated Gpa1 is efficiently localized to membranes. **A** *MATa/α* diploid, a *MATa* wild-type haploid, and the *MATa* wild-type haploid strain transformed with a mutated allele of *GPA1* whose product cannot be myristoylated were metabolically labeled with [35 S]methionine, and crude membrane and cytosolic fractions were prepared. Aliquots normalized by cell equivalents were immunoprecipitated and electrophoresed. Molecular mass is indicated in kilodaltons (*left*). **(A)** Proteins immunoprecipitated with an affinity-purified anti-Gpa1 polyclonal antibody. The arrow marks the myristoylated form of Gpa1; the asterisk indicates the nonmyristoylated species. **(B)** Proteins immunoprecipitated with a polyclonal antibody raised against yeast alcohol dehydrogenase (Adh).

the nonmyristoylated form of the protein. Alternatively, Gpa1 may undergo an acylation/deacylation/reacylation cycle; the disappearance of the faster-migrating species of Gpa1 in the absence of Nmt1 activity might be the result of demyristoylation of an otherwise stable G α protein. Although amino-terminal myristoylation is generally regarded as a stable, cotranslational modification, demyristoylation of a 68-kD protein in *Dictyostelium discoideum* has been reported (Silva and Klein 1990). We tested this possibility by labeling wild-type and *cdc72-2* mutant cells with [35 S]methionine at 23°C, then blocking further protein synthesis with cycloheximide, and concomitantly inactivating Nmt1 by shifting the cultures to 38°C. Hourly time points were taken, and the fate of the existing Gpa1 was analyzed by immunoprecipitation from whole-cell extracts and electrophoresis, as before. In wild-type cells, myristoylated Gpa1 did not decrease during the course of the experiment, indicating that Gpa1 normally has a half-life of >4 hr (Fig. 6, lanes 2–6). Gpa1 is equally stable in mutant cells. Both forms of the protein persisted at their initial levels for the duration of the experiment (Fig. 6, lanes 7–11). This result contradicts the notion that the myristoyl moiety on Gpa1 is labile, as the myristoylated form of Gpa1 is stable in the absence of Nmt1 activity. We obtained a similar result when we employed a pulse-chase labeling protocol instead of blocking protein synthesis with cycloheximide, thus eliminating the possibility of a drug sensitive demyristoylation activity (data not shown).

Discussion

Failure to myristoylate Gpa1 results in the activation of the pheromone response

We have used a conditional allele of the yeast gene

NMT1 to examine the effects of deficient NMT activity in yeast cells. Despite the global nature of this perturbation, a subset of cell types displayed a very specific defect. We identified the pheromone-responsive G α protein (Gpa1) as the most sensitive target of Nmt1 in mating-competent cells, and were able to assess its function in situ with and without the myristate moiety. Failure to attach myristate to Gpa1 results in activation of the mating response, as shown by arrest in the G $_1$ phase of the cell cycle, projection formation, and the induction of a mating-specific gene. The genetic evidence clearly implicates Gpa1 as the substrate of Nmt1 whose dysfunction results in the mating response phenotypes. Null mutations in gene products immediately downstream of Gpa1 in the pheromone signaling pathway (G β and G γ) are epistatic to Nmt1 deficiency, whereas the consequences of this deficiency are the same with or without the receptor that transmits the pheromone-induced signal to Gpa1. In addition, overexpression of Gpa1 mitigates the effects of inadequate Nmt1 activity, again suggesting that the pheromone responsive G α protein, and not another gene product acting at the same level, is the key target of Nmt1 in mating competent cells.

It is noteworthy that cells carrying a temperature-sensitive allele of *NMT1* (*cdc72*) arrest on the first cell cycle when shifted to the restrictive temperature. Our data indicate that the myristoylated form of Gpa1 largely disappears during this time period (Fig. 4). Because Gpa1 has a half-life that is greater than one generation time, and because the myristoyl moiety does not appear to be labile (Fig. 6), we conclude that the apparent loss of myristoylated Gpa1 in the absence of Nmt1 activity is a simple result of its decreasing representation as a part of the total cellular protein. In the Western blot shown in Figure 4, for example, each lane represents an equivalent amount of cellular extract; myristoylated Gpa1 must therefore constitute a proportionately smaller part of the

Figure 6. Amino-terminal myristoylation of Gpa1 is a stable modification. Wild-type and *cdc72-2* mutant cells were metabolically labeled with [35 S]methionine at 23°C for 3 hr. At zero time, the cells were treated with 10 μ g/ml of cycloheximide and concomitantly shifted to 38°C. Cells were harvested at 0, 1, 2, 3, and 4 hr, as indicated. Whole-cell extracts were prepared and aliquots normalized by cell equivalents were immunoprecipitated with an affinity-purified anti-Gpa1 antibody. Radiolabeled protein immunoprecipitated from a *MATa/α* diploid extract was also immunoprecipitated as a negative control for Gpa1 expression. The arrow marks the myristoylated form of Gpa1; the asterisk indicates the nonmyristoylated species. Molecular mass is indicated in kilodaltons (*left*).

total protein as *cdc72* mutant cells continue to synthesize polypeptides (including Ste4 and nonmyristoylated Gpa1) at the restrictive temperature. The dilution of myristoylated Gpa1 is most likely accelerated by the transcriptional induction of *GPA1* in response to pheromone pathway activation (Jahng et al. 1988). In contrast, the abundance of the myristoylated form of Gpa1 did not decrease with respect to the total protein when *cdc72* mutant cells were shifted to the restrictive temperature and protein synthesis was concomitantly blocked (Fig. 6). Given the tight stoichiometric relationships between the G protein subunits, a first cycle arrest as a result of dilution of myristoylated Gpa1 is quite plausible. It is known that a slight excess of G_{β} relative to G_{α} (as little as one extra copy of *STE4*) is sufficient to cause a partial activation of the pheromone response pathway (Cole and Reed 1991). In *cdc72* mutant cells growing at permissive temperature, approximately one-half of the Gpa1 is nonmyristoylated. These cells exhibit signs of a partially induced mating response. Therefore, upon thermal inactivation of the defective Nmt1, it is not surprising that the ratio of $G_{\beta\gamma}$ to myristoylated G_{α} increases rapidly enough to fully induce the mating response and cause G_1 arrest.

Localization of Gpa1 to membranes does not require Nmt1 activity

The basis for the association of G protein heterotrimers with the cytoplasmic surface of the plasma membrane is not known. On the basis of their cDNA sequence analyses, mammalian G_{α} proteins are predicted to be relatively hydrophilic (Gilman 1987; Jones and Reed 1987; Lochrie and Simon 1988). Consistent with this prediction, purified α subunits do not require detergent to remain in aqueous solution (Sternweis 1986). Since G_{α} proteins associate with phospholipid vesicles only if the vesicles contain $G_{\beta\gamma}$ complexes, it has been suggested that $G_{\beta\gamma}$ serves as a membrane anchor for G_{α} (Sternweis 1986). This idea is contradicted by the observation that G_{α} subunits remain associated with the membrane fraction even when expressed at a considerably higher level than $G_{\beta\gamma}$ (Simonds et al. 1989; Mumby et al. 1990). Moreover, the α subunits of heterotrimeric G proteins do not become soluble even when they are activated and release $G_{\beta\gamma}$ (Sternweis 1986; Buss et al. 1987). Clearly, G_{α} proteins must have other means of maintaining their membrane association, independent of $G_{\beta\gamma}$ interaction.

With the exception of α_s , all of the mammalian G_{α} proteins tested thus far are myristoylated and membrane localized when transiently expressed in COS cells. Mutations that abolish the myristoylation of α_i and α_o , as well as that of pp60^{src}, cause these proteins to accumulate in the cytoplasm (Cross et al. 1984; Jones and Reed 1987; Mumby et al. 1990). This observation has led to the proposal that myristoylation is required for membrane localization of at least some G_{α} subunits. The intrinsic hydrophobicity of myristic acid may promote interaction with membrane lipids, other membrane proteins, or both. There is not a simple causative

relationship between amino-terminal myristoylation and membrane localization, however. Not all myristoylated proteins are associated with membranes (Olson et al. 1985), and α_s is membrane localized even though it lacks this modification (Jones et al. 1990; Mumby et al. 1990).

Like its mammalian counterparts, Gpa1 is a relatively hydrophilic protein that is amino-terminally myristoylated and found in association with the membrane fraction of cellular lysates. However, neither the initial localization to, nor the ongoing association of Gpa1 with this membrane fraction depends on its myristoylation. Our results clearly show that Gpa1 remains membrane associated when Nmt1 is inactivated in *cdc72-2* mutant cells (Fig. 3), and that a mutant form of Gpa1 that cannot be myristoylated is nevertheless found in the membrane fraction (Fig. 5). It is possible that the localization of nonmyristoylated Gpa1 to membranes depends on its binding to $G_{\beta\gamma}$ or the pheromone receptor, proteins with which Gpa1 is presumed to interact. Two results argue against this idea. First, nonmyristoylated Gpa1 does not become soluble when the cells producing it are treated with pheromone (data not shown). By analogy with mammalian systems, stimulation with agonist is expected to cause the release of the G_{α} protein from both the receptor and $G_{\beta\gamma}$. Second, nonmyristoylated Gpa1 is found primarily in the membrane fraction of cells lacking functional copies of either the *STE2* (receptor) or *STE4* (G_{β}) genes (data not shown).

We cannot assume that the nonmyristoylated Gpa1 that we find in our crude membrane fractions is folded properly and has reached its proper position in the cell. The aberrant species of G_{α} may accumulate in vesicles or associate with insoluble material that cofractionates with the lipid bilayer. Nevertheless, although our fractionation procedure does not yield plasma membrane exclusively, it is similar to the protocol applied in assessing the localization of mammalian G_{α} proteins. Yet, blocking the myristoylation of α_i and α_o causes these proteins to accumulate in the cytosol. Relatively little nonmyristoylated α_i and α_o is found in the crude membrane pellet. Assuming that our membrane preparation procedure yields a pellet fraction of similar purity, our results suggest that Gpa1 is localized by mechanisms distinct from those that dictate the membrane association of α_i and α_o . What are these mechanisms? An observation that may pertain to this question is that Gpa1 synthesized in *Escherichia coli*, which comigrates with the nonmyristoylated form of Gpa1 produced in yeast, is soluble (D. Stone, E. Meldrum, and S. Reed, unpubl.). Because nonmyristoylated Gpa1 is found in the membrane fraction of yeast lysates, it may interact with a membrane-bound protein, or its binding may depend on the composition of the yeast lipid bilayer. Alternatively, Gpa1 may undergo an additional modification or modifications (besides myristoylation) in yeast but not in bacterial cells.

A recent study of α_s localization may provide another clue as to how nonmyristoylated Gpa1, in contrast to nonmyristoylated α_i and α_o , is anchored to membranes. Using a reconstitution assay, Audigier et al. (1990)

showed that the carboxy-terminal 1 kD of α_s is required for its attachment to membranes. Removal of a 9-kD amino-terminal portion of α_s , on the other hand, had no effect on its localization in this system. It is possible that α_s and Gpa1 employ similar mechanisms to effect stable membrane association. Neither protein requires amino-terminal myristoylation. Rather, each may rely on the intrinsic structure or modification of its carboxy-terminal domain for proper localization.

Why is nonmyristoylated Gpa1 unable to negatively regulate the pheromone response pathway?

In *S. cerevisiae*, the pheromone responsive G_{α} protein negatively regulates the mating signal, presumably by binding to $G_{\beta\gamma}$ until the appropriate stimulus is received. The inability of nonmyristoylated Gpa1 to inhibit the pheromone response pathway must ultimately be the result of an inability to sequester the signal transducing $G_{\beta\gamma}$ dimer. Such a defect could result in one of two fundamental ways: either nonmyristoylated Gpa1 is mislocalized, or it has a decreased affinity for $G_{\beta\gamma}$. We favor the latter hypothesis. Our finding that nonmyristoylated Gpa1 is membrane associated, although short of proving proper localization, suggests that myristoylation serves a purpose other than as a membrane anchor. Moreover, there is precedent for the direct involvement of myristate in protein-protein interactions. The capsid protein VP4 of poliovirus is myristoylated, and electron density maps of the virion indicate that myristate is an integral part of the protein shell, where it is thought to stabilize the interaction between VP3 and VP4 (Chow et al. 1987). Of greater relevance, Linder et al. (1991) have recently demonstrated that α_o has a greatly increased affinity for $G_{\beta\gamma}$ when it is myristoylated than when it is not. In light of this finding, as well as our results, it is tempting to speculate that the primary role of the myristate moiety is to provide specificity in the interactions of G_{α} proteins with other signaling molecules. Thus, the activation of the pheromone response in *cdc72* mutant yeast cells might be attributable to the compromised ability of Gpa1, lacking its amino-terminal modification, to bind $G_{\beta\gamma}$. The decreased affinity for $G_{\beta\gamma}$ might result from an altered conformation of nonmyristoylated G_{α} , or because the myristoyl moiety constitutes part of the $G_{\beta\gamma}$ binding site. Alternatively, the myristoyl tail may orient G_{α} on the face of the membrane so that it is best able to interact with $G_{\beta\gamma}$.

In addition to the role that the inactive (GDP-bound) form of Gpa1 plays in negatively regulating the pheromone response (binding of $G_{\beta\gamma}$), the active (GTP-bound) form of Gpa1 is thought to stimulate an adaptive signaling pathway. Interestingly, a mutant form of Gpa1 that induces a hyperadaptive response to pheromone is unable to do so when a second mutation that abolishes myristoylation is created (D. Stone, H. Stratton, and S. Reed, unpubl.). The activated form of Gpa1 may need to interact with $G_{\beta\gamma}$ to stimulate adaptation, or perhaps nonmyristoylated Gpa1 has a decreased affinity for the putative adaptation effector.

Nmt1 modifies at least one substrate essential to the vegetative growth of all cell types

Although Gpa1 is the most critical target of Nmt1 in haploid cells, it is clear that there is at least one other essential substrate of this enzyme. In cells that do not express the mating pheromone response pathway, loss of Nmt1 activity causes a nonspecific growth defect: heterogeneous arrest on the first cell cycle. This occurs at a restrictive temperature significantly higher than that required to induce the synchronous arrest seen in mating-competent cells. Overexpressing Gpa1 in haploid *cdc72* cells relieves the mating response phenotypes—induction of *FUS1*, G_1 arrest, and projection formation—but not the inability of the mutant cells to proliferate. Haploid *cdc72* cells overexpressing Gpa1 arrest throughout the cell cycle, as do *MATa/α cdc72/cdc72* diploids. We conclude that the myristoylation of Gpa1 is the limiting defect in *cdc72* mutant cells that are competent to mate, probably because of the tight stoichiometric relationship between the G protein subunits. However, other essential functions dependent on Nmt1 activity are uncovered when the mating response pathway is turned off. Two additional observations—that the *NMT1* transcript is expressed equally in all cell types (Reed et al. 1988) and that numerous *S. cerevisiae* proteins can be rapidly labeled with [3 H]myristate (Towler and Glaser 1986; this paper; data not shown)—are consistent with the suggestion that Nmt1 activity is required for more than one function in vegetatively growing cells.

Prior to this work, the role of amino-terminal myristoylation has been studied by blocking the acylation of individual substrates, one at a time. Typically, these experiments are performed by transiently expressing mammalian G_{α} proteins in heterologous tissue culture cell lines, or by adding protein synthesized in vitro to membrane preparations. These procedures have obvious limitations. It is not possible to preserve important stoichiometric relationships, for example, and the effects of myristoylation on native G protein signaling pathways cannot be assessed. The identification in yeast of conditional mutations in *N*-myristoyl transferase has allowed us to examine, for the first time, the effects of inactivating this enzyme on the function of a G protein-mediated signal transduction pathway in situ. The availability of conditional alleles of *NMT1* provides us with a valuable tool with which to identify other substrates of *N*-myristoyl transferase, as well as the proposed myristoyl protein receptors and enzymes required for processing the amino-termini of myristoylated proteins.

Materials and methods

Yeast strains, media, and microbiological techniques

All yeast strains used in this study are isogenic derivatives of strain 15Da (*MATa ade1 his2 leu2-3,-112 Δura3 Δtrp1*) (Cole et al. 1990), generated from BF264-15D (Reed et al. 1985) by disruption of the *URA3* locus, and strain 381G (*MATa ade2-1 cry1 his4-580 lys2 tyr1 trp1*) (Hartwell 1980). Both rich medium (YEPD) and synthetic minimal media (SD) supplemented with

various nutrients to allow cell growth and plasmid maintenance were prepared as described by Sherman et al. (1986). For induction of *GPA1* in cells containing the *GAL1p::GPA1* plasmid (Cole et al. 1990), 2% galactose was added to cells grown in sucrose-based media (2%). Genetic manipulation of yeast cells was as described by Mortimer and Hawthorne (1969). Transformation of yeast cells was by the alkali cation method of Ito et al. (1983). Formation of mating-type homozygous diploids was by spheroplast fusion as described by Katz et al. (1987). The growth rate of yeast cultures was determined by counting cells in a hemacytometer using a Leitz phase-contrast microscope. All photographs were taken with a Zeiss Axiophot photomicroscope fitted with differential interference contrast (Nomarski) optics, with a 100× objective and Kodak Tri-X pan film. *E. coli* strains used to propagate plasmids were JA226 and DH5α. Recombinant DNA techniques were essentially as described by Sambrook et al. (1989).

Preparation of polyclonal antisera against Gpa1 peptides and recombinant Gpa1 protein

All methods for the generation and purification of antibodies were essentially as described by Cole and Reed (1991). The *GPA1*-derived peptide EQSLQLEKQRDKNE-amide (a generous gift of Dr. Richard Houghton) was coupled to the carrier protein keyhole limpet hemocyanin (Sigma) with the heterobifunctional cross-linking agent *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS), as described in Harlow and Lane (1988), with the exception that free amino groups on the peptides were blocked by treatment with 0.1 sodium borohydride in ice-cold 0.1 M sodium borate buffer. A New Zealand white rabbit was subcutaneously injected with peptide-coupled carrier. The first injection was with 200 µg of antigen in PBS (150 mM NaCl, 10 mM sodium phosphate at pH 7.6) mixed with an equal volume of Freund's complete adjuvant. Subsequent boosts were with 100 µg of antigen in PBS mixed with an equal volume of Freund's incomplete adjuvant. Boosts were at 2-week intervals, with bleeding at 10–14 days after boosting.

Anti-peptide antisera were affinity purified with bacterially produced recombinant Gpa1 (see below) coupled to CNBr-Seph-*arose* CL-4B, essentially as described by Wittenberg et al. (1987). Two to three milliliters of crude serum were slowly cycled through the Gpa1-Seph-*arose* column for 1.25 hr. The column was then washed with 10 volumes of PBS (pH 7.6), 10 volumes of 0.1 M sodium acetate, 1 M NaCl (pH 4.8), 10 volumes of 0.1 M NaHCO₃, 1 M NaCl (pH 7.6) and, finally, 10 volumes of PBS (pH 7.6). Elution of antibody was with 0.1 M glycine (pH 2.5) into a volume of 1 M Tris-HCl (pH 8.0) sufficient to neutralize the glycine.

Antisera against recombinant Gpa1 protein were prepared using the method of Rosenberg et al. (1987). A *NdeI* site was engineered precisely at the initiation codon of the *GPA1*-coding sequence using the polymerase chain reaction (PCR). Taking advantage of an *EcoRI* site in the 3'-flanking region of *GPA1*, a *NdeI*-*EcoRI* fragment containing the *GPA1*-coding sequence was placed under the control of the T7 polymerase promoter in the vector pRK172 (Rosenberg et al. 1987). This construct was transformed into *E. coli* strain BL21 (DE3). Treatment of this strain with 0.4 mM IPTG leads to rapid, high-level accumulation of Gpa1 protein. This bacterially produced protein was enriched from the insoluble fraction of bacterial extracts as described by Kleid et al. (1981). Purified recombinant Gpa1 was obtained by preparative SDS-PAGE followed by electroelution. Injection of rabbits and affinity purification of antisera was as described above for the Gpa1 peptide antisera.

Quantitative mating assays

Mating assays were performed essentially as described by Reid and Hartwell (1977) except that conjugation mixtures consisted of 10⁶ experimental cells and 10⁷ tester cells. After preincubating the strains to be assayed at permissive (23°C) or restrictive temperatures (36°C) for 2 hr, the cells were mixed and filtered onto sterile nitrocellulose disks. These disks were placed onto YEPD plates and incubated for 4 hr at 23°C or 36°C. Cells were then resuspended in sterile water, serially diluted, and plated onto both rich medium and medium selective for diploids formed by mating of the two strains (containing complementary auxotrophic markers). The frequency of mating was then calculated as the number of colonies formed on selective medium divided by the number of colonies formed on rich medium. The mating efficiency of the various mutant strains is expressed as a percentage of the mating frequency observed in the wild-type cross.

FUS1 expression assays

The expression of the pheromone-inducible *FUS1* transcript was assayed by measuring β-galactosidase levels in cells containing a *FUS1::lacZ* hybrid gene. Wild-type and mutant strains transformed with the *FUS1::lacZ* reporter vector (pSB231) (Trueheart et al. 1987) were grown to an A₆₀₀ of 0.5 in selective medium. Cultures were divided and shifted to 37°C for 3 hr, treated with 2 µg/ml of α-factor for 3 hr at 23°C or left untreated and growing at 23°C. Cells were then harvested and β-galactosidase activity was determined as described previously (Slater and Craig 1987).

Membrane preparations (cellular fractionation)

To prepare crude membrane and cytosolic fractions for immunoblotting, ~2 × 10⁸ cells were harvested and washed in buffer B [10 mM Tris acetate (pH 8.0), 1 mM Mg acetate, 0.1 mM EDTA, 10% glycerol] (Blumer and Thorner 1990). Cell pellets were resuspended in 200 µl of buffer B with the addition of protease inhibitors [0.025% PMSF, 5% (vol/vol) aprotinin (Sigma), and 10 µg/ml of leupeptin and pepstatin], and 1 mM AlCl₃, 10 mM NaF, 10 mM MgCl₂, and 10 µM GTPγS. Acid-washed glass beads (0.45 mm) were added to the suspensions, and the mixtures were vortexed at high speed four times for 2 min. The lysates were spun in a clinical centrifuge (2000 rpm) to remove the glass beads, and then for 10 min in a microfuge (12,000g) to pellet cell debris. The cleared lysates were centrifuged at 100,000g for 90 min, after which the supernatants were recentrifuged (100,000g, 90 min), and the supernatants from this spin were reserved as the cytosolic fractions. The pellets were washed with buffer B plus protease inhibitors, and respun (100,000g, 90 min). To extract membrane proteins, the pellets were resuspended in 200 µl of buffer P (50 mM NaPO₄ buffer at pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 1 mM β-mercaptoethanol) (Powers et al. 1986) and sonicated for 15 sec. After a 30 min incubation on ice, the extracts were microfuged for 30 min (12,000g), and the detergent solubilized material was reserved as the crude membrane fractions.

Membrane and cytosolic fractions were prepared for immunoprecipitation as described above except that the first microfuge spin was omitted and proteins were extracted from the 100,000g pellets by boiling them for 4 min in denaturing lysis buffer (see below), and reserving the material that was not pelleted in a subsequent 30-min microfuge spin (12,000g).

Immunoblots

An amount of protein from crude membrane and cytosolic fractions equal to an A_{280} of ~ 0.2 , and normalized between strains by cell equivalents, was electrophoresed on a discontinuous SDS-polyacrylamide gel and electroblotted to nitrocellulose (Schleicher & Schuell) as described by Kyhse-Anderson (1984). Blots were then blocked with 2.5% nonfat dry milk, 2.5% BSA in Tris-buffered saline (20 mM Tris-HCl at pH 7.5, 500 mM NaCl) overnight. To increase the signal-to-noise ratio, a triple antibody detection method was employed. Blots were first incubated with dilute affinity-purified peptide antibody in 0.2 $\times$ blocking solution in TBS for 4 hr, followed by several washes with TBS plus 0.1% Tween, and a 2-hr incubation with a 1 : 5000 dilution of goat anti-rabbit antibody (Sigma). After several more washes with TBS plus 0.1% Tween, alkaline phosphatase-conjugated anti-goat IgG (Sigma) was then used to identify antigen-bound antibody on the blots according to the manufacturer's instructions.

Biosynthetic labeling and immunoprecipitations

For bulk labeling of proteins, cells were grown in synthetic medium (SD) lacking methionine to mid-log phase (1×10^7 cells/ml). Three hours prior to harvest, 250 μ Ci/ml of [35 S]methionine (Tran 35 S-label, ICN Radiochemicals) was added per milliliter of culture. To specifically label myristoylated proteins, wild-type cells carrying the *GAL1* promoter vector (YCpG2) or the *GAL1p::GPA1* fusion vector (YCpG2/GPA1) [Cole et al. 1990] were grown to mid-log phase in sucrose-based synthetic medium. The *GAL1* promoter-driven expression of Gpa1 was then induced by the addition of 2% galactose to the culture medium. Cells were allowed to grow for an additional 4 hr, with 500 μ Ci/ml of [3 H]myristate added 2 hr before harvest. Fifteen minutes before the addition of label, cells were treated with 2 μ g/ml of cerulenin, an inhibitor of yeast fatty acid synthesis that enhances the labeling of acylated proteins severalfold [Omura 1976].

Whole-cell extracts were prepared by resuspending cell pellets in lysis buffer [50 mM Tris-HCl (pH 7.5), 1% SDS, 10 mM sodium pyrophosphate, 5 mM EDTA, 5 mM EGTA, 0.1 mM sodium vanadate, 0.025% PMSF, 5% (vol/vol) aprotinin (Sigma), and 10 μ g/ml of leupeptin and pepstatin]. A mixture of ions and a nonhydrolyzable analog of GTP were also added to the lysis buffer to guard against the proteolysis of Gpa1 (1 mM AlCl $_3$, 10 mM NaF, 10 mM MgCl $_2$, and 10 μ M GTP γ S) [D. Stone, E. Meldrum, and S. Reed, unpubl.]. Acid-washed glass beads were added, and the suspension was vortexed at high speed for 2 min. The crude lysates were then boiled for 4 min. After the glass beads were removed, the lysates were spun in a microfuge at 12,000g for 5 min to remove cell debris. An equal volume of 2 $\times$ TNT (2% Triton X-100, 100 mM NaCl, 40 mM Tris-HCl at pH 7.5) was added, as well as 20-fold excess of unlabeled extract prepared for a *MATa*/ α diploid strain (which does not express Gpa1), SDS to a final concentration of 0.2% (0.1% for membrane and cytosolic fractions), protease inhibitors, and affinity-purified polyclonal antibody raised against recombinant Gpa1. For immunoprecipitation of alcohol dehydrogenase (Adh), 5 mg/ml of BSA was substituted for the *MATa*/ α cell extract.

Immunoprecipitates were allowed to form at 0°C for 2 hr except when noted. Antibody-antigen complexes were bound with fixed *Staphylococcus aureus* cells (Pansorbin, Calbiochem) for 30 min, washed three times with TNTBPS 0.1% (1 $\times$ TNT, 2 mg/ml of BSA, 5 mM EDTA, 5 mM EGTA, 10 mM sodium vanadate, 0.1% SDS), and once with 20 mM Tris-HCl (pH

7.5). Proteins were eluted from *S. aureus* cells by boiling in sample buffer (0.1 M Tris-HCl at pH 6.8, 2% SDS, 2% β -mercaptoethanol, 20% glycerol) for 4 min and then separated by SDS-PAGE. Prior to vacuum-drying, gels were treated with Amplify (Amersham), according to the manufacturer's instructions. [35 S]-labeled and [3 H]-labeled proteins were visualized by autoradiography without intensifying screens on XAR-5 X-ray film (Kodak).

PCR mutagenesis of *Gpa1*

The oligonucleotide 5'-GGGGATCCATAATGGCGTGTA-CAG-3' was used to amplify a portion of the *GPA1* gene by using standard PCR techniques [Saiki et al. 1988]. This oligonucleotide substitutes alanine for glycine at position 2 of the putative Gpa1 polypeptide by making a single G $\rightarrow$ C transversion. A small fragment of the mutant sequence extending from the artificial *Bam*HI site inserted just 5' of the initiation codon to a *Hind* III site at position 388 was inserted back into the wild-type *GPA1* gene. The entire construct was then transferred to a centromere vector containing the selectable *TRP1* marker, YCplac 204 [Gietz and Sugino 1988].

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