Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 9688–9692, October 1993
Cell Biology

Pheromone action regulates G-protein α-subunit myristoylation in the yeast *Saccharomyces cerevisiae*

(posttranslational modification/mutants/adaptation)

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Communicated by Randy Schekman, July 19, 1993

ABSTRACT **Myristic acid (C14:0) is added to the N-terminal glycine residue of the α subunits of certain receptor-coupled guanine nucleotide-binding regulatory proteins (G proteins). The Gα subunit (*GPA1* gene product) coupled to yeast pheromone receptors exists as a pool of both myristoylated and unmyristoylated species. After treatment of *MAT*a cells with α factor, the myristoylated form of Gpa1p increases dramatically, and the unmyristoylated form decreases concomitantly. This pheromone-stimulated shift depends on the function of *STE2* (α-factor receptor), *STE11* (a protein kinase in the response pathway), and *NMT1* (myristoyl-CoA:protein *N*-myristoyltransferase) genes and uses the existing pool of fatty acids (is not blocked by cerulenin). Myristoylated Gpa1p persists long after pheromone is removed. Because myristoylation is essential for proper Gα–Gβγ association and receptor coupling, pheromone-dependent stimulation of Gpa1p myristoylation may be an important contributing factor in adaptation after signal transmission.**

The two haploid cell types, *MAT*a and *MAT*α, of *Saccharomyces cerevisiae* secrete peptide pheromones that trigger responses (including gene induction and growth arrest) that lead to mating and formation of *MAT*a/*MAT*α diploid cells (1, 2). Like many extracellular stimuli in mammalian cells, the yeast pheromones bind to seven-transmembrane-segment receptors in the plasma membrane and promote dissociation of a receptor-coupled G protein into its α and βγ subunits (3, 4). In yeast, the Gβγ moiety, comprised of the *STE4* and *STE18* gene products (Ste4p and Ste18p) (5), rather than Gα, the *GPA1* (also called *SCG1*) gene product (Gpa1p) (6, 7), activates the downstream cascade of events (8–11). Thus, the primary role of Gpa1p is to associate reversibly with the Ste4p–Ste18p complex and thereby regulate the level of free Gβγ moiety (12).

Proteins that participate in signal transduction often carry posttranslational modifications. Examples of regulatory proteins that are myristoylated on their N-terminal glycine residue (13, 14) include $pp60^{v\text{-}src}$ (15), catalytic subunit of cAMP-dependent protein kinase (16), and regulatory subunit of phosphoprotein phosphatase 2B (calcineurin) (17). Mammalian regulatory $G_o\alpha$ and inhibitory $G_i\alpha$ subunits (but not stimulatory $G_s\alpha$ subunit) are N-myristoylated (18, 19), and α subunit of retinal G protein, transducin, is heterogeneously acylated (20, 21). N-myristoylation of $G_o\alpha$ and $G_i\alpha$ subunits is required for their association with membranes and for their high-affinity binding to Gβγ moiety *in vitro* (22–25). Likewise, yeast Gpa1p is N-myristoylated, and myristoylation is necessary for its efficient interaction with Gβγ *in vivo* (26).

Activation of mammalian macrophages by cytokines and of other cell types by various extracellular stimuli induces N-myristoylation of a protein kinase C substrate (MARCKS) (27, 28) and unidentified proteins of 42, 45, and 48 kDa (29, 30). We sought to determine whether N-myristoylation of a Gα subunit is also modulated by hormone action using *S. cerevisiae* because (*i*) the number of myristoylated species in yeast is low (31); (*ii*) N-myristoyltransferase was first purified from this source (32, 33), and its gene (*NMT1*) has been characterized (34, 35); (*iii*) Gpa1 is N-myristoylated (26) and is the only Gα subunit coupled to the pheromone receptors (36); and (*iv*) by using conditional *nmt1* mutations (26, 34, 35) or mutations that eliminate or replace Gly-2 in Gpa1p (26, 37), lack of N-myristoylation of Gpa1p has been shown to result in constitutive activation of the pheromone-response pathway, presumably due to Gβγ release.

Because N-myristoylation is required for Gpa1p function, pheromone control of this modification would provide a potentially important feedback mechanism for regulating the state of assembly of the heterotrimeric G-protein complex and its capacity to couple to its cognate receptor.

MATERIALS AND METHODS

Strains, Media, and Transformation. *S. cerevisiae* strains were as follows: YPH499 (*MAT*a *ura3-52 lys2-801^{am} ade2-101^{oc} trp1-Δ63 his3-Δ200 leu2-Δ1* (38); DMY400 (YPH499 *sst2-Δ2*) [from D. Ma, this laboratory (Berkeley)]; JGY11 (*MAT*a *ste11^{ts} LEU2 ade2^{oc} his$^-$ lys2 trp1 ura3-52*) (from J. Gowen, this laboratory), derived from a cross of YPH500 (*MAT*α) (38) and 381G-44B (*MAT*a *ste11^{ts}*) (39); MHY6 (YPH499 *ste18Δ::LEU2*) (40); JDY3 (YPH499 *ste12Δ::LEU2*) (41); DK102 (YPH499 *ste2Δ::HIS3 sst1-Δ5*) (from D. Kaim, this laboratory); YB332 (*MAT*a *ura3 his3-Δ200 ade2 lys2-801^{am} leu2*) and YB334 (YB332 *nmt1-72^{ts}*) (from J. Gordon, Washington University School of Medicine, St. Louis) (35); and DJ803-11-1 (*MAT*a *ura3 ste5-3^{ts} bar1-1 leu2 ade2 can1 cyh2 TYR1*) and DJ803-2-1 (DJ803-11-1 *scg1::lacZ/LEU2 ADE2*) (from D. Jenness, University of Massachusetts Medical School, Worcester, MA) (8). Most experiments were done with strain DMY400 because lower doses of α factor could be used (42), but qualitatively similar results were obtained when unrelated SST2$^+$ strains (for example, strain YB332) were used. Standard methods for the growth, DNA-mediated transformation, and genetic manipulation of yeast were used (43, 44).

Reagents for SDS/PAGE and electroblotting were from Bio-Rad. Nitrocellulose (0.2-μm pore size) was from Schleicher & Schuell. Cerulenin (Calbiochem) and cycloheximide (Sigma) were prepared as concentrated (1000 times) stock solutions in ethanol and stored at −80°C. Synthetic α factor

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(Star Biochemicals, Torrance, CA) was dissolved in HPLC-grade water (1 mg/ml) and stored at −20°C.

Preparation of Anti-Gpa1p Antiserum. A decapeptide (H_2N-QQNLKKIGII-COOH) corresponding to the C-terminal end of Gpa1p (6, 7) was synthesized (by C. G. Unson, Rockefeller University, New York), conjugated to keyhole limpet hemocyanin using glutaraldehyde, and used as the immunogen to raise antisera in rabbits by methods described in detail elsewhere (45). Anti-Gpa1p antibodies were isolated by immunoaffinity chromatography on a column containing the decapeptide immobilized on Affi-Gel 15 beads (Bio-Rad) using described procedures (46).

Protein Mobility-Shift Assay. Yeast cells were grown routinely at 30°C in yeast extract/peptone/dextrose medium (43). Cultures of each temperature-sensitive strain (and its corresponding wild-type parent) were grown at a permissive temperature, and after reaching midexponential phase, portions were shifted to a restrictive temperature for 2 hr (24°C → 37°C, for strains JGY11, YPH499, YB332, and YB334; 37°C → 24°C, for strains DJ803-11-1 and DJ803-2-1). Before pheromone treatment, cultures were treated for 30 min with either cycloheximide at 10 μg/ml, cerulenin at 2 μg/ml, or solvent alone (0.1% ethanol). After drug treatment, the cultures were exposed to 5 μM α factor for an additional 30–60 min, as indicated. Growth was stopped by addition of 10 mM NaN_3 and chilling on ice. Cells were harvested by centrifugation (4°C) and washed once with ice-cold 10 mM NaN_3. Cell density was determined spectrophotometrically, and an equivalent number of cells (≈30 A_{600nm} units) were transferred to a 1.5-ml Eppendorf tube and collected by brief centrifugation. The resulting cell pellet was resuspended in 300 μl of lysis buffer (0.1 M Tris·HCl, pH 6.8/2% SDS/2% 2-mercaptoethanol/20% (vol/vol) glycerol/0.003% bromophenol blue), boiled for 10 min, and further disintegrated by vigorous mixing with glass beads (0.50-mm diameter) for 4 min. Resulting whole-cell extracts were resolved by discontinuous SDS/PAGE (47) using a Mini-Protean (Bio-Rad) apparatus, transferred electrophoretically to nitrocellulose paper (48), and probed (49) by using polyclonal anti-Gpa1p antibodies (3 μg/ml), horseradish peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad; 1:10,000 dilution), and a chemiluminescence detection system (ECL; Amersham).

RESULTS

Two Immunoreactive Species of Gpa1p. Measurement of protein myristoylation typically requires radiolabeling and immunoprecipitation of an over-expressed protein (17). However, the myristoylated and unmyristoylated species of Gα subunits often are separable by gel electrophoresis (23, 24). Resolution of these forms is particularly striking for yeast Gpa1p (26). Indeed, we found that immunoblot analysis could be applied to visualize conveniently the relative abundance of the modified and unmodified forms of Gpa1p, even at their endogenous level, using an antiserum raised against a synthetic decapeptide corresponding to the C terminus of Gpa1p and chemiluminescence detection. Proteins with apparent molecular masses of 54 and 56 kDa were among the most prominent bands detected (Fig. 1). These two species represented different forms of Gpa1p because both migrated near the predicted molecular mass of the *GPA1* gene product (54.1 kDa) and were absent in mutant cells lacking a functional *GPA1* gene (Fig. 1). Moreover, the same bands cross-reacted with polyclonal anti-Gpa1p antiserum raised against a LacZ–Gpa1p fusion protein (50) and were overproduced in cells carrying the *GPA1* gene on a multicopy plasmid (data not shown). Only the 54-kDa species can be metabolically labeled with [^{3}H]myristate (26); and conversely, the 56-kDa species accumulates when cells carrying a temperature-sensitive mutation (*nmt1-72*) in the *N*-myristoyltransferase are shifted

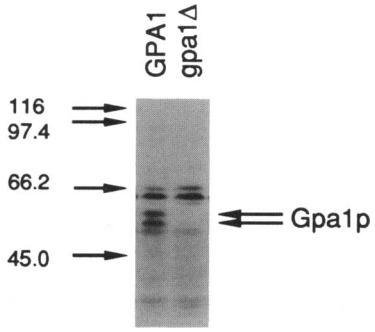

FIG. 1. Detection of two forms of Gpa1p by immunoblotting. Strains DJ803-11-1 (*MAT***a** *GPA1 ste5^{ts}*) and DJ803-2-1 (*MAT***a** *gpa1Δ ste5^{ts}*) were grown at 37°C, harvested, washed, extracted, fractionated by SDS/PAGE, and analyzed by using polyclonal anti-Gpa1p antibodies. At 37°C, inactivation of thermolabile Ste5p prevents constitutive growth arrest that would ordinarily result from the absence of Gpa1p (8). Arrows, unmyristoylated (≈56 kDa) and myristoylated (≈54 kDa) Gpa1p. Molecular size markers (in kDa) are at left.

to the restrictive temperature (26, 35). Thus, the 54-kDa form represents myristoylated Gpa1p, and the 56-kDa form represents unmyristoylated Gpa1p.

Pheromone Treatment Affects Gpa1p Myristoylation. The difference in electrophoretic mobility between myristoylated and unmyristoylated Gpa1p was used to follow this modification under various conditions. During balanced growth, there was an approximately equimolar ratio of the two forms (Fig. 2). After treatment of *MAT***a** cells with α factor, however, the proportion of the myristoylated species noticeably increased, even after periods as brief as 10 min (Fig. 2). By 1 hr after exposure to pheromone, the pool of Gpa1p was converted nearly quantitatively to the myristoylated form.

Pheromone-Induced Myristoylation Requires New Protein Synthesis. The substrates for Gpa1p myristoylation are myristoyl-CoA and des-Met-Gpa1p (13, 14). Myristoylation of most proteins in mammalian cells is rapidly blocked by protein synthesis inhibitors, suggesting that modification may occur concomitantly with nascent chain synthesis (51–53). However, examples of apparent posttranslational myristoylation of proteins also have been reported in slime mold (54), yeast (55), and human (56) cells. To distinguish between these two different modes of modification, *MAT***a** cells were treated with cycloheximide for 30 min before exposure to α factor. In the absence of pheromone administration, cycloheximide treatment significantly reduced the levels of both myristoylated and unmyristoylated Gpa1p (Fig. 3), indicating that both forms are more rapidly degraded than the bulk of cellular protein (because equal amounts of protein were loaded in each lane). When cells were exposed to α factor after treatment with cycloheximide, the dramatic increase in

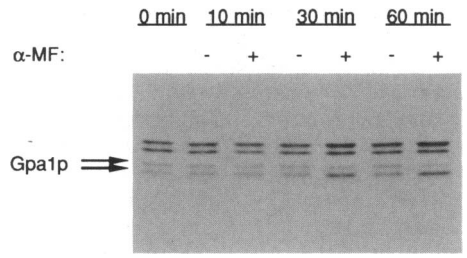

FIG. 2. Time course of pheromone-stimulated myristoylation of Gpa1p. Strain DMY400 (*MAT***a** *GPA1 sst2-Δ2*) was grown in yeast extract/peptone/dextrose at 30°C and split into two equal portions. One sample was treated with 5 μM α factor (α-MF) for the indicated times (+), and the other received only H_2O (−). Extracts were prepared and analyzed as described in the legend for Fig. 1.

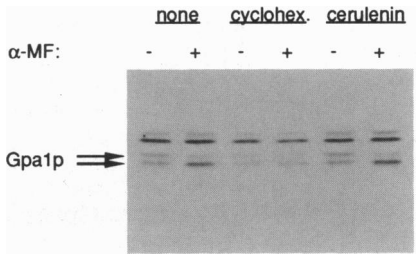

FIG. 3. Effect of metabolic inhibitors on Gpa1p myristoylation. Strain DMY400 was grown at 30°C, split into three equal portions, incubated for 30 min with cycloheximide (cyclohex.) at 10 μg/ml, cerulenin at 2 μg/ml, or solvent (ethanol) alone (none). Then samples were either treated with 5 μM α factor (+) or mock-treated (−) for 30 min. Each sample was analyzed as described in the legend for Fig. 1.

the myristoylated form normally induced by pheromone was almost completely blocked (Fig. 3). However, the ratio of myristoylated-to-unmyristoylated Gpa1p was slightly higher after α-factor treatment than after mock treatment, suggesting that at least some myristoylation might occur posttranslationally.

These observations suggest that the α-factor-stimulated accumulation of myristoyl-Gpa1p results primarily from two processes. (*i*) Both myristoylated and unmyristoylated Gpa1p are rapidly degraded and must be continuously replenished. (*ii*) The Gpa1p newly synthesized after α-factor addition is more efficiently myristoylated than the preexisting protein. Hence, the net effect of pheromone treatment is to shift the pool of Gpa1p from partially myristoylated to mostly myristoylated.

In contrast to the effect of cycloheximide, neither Gpa1p synthesis nor α-factor-dependent enhancement of myristoylation was prevented by treatment with cerulenin, an inhibitor that efficiently blocks *de novo* fatty acid biosynthesis (57, 58) (Fig. 3). Thus, the existing pool of myristoyl-CoA appeared adequate to accommodate new Gpa1p synthesis for at least 30 min.

Myristoylation Is a Stable Modification of Gpa1p. In principle, α factor could stimulate the accumulation of myristoylated Gpa1p by inhibiting its demyristoylation. Unlike other types of fatty acylations of proteins (59), however, N-myristoylation does not seem reversible (60). To determine whether pheromone-stimulated Gpa1p myristoylation is a stable modification, cells were treated with α factor for 30 min, washed thoroughly to remove the pheromone, and then harvested immediately or grown for an additional 1.5 hr with or without freshly added pheromone. After the initial pheromone treatment, the myristoylated form of Gpa1p was the predominant species and persisted, even when cells were incubated for an extended period without α factor (Fig. 4).

A Functional Pheromone Response Pathway Is Required for Stimulation of Gpa1p Myristoylation. To determine whether stimulation of Gpa1p myristoylation is mediated via the known mating signal-transduction cascade (1, 2), the forms of Gpa1p in a series of nonmating (*ste*) mutants were examined before and after pheromone stimulation.

Unlike wild-type *MAT***a** cells, where a pronounced increase in myristoyl-Gpa1p occurred after treatment with α factor, no shift was seen (Fig. 5*A*) in an otherwise isogenic *MAT***a** *ste2*Δ strain (which lacks the α-factor receptor) (61). Thus, α factor must bind to its receptor to initiate the events that lead to increased myristoylation of Gpa1p. Pheromone-dependent stimulation of Gpa1p myristoylation in a ste18Δ mutant (which lacks Gγ subunit) (5) and in a ste12Δ mutant (which lacks the transcription factor required for pheromone induction of gene expression) (62) was difficult to assess because of the significant reduction in Gpa1p expression

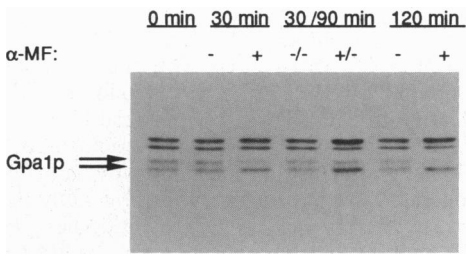

FIG. 4. Stability of Gpa1p myristoylation. Strain DMY400 was grown at 30°C, and a sample was withdrawn (0 min). The remainder was split into two portions and incubated for 30 min with or without 5 μM α factor (α-MF). Control (−) and pheromone-treated (+) cultures were each split into three equal portions. (*i*) One set (30 min) was harvested immediately. (*ii*) The second set (30/90 min) was washed, and both the cells not previously exposed to α factor (−/−) and those previously exposed to α factor (+/−) were resuspended in fresh medium lacking α factor and incubated for an additional 90 min. (*iii*) The third set (120 min) was washed, and the cells never exposed to α factor were resuspended in fresh medium lacking α factor (−); those cells that had been exposed to α factor were resuspended in fresh medium with 5 μM α factor (+), and both samples were incubated for an additional 90 min. All samples were analyzed as described in the legend for Fig. 1.

caused by these mutations (Fig. 5*A*). The *GPA1* gene is a pheromone-responsive gene (37), and even its basal level of expression depends on an intact pheromone-response pathway (1, 40).

To circumvent this problem, a *MAT***a** strain carrying a temperature-sensitive mutation in the *STE11* gene, which encodes a protein kinase (63) essential for the signaling pathway (39), was used (Fig. 5*B*). When propagated at permissive temperature and treated with pheromone, both the ste11^{ts} strain and a congenic normal strain displayed an increase in the myristoylated form of Gpa1p. When shifted to

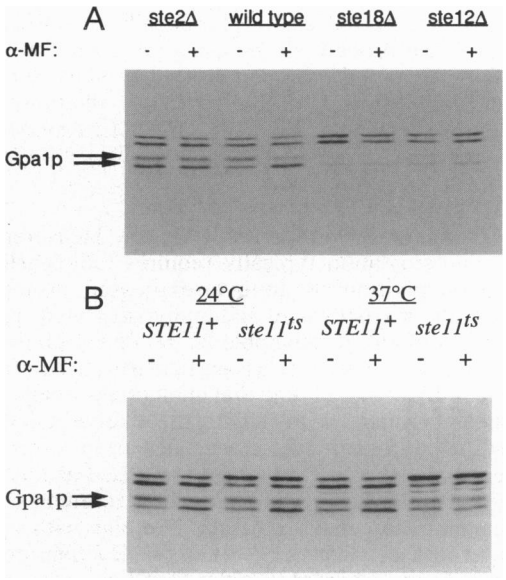

FIG. 5. Perturbation of the mating signal-transduction pathway prevents pheromone stimulation of Gpa1p myristoylation. (*A*) Each mutant strain indicated (ste2Δ, ste18Δ, and ste12Δ) and its otherwise isogenic normal control strain (wild type) were incubated in the absence (−) or presence (+) of 5 μM α factor (α-MF) for 30 min. (*B*) Congenic STE11 and ste11^{ts} strains were grown at 24°C and split into two equal portions, one of which was shifted to 37°C and the other of which remained at 24°C. Two hours after the shift, each culture was further split into two equal portions; one was treated with 5 μM α factor (+) and the other was mock-treated (−); both portions were then incubated for an additional 60 min. All samples were analyzed as described in the legend for Fig. 1.

the restrictive temperature and then exposed to pheromone, the wild-type strain still exhibited the same increase in the proportion of myristoylated Gpa1p, whereas the ste11^{ts} strain did not (Fig. 5*B*).

Pheromone-Induced Gpa1p Myristoylation Requires the *NMT1* Gene Product. In growing cells, myristoylation of Gpa1p requires a functional *NMT1* gene (14, 26). To determine whether an active *NMT1* gene product (Nmt1p) is also required for myristoylation of Gpa1p in response to pheromone, a strain carrying a temperature-sensitive allele (*nmt1-72*) was treated with α factor. In the wild-type control, a pheromone-dependent increase in myristoylated Gpa1p was observed at both 24° and 37°C (Fig. 6). In the otherwise isogenic nmt1-72 strain, high levels of the 56-kDa unmyristoylated form of Gpa1p were accumulated at both 24°C and 37°C, suggesting that the mutant enzyme was quite defective, even at the lower temperature. Nonetheless, at 24°C, the mutant cells displayed a modest, but detectable, increase in the 54-kDa form of Gpa1p in response to pheromone; whereas at 37°C the level of the unmyristoylated form did not increase perceptibly (Fig. 6).

DISCUSSION

Because Gpa1p is a negative regulator of the pheromone signaling pathway (6, 7), changes in its level of expression should affect pheromone responsiveness. Indeed, forced overproduction of Gpa1p attenuates pheromone response (6, 12). Although GPA1 mRNA increases in response to α factor (37), we found that, due to rapid turnover of Gpa1p, the primary effect of *GPA1* induction is not an elevation in total Gpa1p but rather is a marked increase in the fraction of Gpa1p myristoylated. Hence, myristoylation presumably occurs cotranslationally during synthesis of new Gpa1p. In agreement with this conclusion, the pheromone-dependent shift requires the pheromone-response pathway, as well as a functional *NMT1* gene. The myristoylated Gpa1p persists long after withdrawal of the pheromone stimulus.

There are a number of mechanisms by which α factor might stimulate myristoylation. (*i*) The catalytic activity of Nmt1p could be altered by one of the protein kinases (or some other protein) that becomes activated during the pheromone response. Consistent with this possibility, when protein synthesis was inhibited, pheromone treatment still caused a modest, but detectable, increase in the ratio of myristoylated-to-unmyristoylated Gpa1p. (*ii*) α-Factor action could also enhance the transcription, translation, or stability of Nmt1p. (*iii*) α-Factor-induced activation of its receptor should convert Gpa1p to its GTP-bound state, and perhaps this conformation is a better substrate for myristoylation. Analogously, treatment of either intact neutrophils with a chemoattractant peptide or neutrophil extracts with guanosine 5′-[γ-thio]triphosphate promotes carboxylmethylation of the Ras-related protein p22^{rac2} (64). However, mutations in Gpa1p that should stabilize its GTP-bound state have no detectable effect on the ratio of myristoylated-to-unmyristoylated Gpa1p either before or after exposure to pheromone (H.G.D. and J.T., unpublished results).

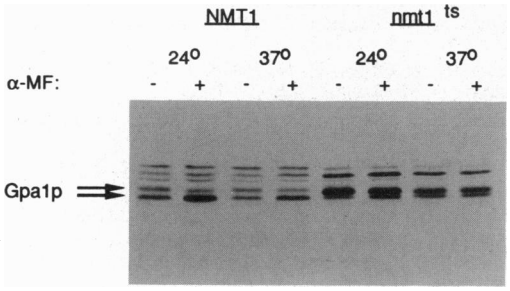

FIG. 6. A functional *NMT1* gene is required for both basal and pheromone-stimulated myristoylation of Gpa1p. Strain YB334 (*MAT***a** *nmt1-72^{ts}*) and isogenic parental strain YB332 (*MAT***a** *NMT1*) were grown at 24°C and split into two equal portions. One portion was shifted to 37°C, and the other portion was left at 24°C. Two hours after shift, each culture was split into two equal portions and incubated either without (−) or with (+) 5 μM α factor (α-MF) for an additional 60 min. Samples were analyzed as described in the legend for Fig. 1.

In cells carrying the *nmt1-72^{ts}* mutation grown at 24°C, only a modest pheromone-induced increase in myristoylated Gpa1p was seen, suggesting that the mutant enzyme was already largely inactive. Conversely, some myristoyl-Gpa1p was still detectable (although its level did not change in response to pheromone), even after the mutant strain was shifted to 37°C for 2.5 hr. This population presumably represents that fraction of Gpa1p already myristoylated before the temperature shift, and its persistence confirms that N-myristoylation of Gpa1p is a stable modification. Most significantly, the overall level of Gpa1p was elevated in the nmt1-72 strain. Because myristoylation of Gpa1p is required for its high-affinity binding to the Gβγ moiety (24, 26), when Gpa1p is not myristoylated, the signal-transduction pathway is partially activated and, consequently, expression of *GPA1* is elevated because it is a pheromone-inducible gene (37). The nmt1-72 mutant can grow at permissive temperature because presumably the elevated level of unmyristoylated Gpa1p overcomes its intrinsically weaker affinity for the Gβγ moiety. Although Nmt1p appears to be the enzyme responsible for myristoylation of Gpa1p, deficiency of Nmt1p activity has pleiotropic effects on the cell. It is possible, therefore, that some other process that requires a myristoylated component accounts for the observed elevation in Gpa1p expression.

Because free Gβγ moiety is responsible for initiating downstream signaling, recapture of Gβγ by Gpa1p is critical for squelching signal transmission. Thus, the pheromone stimulation of Gpa1p modification that we have observed should dampen subsequent signaling and, given its kinetics, contribute significantly to long-term adaptation in this signaling system.

Whether N-myristoylation of Gpa1p plays other roles is not yet known. Myristoylation could dictate the subcellular localization of Gpa1p and, hence, the frequency with which it encounters its cognate receptor (Ste2p) and its cognate Gβγ (Ste4p–Ste18p), which are both plasma-membrane associated. Mutations that prevent N-terminal myristoylation of mammalian Gα subunits, which are normally membrane associated, result in their accumulation in the cytoplasm (23, 26). However, both myristoylated and unmyristoylated Gpa1p have been reported to partition with the membrane fraction (26).

Our findings raise the possibility that hormone action may affect N-myristoylation of Gα subunits in animal cells. On the basis of their apparent size, the unidentified mammalian proteins of 42–48 kDa for which myristoylation is potentiated by a variety of extracellular stimuli (29, 30) could be Gα subunits. In this regard, it is noteworthy that myristoylation seems necessary for the tumorigenic potential of mutationally activated inhibitory $G_{i2}\alpha$ subunit (65). Likewise, N-myristoylation of pp60$^{v\text{-}src}$ is required for its plasma-membrane association and oncogenicity (15). Also, myristoylation of Gag proteins seems critical for replication and capsid assembly of human immunodeficiency virus type 1 and other retroviruses (13, 14). Better understanding of the role of extracellular signals in regulating N-myristoylation of proteins may lead to additional approaches for control of oncogenic transformation and retroviral infection.

We are grateful to P. Casey, J. Gordon, L. Hartwell, P. Hieter, D. Jenness, R. Johnson, C. G. Unson, M. Whiteway, and members of

our laboratory for providing research materials and useful advice. This work was supported by Postdoctoral Fellowship 61-831 from the Jane Coffin Childs Memorial Fund for Medical Research (to H.G.D.), by National Institutes of Health Research Grant GM21841 (to J.T.), and by facilities provided through the Cancer Research Laboratory of the University of California at Berkeley.

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