MOLECULAR AND CELLULAR BIOLOGY, July 1991, p. 3603–3612
0270-7306/91/073603-12$02.00/0

Vol. 11, No. 7

Significance of C-Terminal Cysteine Modifications to the Biological Activity of the *Saccharomyces cerevisiae* a-Factor Mating Pheromone

STEVAN MARCUS,[1] GUY A. CALDWELL,[1] DAVID MILLER,[1] CHU-BIAO XUE,[2] FRED NAIDER,[2] AND JEFFREY M. BECKER[1]*

Department of Microbiology and Program in Cellular, Molecular, and Developmental Biology, University of Tennessee, Knoxville, Tennessee 37996,[1] and Department of Chemistry, College of Staten Island, City University of New York, Staten Island, New York 10301[2]

Received 9 October 1990/Accepted 22 April 1991

We have undertaken total synthesis of the *Saccharomyces cerevisiae* a-factor (NH_2-YIIKGVFWDPAC[*S*-farnesyl]-$COOCH_3$) and several Cys-12 analogs to determine the significance of S-farnesylation and carboxy-terminal methyl esterification to the biological activity of this lipopeptide mating pheromone. Replacement of either the farnesyl group or the carboxy-terminal methyl ester by a hydrogen atom resulted in marked reduction but not total loss of bioactivity as measured by a variety of assays. Moreover, both the farnesyl and methyl ester groups could be replaced by other substituents to produce biologically active analogs. The bioactivity of a-factor decreased as the number of prenyl units on the cysteine sulfur decreased from three to one, and an a-factor analog having the *S*-farnesyl group replaced by an *S*-hexadecanyl group was more active than an *S*-methyl a-factor analog. Thus, with two types of modifications, a-factor activity increased as the *S*-alkyl group became bulkier and more hydrophobic. *MAT*a cells having deletions of the a-factor structural genes (*mfa1 mfa2* mutants) were capable of mating with either *sst2* or wild-type *MAT*α cells in the presence of exogenous a-factor, indicating that it is not absolutely essential for *MAT*a cells to actively produce a-factor in order to mate. Various a-factor analogs were found to partially restore mating to these strains as well, and their relative activities in the mating restoration assay were similar to their activities in the other assays used in this study. Mating was not restored by addition of exogenous a-factor to a cross of a wild-type *MAT*α strain and a *MAT*aste6 mutant, indicating a role of the *STE6* gene product in mating in addition to its secretion of a-factor.

Numerous proteins are now known to be modified post-translationally by addition of prenyl groups. The consensus sequence for prenylation appears to be the C-terminal -CA AX box, where C is cysteine, A is an aliphatic amino acid, and X is any amino acid (18, 19); the modification involves addition of the prenyl group to the cysteine sulfur, followed by proteolysis of the C-terminal -AAX (39, 55). Examples of prenylated proteins include yeast and mammalian RAS (6, 54, 55), the γ subunit of yeast and mammalian G-proteins (16, 38), nuclear lamins (61), and various fungal mating pheromones (2, 31, 51, 52). In some cases, but not all, the carboxyl group of the C-terminal cysteine is also methyl esterified (2, 11, 13). Recent studies (14, 37, 50) suggest that other mammalian proteins are prenylated, indicating that this type of posttranslational modification may be common to many proteins in eukaryotic organisms.

In efforts to elucidate the biological significance of prenylation, it has been shown that this modification is required for proper localization and function of mammalian and yeast RAS (48, 55) and for maximal biological activity of several fungal mating pheromones (2, 59, 63). In addition, prenylation of the *Saccharomyces cerevisiae* a-factor seems to be required for secretion of this peptide mating pheromone (48, 54). The significance of the second modification, the carboxy-terminal methyl ester, found on some prenylated proteins is not as well understood. It is known, however, that this modification is important for the biological activity of some fungal pheromones (2, 17, 63). In addition, it has been shown that C-terminal methyl esterification is not required for secretion of a-factor (41). The purpose of this work was to further study the significance of prenylation and methyl esterification to the biological activity of the *S. cerevisiae* a-factor.

Conjugation between *S. cerevisiae* a (*MAT*a) and α (*MAT*α) haploid cells to form a/α diploids requires the reciprocal action of a-factor, produced by *MAT*a cells, and α-factor, a peptide pheromone secreted by *MAT*α cells (12, 25, 26). These pheromones induce in their target cells similar responses which are required for mating. Growth arrest occurs at G_1 of the cell cycle (5, 23), cell surface agglutinins are produced (15), transcription of several genes is induced (1, 33, 60), and the cells undergo a morphological alteration termed shmooing (36, 57). α-Factor, a tridecapeptide, is processed via the well-characterized yeast secretory pathway, and considerable studies on structure-activity relationships have been published (47). On the other hand, structure-activity relationship studies on a-factor, which is apparently secreted by a novel secretory pathway which has not been fully characterized (4, 28, 34, 39, 41, 43, 54, 55), have only recently been initiated. Anderegg et al. (2) found that replacement of the farnesyl group of a-factor by a methyl group (*S*-methyl a-factor) caused only a moderate decrease in biological activity. These workers concluded that the farnesyl group of a-factor was not required for biological activity. We have previously shown that replacement of the farnesyl group of a-factor by a hydrogen atom (nonfarnesylated a-factor) results in an approximately 800-fold decrease in biological activity (63).

While a-factor in the absence of *MAT*a cells is capable of

* Corresponding author.

inducing a biological response in *MAT*α cells, it had been suggested that *MAT***a** cells must actively produce **a**-factor in order to mate (44). In those studies, *MAT***a** cells having deletions of the **a**-factor structural genes (*mfa1 mfa2* mutants) were not capable of mating with wild-type (*SST2*$^+$) *MAT*α cells, even in the presence of exogenous **a**-factor. This result led the authors to hypothesize that the **a**-factor found extracellularly is not sufficient for mating but rather that a *MAT***a** cell-bound form of **a**-factor might be required for mating. Recent studies by Jackson and Hartwell further demonstrated the importance of both **a**- and α-factor production in a process they termed courtship (29, 30), a process by which opposite mating types select the mating partner which produces the most pheromone.

To further study **a**-factor structure-activity relationships, we synthesized several **a**-factor analogs and examined the role of C-terminal cysteine modifications in the biological activity of this lipopeptide mating pheromone. In this report, we show by use of four different and sensitive biological assays that removal of either the farnesyl group or the carboxy-terminal methyl ester of **a**-factor leads to a similar reduction in the bioactivity of the modified pheromones. In addition, we show that the farnesyl group is not specifically required for high biological activity, since replacement of this group with any of several different hydrophobic hydrocarbon moieties resulted in analogs with biological activity close to that of **a**-factor. We further show that addition of exogenous **a**-factor allows *MAT***a** *mfa1 mfa2* mutants to mate with *sst2* or wild-type (*SST2*$^+$) *MAT*α cells, demonstrating that it is not absolutely essential for *MAT***a** cells to actively produce **a**-factor in order to mate.

MATERIALS AND METHODS

Strains. Halo and shmoo assays were carried out with wild-type *S. cerevisiae* X2180-1B (*MAT*α) (Yeast Genetics Stock Center) or a mutant supersensitive to **a**-factor, RC757 (*MAT*α *sst2-1 rme1 his6 met1 can1 cyh2*) from Russell Chan (7, 8). *FUS1-lacZ* assays were carried out with *S. cerevisiae* LM23-116az [*MAT*α *FUS1*::*lacZ leu2 lys5 met1 ura3-52 ste2*::*TRP1*] from Lorraine Marsh. Mating experiments were done with *S. cerevisiae* RC757 (above), CR11 (*MAT*α *cry1-11 thr4 his6 lys1*) from Wolfgang Duntze (56), SM1058 (*MAT***a** *trp1 his4 leu2 ura3 can1*) and SM1229 (isogenic to SM1058 but *mfa1*Δ::*LEU2 mfa2*Δ::*URA3*) from Susan Michaelis (44), and JPY200 [*MAT***a** *gal2 his3*Δ*200 leu2-3,112 lys2-801 trp-1 ura3-52*] and JPY201 (isogenic to JPY200 but *ste6*Δ*-1*::*HIS3*) from John McGrath (43).

Synthesis and characterization of a-factor and Cys-12 analogs. The **a**-factor (NH_2-YIIKGVFWDPAC[*S*-farnesyl]-$COOCH_3$) and **a**-factor analogs used in this study were synthesized either by solution-phase peptide synthesis or by a combination of solid-phase and solution-phase peptide syntheses; the structures of all analogs are shown in Fig. 1. Detailed protocols for the synthesis and characterization of **a**-factor, nonfarnesylated **a**-factor, and nonmethylated **a**-factor have been published previously (62, 63). Details of the synthesis and characterization of all other analogs will be published separately. All peptides were purified to greater than 98% homogeneity by reversed-phase high-pressure liquid chromatography (HPLC) and were characterized by amino acid analysis, 400 MHz ^1H nuclear magnetic resonance spectroscopy, and fast atom bombardment mass spectroscopy. **a**-Factor and **a**-factor analogs were dissolved directly at 1 to 5 mg/ml in methanol for use as stock solutions in all biological assays.

Shmoo assays. RC757 and X2180-1B were grown at 30°C with shaking to early log phase in YEPD-BSA (1% yeast extract, 2% peptone, 2% dextrose with bovine serum albumin [BSA, 0.2 mg/ml] in 0.05 M citrate, pH 4.5). The cells were harvested by centrifugation at 1,000 × *g*, washed twice with sterile distilled water, and resuspended to 4×10^6 cells per ml in YEPD-BSA. Dilution series (1:2) of **a**-factor and **a**-factor analogs were prepared in siliconized borosilicate glass tubes with YEPD-BSA as the diluent. Five hundred microliters of cell suspension was added to 500 μl of each of the **a**-factor and **a**-factor analog dilutions, and the resulting cell suspensions were incubated for 3.5 h at 30°C with shaking. At the completion of the incubation period, the cells were placed on ice, and 10-μl portions of each suspension were placed in a hemacytometer and observed microscopically to quantitate the total number of cells, percent shmoos (elongated, pear-shaped cells [36, 57]), and percent unbudded cells. The endpoint of activity in this assay was defined as the concentration of pheromone or pheromone analog which caused approximately 50% of the cells to shmoo. At higher concentrations of pheromone, 100% of treated cells were shmoos. The profiles of the response curves to all analogs were highly similar, so that the amounts of pheromone causing 10% shmoos, 50% shmoos, and 100% shmoos were nearly identical for all pheromones tested.

Halo assay. YEPD (YEPD [pH 4.5] with 2% Bitek agar [Difco]) plates were overlaid with 4 ml of RC757 cells (2.5×10^5 cells per ml) in Noble agar (0.825%). Five-microliter portions of **a**-factor solutions at various concentrations in BSA (0.2 mg/ml of water) were then spotted onto the overlay. The plates were incubated at 30°C for 24 to 36 h and then observed for clear zones (halos), an indication of the arrest of cell growth, at the various sample locations (Fig. 2).

***FUS1-lacZ* induction assay.** *S. cerevisiae* LM23-116az was grown overnight in YEPD at 30°C. Cells were diluted to a concentration of 5×10^6/ml and grown to 1×10^7 to 2×10^7/ml at 30°C. Cells were concentrated by centrifugation and then resuspended at 10^8/ml. Induction was performed by adding 0.5 ml of **a**-factor, **a**-factor analog, or α-factor dilutions or water to 4.5 ml of concentrated cells. These mixtures were vortexed and placed at 30°C with shaking for 2 h. After this time, cells were harvested in a tabletop centrifuge, and each pellet was resuspended in 0.5 ml of Z buffer (16.1 g of $Na_2HPO_4 \cdot 7H_2O$, 5.5 g of $NaH_2PO_4 \cdot H_2O$, 0.75 g of KCl, 0.246 g of $MgSO_4 \cdot H_2O$, 2.7 ml of β-mercaptoethanol per 1,000 ml). For each sample, the OD_{600} was recorded, and 1:5 and 1:10 dilutions of cells in Z buffer in a total volume of 1.0 ml were prepared. Yeast cells were permeabilized by the addition of 75 μl of 0.1% sodium dodecyl sulfate and 60 μl of chloroform, followed by vortexing for 15 s. After equilibration to 30°C, the mixtures were assayed for β-galactosidase activity. Assays were initiated by the addition of 0.2 ml of *O*-nitrophenyl-β-D-galactopyranoside (4 mg/ml in 0.1 M KPO_4 [pH 7]) to each sample. After the appearance of a medium yellow color (10 min to 4 h), reactions were stopped by adding 0.5 ml of 1 M Na_2CO_3. Cells from all assay mixtures were pelleted, the supernatant fractions were read at OD_{420} and OD_{550}, and units of β-galactosidase activity were calculated (21, 45, 49). The endpoint of activity in this assay was defined as the concentration of pheromone required to induce a response in β-galactosidase units that was twofold greater than the basal level observed in either untreated LM23-116az cells or LM23-116az cells treated with α-factor (1 μg/ml). Endpoints were calculated as the average value from three separate trials, with each trial being performed in duplicate. In all *FUS1-lacZ* assays performed,

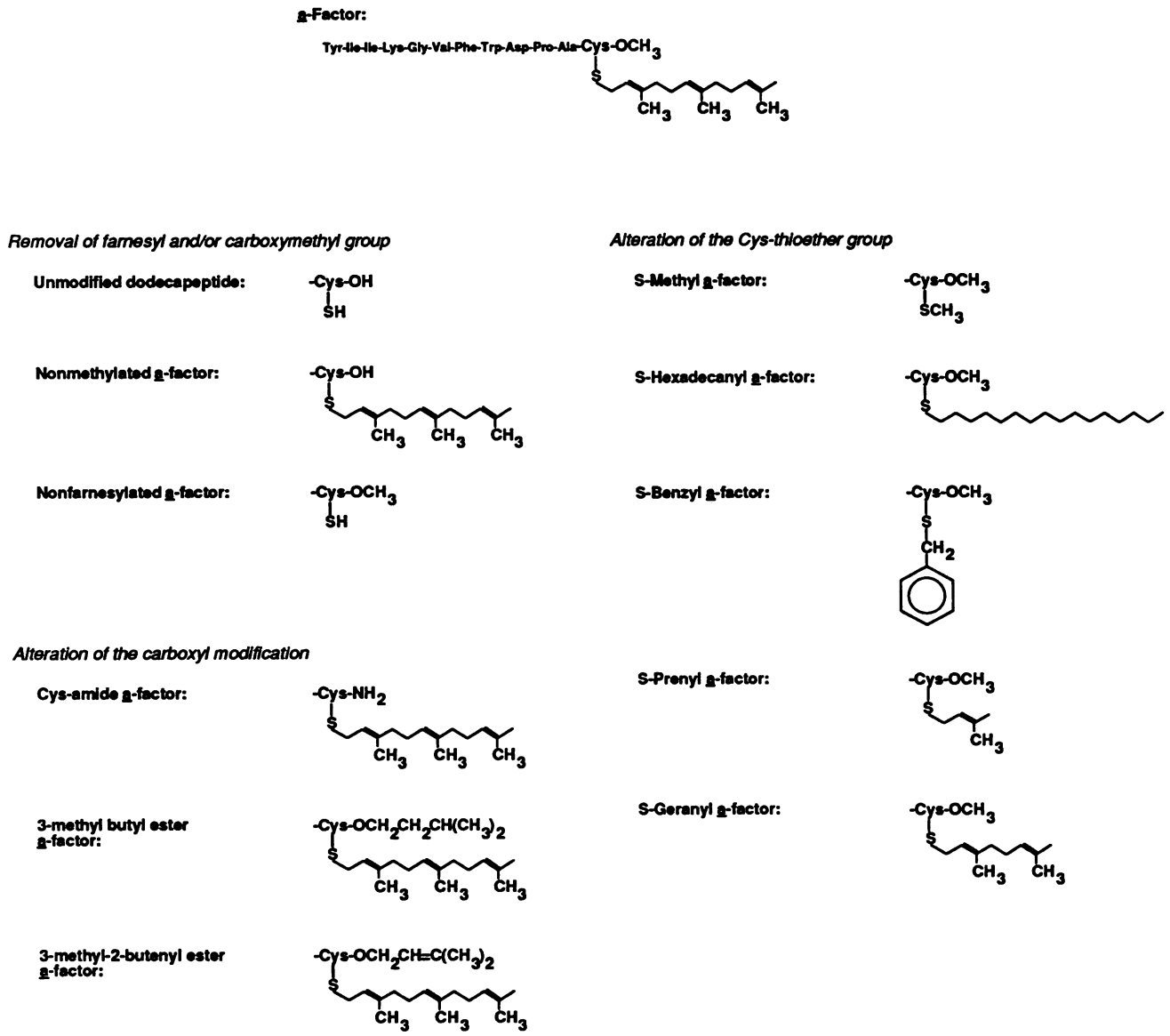

FIG. 1. Structures of *S. cerevisiae* a-factor and various Cys-12 analogs used in this study. All analogs are denoted only by the Cys modification in this figure. All of the analogs maintained the full peptide backbone of YIIKGVFWDPAC.

the level of β-galactosidase induction correlated to the concentration of pheromone used, so that a gradual increase in β-galactosidase units resulted from a corresponding increase in the concentration of pheromone tested.

Mating restoration assay. The mating restoration assay used in this study was a modification of the procedure developed by Horecka and Sprague (27). For dose-response assays, *S. cerevisiae* RC757 and SM1229 were grown to late log phase in YEPD at 30°C with vigorous shaking. Cells were pelleted by centrifugation, washed two times with cold YEPD, and then resuspended in cold YEPD at 10^7/ml. Equal volumes of the two suspensions were mixed together, and this mixed cell suspension was diluted 1:3 with sterile distilled water. A 900-μl amount of the diluted mixed suspension was plated onto SD medium (yeast nitrogen base without amino acids [Difco], 2% dextrose, and 2% Bitek agar [Difco]) for selection of diploids. The plates were incubated at room temperature for about 1 h prior to spotting of a-factor. a-Factor and a-factor analog dilutions were prepared with BSA (2 mg/ml) as the diluent, and each dilution was spotted onto the mixed lawn of cells. The plates were incubated at 30°C for 2 days, after which constellations of diploid colonies were apparent at the positions where a-factor was spotted onto the mixed lawn (Fig. 3). The endpoint of a-factor activity was defined as the lowest amount of a-factor which produced a constellation of diploid colonies.

Mating efficiency assays were performed by a modification of the dose-response assay discussed above and the mating assay used by Michaelis and Herskowitz (44). *S. cerevisiae* *MAT*a and *MAT*α strains were grown to late log phase in YEPD at 30°C with vigorous shaking. Cells were pelleted by centrifugation and then washed two times with cold YEPD. *MAT*α cells were resuspended in cold YEPD at 10^8/ml. Wild-type (SM1058 and JPY200) *MAT*a cells were resus-

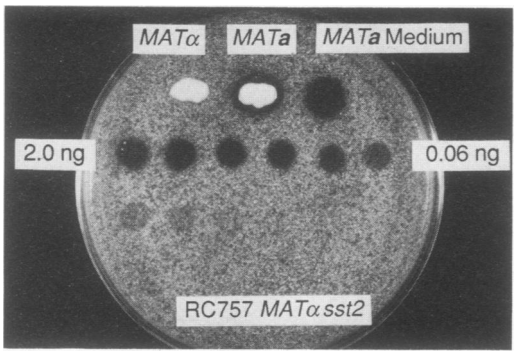

FIG. 2. Halo assay of synthetic a-factor. A lawn of RC757 *MAT*α cells was prepared as described in Materials and Methods. A 1:2 dilution series of synthetic a-factor was prepared in BSA (0.5 mg/ml), and 5 μl of each dilution was spotted on the lawn. Pheromone bioactivity resulted in the formation of clear zones, or halos, at the areas where samples were spotted, an indication of growth arrest. X2180-1A (*MAT***a**) and X2180-1B (*MAT*α) were patched, and 5 μl of concentrated MATa culture medium was spotted onto the lawn, as indicated, all as controls. As indicated, the endpoint of a-factor activity was 0.06 ng.

pended in cold YEPD at concentrations of 10^5/ml for determination of normal wild-type mating efficiency and 10^8/ml for determination of mating efficiency in the presence of exogenous a-factor. *mfa1 mfa2* (SM1229) and *ste6* (JPY201) *MAT***a** strains were resuspended in cold YEPD at 10^7/ml for crosses with RC757 and 10^8/ml for crosses with CR11. Equal volumes of *MAT***a** and *MAT*α suspensions were mixed together, and then the mixed cell suspensions were diluted 1:3 with sterile distilled water. The remainder of the assay was the same as that used for the dose-response assays. The efficiency of mating was calculated as the ratio of the number of diploid colonies formed at the affected area (constellation of diploid colonies) to the total number of *MAT***a** cells calculated to be present in that area (as calculated from the total number of *MAT***a** cells plated). For example, if the diameter of a constellation of diploids was 10 mm and it contained 400 colonies, then the diploid density was 5 diploids per mm^2. If a total of 1.5×10^6 *MAT***a** cells (from 900 μl of a 1:3 dilution of the mixture of SM1229 and RC757 cells) were plated on an 85-mm-diameter agar plate, the density of *MAT***a** cells was 264 cells per mm^2. The mating efficiency was then calculated as the observed colony density in the diploid constellation (5 diploids per mm^2) divided by the density of *MAT***a** cells (264 potential diploids per mm^2), which gives a value of 1.9×10^{-2}. In some cases, a dissecting microscope was used to visualize accurately the individual colonies in a small area. Under the conditions described (1.5×10^7 *MAT*α cells per 85-mm-diameter petri plate [2,643 cells per mm^2]), wild-type strains (CR11 × SM1058) mated with an average efficiency of about 20%, as determined in three independent trials. However, mating efficiency increased to about 60% when 5×10^7 *MAT*α cells per plate were used (8,811 cells per mm^2). In order to compare and calibrate the above-outlined mating assay to the filter mating assay (24) used by some other investigators, we also crossed CR11 and SM1058 to measure the mating efficiency by that method. The mating efficiency of these strains in the filter mating assay was 24%, which correlated closely with our values from the mating restoration assay used in this study.

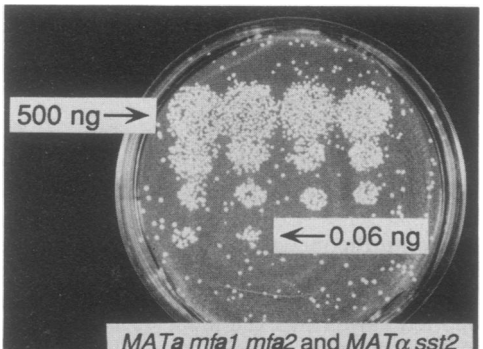

FIG. 3. Mating assay with synthetic a-factor. A mixed lawn of RC757 and SM1229 was prepared on minimal medium for selection of diploids as described in Materials and Methods. A 1:2 dilution series of synthetic a-factor was prepared in BSA (0.5 mg/ml), and 5 μl of each dilution was spotted onto the mixed lawn of cells. The plates were incubated at 30°C for 2 to 3 days, after which constellations of diploid colonies were apparent at the positions where a-factor was spotted onto the mixed lawn. As indicated, the endpoint of a-factor activity was 0.06 ng.

TABLE 1. Biological significance of the carboxy-terminal methyl ester and farnesyl groups of a-factor

a-Factor or analog	% of wild-type activity[a]		
	Halo assay	Shmoo assay[b]	*FUS1-lacZ* assay
a-Factor (wild type)	100	100	100
Nonmethylated a-factor	1.5	0.8	0.05
Nonfarnesylated a-factor	0.1	0.1	0.1
S-Methyl a-factor	0.2	0.4	NT[c]
Unmodified a-factor peptide	0.0025	NT	NT

[a] Wild-type a-factor activity in each assay was as follows: halo assay, 0.06 ng; shmoo assay, 0.04 ng/ml; *FUS1-lacZ* assay, 0.03 ng/ml. Each value represents an average of at least three determinations, with a variation of not more than twofold for each value reported.

[b] The tester strain used in this shmoo assay was RC757 (*sst2*).

[c] NT, not tested.

RESULTS

Biological significance of the carboxy-terminal methyl ester and farnesyl groups of a-factor. For our analyses of the biological activity of a-factor and Cys-12 analogs, we used four different assays: the halo assay, an agar diffusion assay which measures pheromone response based on growth arrest of α cells (Fig. 2); the shmoo assay, an assay which measures pheromone response by morphological alteration (shmooing) and growth arrest of α cells; the *FUS1-lacZ* induction assay, an assay which measures pheromone response by induction of a *FUS1-lacZ* reporter gene (42, 58); and the mating restoration assay, which is discussed in more detail below. To address the significance of the farnesyl and methyl ester modifications to the biological activity of a-factor, we tested a-factor, unmodified a-factor dodecapeptide, nonfarnesylated a-factor, and nonmethylated a-factor (Fig. 1). As shown in Table 1, for three different and sensitive bioassays, our results clearly indicate that removal of either the farnesyl or methyl ester group leads to approximately a 100- to 1,000-fold reduction in bioactivity. The unmodified a-factor dodecapeptide was virtually inactive, showing an endpoint of activity of 2.5 μg in the halo assay (40,000-fold less active

TABLE 2. Activity of a-factor and various analogs in the shmoo assay with *SST2*$^+$ *MAT*α cells

a-Factor or analog	% of wild-type activity[a]
a-Factor (wild type)	100
Nonmethylated a-factor	6.5
Nonfarnesylated a-factor	1.6
S-Methyl a-factor	6.5
Unmodified a-factor peptide	NA[b]

[a] The *SST2*$^+$ strain used in this shmoo assay was X2180-1B. The assay was carried out as described in Materials and Methods except that YNB was used as the assay medium. Wild-type a-factor activity in this assay was 160 ng/ml. Each value represents an average of at least three determinations, with a variation of not more than twofold for each value reported.

[b] NA, not active at 40 μg/ml.

than a-factor). The endpoint for a-factor response, as defined in Materials and Methods, was approximately the same in the shmoo assay (40 pg/ml) and the *FUS1-lacZ* assay (30 pg/ml). The rank order of activities of the various analogs was approximately the same for all assays.

Our results suggest that the farnesyl group and carboxy-terminal methyl ester play nearly equal roles in the biological activity of a-factor. This conclusion is in contrast to that made by Anderegg et al. (2), who concluded that only the carboxy-terminal methyl ester was required for activity. However, it should be pointed out that in their study, *S*-methyl a-factor, and not nonfarnesylated a-factor (S-H a-factor), was tested. For comparative purposes, we determined that *S*-methyl a-factor was about two- to four-fold more active than nonfarnesylated a-factor in the halo and shmoo assays (Table 1). In our assays, *sst2 MAT*α cells were used, and the assay medium was YEPD. Anderegg et al. (2) used *SST2*$^+$ *MAT*α cells (X2180-1B) and YNB as the assay medium in their a-factor bioactivity assays. We tested the analogs shown in Table 1 under the assay conditions of Anderegg et al. (2) and obtained a rank order of activity (Table 2) that was similar to the rank order obtained when *sst2 MAT*α cells and YEPD were used (Table 1).

Consequences of altering the size and hydrophobicity of the Cys-thioether group of a-factor. To determine whether the farnesyl group of a-factor is specifically required for full bioactivity, we synthesized the following a-factor analogs: *S*-methyl a-factor, *S*-hexadecanyl a-factor, *S*-prenyl a-factor, *S*-geranyl a-factor, and *S*-benzyl a-factor (Fig. 1). Replacement of the farnesyl moiety by a methyl group (*S*-methyl a-factor) produced an analog which was about 200- to 500-fold less active than a-factor and 2- to 4-fold more active than nonfarnesylated a-factor (Tables 2 and 3). Replacement

TABLE 3. Biological activity of a-factor analogs with altered Cys-thioether groups[a]

a-Factor or analog	% of wild-type activity		
	Halo assay	Shmoo assay[b]	*FUS1-lacZ* assay
a-Factor (wild type)	100	100	100
Nonfarnesylated a-factor	0.1	0.1	0.1
S-Methyl a-factor	0.2	0.4	NT[c]
S-Hexadecanyl a-factor	1.5	3.3	15
S-Prenyl a-factor	1.5	6.7	NT
S-Geranyl a-factor	12	50	NT
S-Benzyl a-factor	3	25	NT

[a] See Table 1, footnotes *a*, *b*, and *c*.

TABLE 4. Biological activity of a-factor analogs with altered C-terminal carboxyl groups[a]

a-Factor or analog	% of wild-type activity	
	Halo assay	Shmoo assay
a-Factor (wild type)	100	100
Nonmethylated a-factor	1.5	0.8
Cys-amide a-factor	6	6
3-Methyl butyl ester a-factor	0.8	0.4
3-Methyl-2-butenyl ester a-factor	1.5	0.8

[a] See Table 1, footnotes *a* and *b*.

of the farnesyl group with a hexadecanyl group produced an analog that was 30- to 70-fold less active in the shmoo and halo assays and 7-fold less active in the *FUS1-lacZ* assay. When the sulfur group was modified with an *S*-benzyl moiety, the resulting analog exhibited activity that was 4- to 30-fold lower than that of a-factor in the shmoo and halo assays. The effect of alteration of the length of the Cys-isoprenyl group was determined by analysis of the activity of *S*-prenyl a-factor and *S*-geranyl a-factor. *S*-Prenyl a-factor was found to be 15- to 70-fold less active than a-factor, while *S*-geranyl a-factor was only 2- to 8-fold less active. Indeed, the *S*-geranyl a-factor is the most potent synthetic analog prepared to date.

Consequences of altering the C-terminal carboxy modification of a-factor. Having determined the relative significance of the Cys-thioether modification of a-factor, we focused our attention on the C-terminal methyl ester modification. The following analogs were synthesized and analyzed: Cys-amide a-factor, 3-methyl butyl ester a-factor, and 3-methyl-2-butenyl a-factor (Fig. 1). The Cys-amide a-factor was the most active of this series of analogs, being about 16-fold less active than a-factor (Table 4). 3-Methyl-2-butenyl ester a-factor and 3-methyl butyl ester a-factor did not exhibit activities above that observed for nonmethylated a-factor, each being about 100-fold less active than a-factor.

***MAT*a cells need not actively produce a-factor in order to mate with either *sst2* or wild-type *MAT*α cells.** To investigate further the biological significance of extracellular a-factor, we tested a-factor and several Cys-12 analogs in a recently developed mating restoration assay (27). In this assay, a mixed lawn of *MAT*a cells defective in a-factor production (*mfa1 mfa2* mutants) and *MAT*α cells were prepared on medium selective for growth of diploid cells. In the absence of exogenously added a-factor, *mfa1 mfa2* mutants could not mate with wild-type *MAT*α cells and mated with *sst2 MAT*α cells at very low efficiency (0.038% of wild-type mating level) (Table 5). However, when a-factor was spotted on the lawn, constellations of diploid colonies grew at the locations where the pheromone was spotted (Fig. 3). Although mating was not restored to a wild-type level (Table 5), the results demonstrate that it is not absolutely essential for *MAT*a cells to actively produce a-factor in order to mate with wild-type *MAT*α cells. The endpoint for mating restoration with wild-type *MAT*α cells was about the same as the endpoint obtained in the halo assay for the same cells (2 μg). As shown in Table 6, the proficiency with which various a-factor analogs restored mating correlated, for the most part, with activities found in the halo assay. However, the *S*-prenyl, *S*-geranyl, and *S*-benzyl a-factors exhibited activities higher than those found in the halo assay.

One possible explanation for the inability of exogenous pheromone to restore mating of the *mfa1 mfa2* mutant to a

TABLE 5. Exogenous a-factor restores mating of the *mfa1 mfa2* mutant to either wild-type or *sst2 MAT*α cells

*MAT*α strain	*MAT***a** strain[a]	a-Factor added[b]	% of wild-type mating efficiency[c]
CR11 (wild type)	Wild type	−	100
	mfa1 mfa2	−	$<1.0 \times 10^{-6}$
	Wild type	+	9.5×10^{-2}
	mfa1 mfa2	+	6.8×10^{-2}
RC757 (*sst2*)	Wild type	−	90
	mfa1 mfa2	−	3.8×10^{-2}
	Wild type	+	9.0×10^{-1}
	mfa1 mfa2	+	4.3

[a] The *MAT***a** strains tested were SM1058 (wild type) and SM1229 (*mfa1 mfa2*).
[b] A + indicates that 4 μg of a-factor was spotted onto the lawn.
[c] The mating restoration assay used for determination of mating efficiencies is described in the Materials and Methods section. All values are expressed as percent wild-type mating efficiency, which was normalized to 100%. The actual average mating efficiency of the wild-type strains used in this assay was 21%, as determined in three independent trials.

wild-type level is that a-factor plays an intracellular role that cannot be complemented by exogenous pheromone. Since *ste6* mutants accumulate a-factor intracellularly without secreting the pheromone (34), we tested the ability of a *ste6* mutant to mate with either wild-type or *sst2 MAT*α cells in the mating restoration assay. Exogenous a-factor did not restore mating at all when the *ste6* mutant was crossed with wild-type *MAT*α cells (Table 7). Some degree of restoration was obtained when the *ste6* mutant was crossed with *sst2 MAT*α cells, but the level of restoration was lower than that obtained with the *mfa1 mfa2* mutant.

A second possible reason for the low level of mating restoration by exogenous a-factor is that exogenous pheromone interferes with mating partner selection by *MAT*α cells. Jackson and Hartwell (29, 30) showed that *S. cerevisiae MAT***a** and *MAT*α haploids preferentially select mating partners which produce the highest level of pheromone, suggesting that the cells might respond to pheromone gradients. If this hypothesis is correct, then destruction of the gradient, as would occur after addition of high levels of exogenous pheromone, should markedly decrease the level of wild-type mating. As shown in Table 5, this is indeed the case. When a-factor was added to a mating mixture of wild-type *MAT***a** and *MAT*α cells, the mating efficiency was decreased to nearly the same level as was observed when exogenous a-factor was added to a cross of *mfa1 mfa2* mutants and wild-type *MAT*α cells. Similar decreases in mating efficiency were observed when *sst2 MAT*α cells were used (Table 5).

TABLE 6. Activity of a-factor and various analogs in the mating restoration assay[a]

a-Factor or analog	% of wild-type activity[a]
a-Factor (wild type)	100
Nonmethylated a-factor	1.5
Nonfarnesylated a-factor	0.8
S-Methyl a-factor	1.5
S-Hexadecanyl a-factor	0.8
S-Prenyl a-factor	25
S-Geranyl a-factor	200
S-Benzyl a-factor	25
Cys-amide a-factor	6
3-Methyl butyl ester a-factor	0.4
3-Methyl-2-butenyl ester a-factor	0.8

[a] The mating restoration assay is described in the Materials and Methods section. RC757 (*sst2*) was used as the *MAT*α strain. All values are expressed as percent wild-type activity. Wild-type a-factor activity in this mating restoration assay was 0.06 ng. Each value represents an average of at least three determinations, with a variation of not more than twofold for each value reported.

TABLE 7. Exogenous a-factor does not restore mating of the *ste6* mutant to a wild-type level

*MAT*α strain	*MAT***a** strain[a]	a-Factor added[b]	% of wild-type mating efficiency[c]
CR11 (wild type)	Wild type	−	100
	ste6	−	$<1.0 \times 10^{-6}$
	Wild type	+	2.1×10^{-1}
	ste6	+	$<1.0 \times 10^{-6}$
RC757 (*sst2*)	Wild type	−	90
	ste6	−	4.1×10^{-2}
	Wild type	+	9.3×10^{-1}
	ste6	+	7.1×10^{-1}

[a] The *MAT***a** strains tested were JPY200 (wild type) and JPY201 (*ste6*).
[b] See Table 5, footnote *b*.
[c] See Table 5, footnote *c*. The actual average mating efficiency of the wild-type strains used in this assay was 25%.

DISCUSSION

Prenylation is now recognized as an important posttranslational modification of numerous proteins in eukaryotic organisms. In this article, we have presented the results of a detailed study of structure-activity relationships for a prenylated yeast mating pheromone, the *S. cerevisiae* a-factor. Previous studies on the prenylated peptide pheromones, rhodotorucine *A* and tremerogen *A*-10, of the heterobasidiomycetous yeasts *Rhodosporidium toruloides* and *Tremella mesenterica*, respectively, demonstrated that hydrophobic modification of the C-terminal cysteines of each of these mating pheromones was required for biological activity (17, 59). Detailed studies on analogs of tremerogen *A*-10 demonstrated that decreasing the number of prenyl units from three to one resulted in a corresponding decrease in biological activity (17). In the present study, we have shown that absence of either the farnesyl group (NH_2-YIIKGVFWDPA C-$COOCH_3$) or carboxy-terminal methyl ester (NH_2-YIIKG VFWDPAC[*S*-farnesyl]-COOH) of a-factor results in a similar marked decrease but not loss of biological activity, as determined by four sensitive bioassays. In addition, an analog lacking both the farnesyl group and methyl ester (NH_2-YIIKGVFWDPAC-COOH) was virtually inactive, being 40,000-fold less active than a-factor. These results support those in previous studies on the *S. cerevisiae* a-factor which utilized only the halo assay (63) and suggest that both the farnesyl group and carboxy-terminal methyl ester of a-factor are important for high biological activity. This conclusion is in contrast to that of Anderegg et al. (2), who concluded that only the methyl ester modification of the a-factor peptide was required for high biological activity. However, Anderegg et al. (2) examined an *S*-methyl a-factor rather than S-H (nonfarnesylated) a-factor. Furthermore, they did not test concentrations of a-factor analogs more than fivefold higher than the concentration of a-factor tested. This prevented them from detecting activity from analogs that were significantly less active than a-factor.

Additional a-factor analogs were studied to determine whether *S*-alkyl modifications other than farnesyl would

result in biologically active lipopeptides. We found that aliphatic, benzylic, and geranyl substituents resulted in pheromone analogs exhibiting from 0.2 to 50% of the activity of the natural mating factor (Table 3). As illustrated by the *S*-benzyl analog, an isoprenyl moiety is not required for high biological activity. The bioactivity of the pheromone increased as the length of the prenyl moiety was increased (*S*-farnesyl **a**-factor > *S*-geranyl **a**-factor > *S*-prenyl **a**-factor), and the *S*-hexadecanyl **a**-factor was more active than the *S*-methyl analog. Thus, within these two types of *S*-alkyl modifications, **a**-factor activity was found to increase as the *S*-alkyl group became bulkier and more hydrophobic.

Replacement of the methyl ester by larger, more hydrophobic esters resulted in a marked decrease in activity (Table 4). In contrast, the Cys-amide **a**-factor (Fig. 1) had 6% of the activity of the native pheromone. Thus, the ester oxygen is not specifically required for interaction with the **a**-factor receptor, and some hydrophobic groups are not well tolerated in the ester moiety. We are presently synthesizing additional analogs to investigate this point in more detail.

Some differences in the activity of **a**-factor analogs relative to **a**-factor were found, depending on the assay used for measuring activity. One must bear in mind that while each of the **a**-factor analogs contains the same peptide moiety, they differ in their overall physical characteristics, especially with regard to hydrophobicity, which influences peptide solubility in different media and susceptibility to degradation by a recently identified *MAT*α cell-specific **a**-factor protease (40). Furthermore, the conditions of each assay, as well as the specific physiological response measured, are different. Thus, the threshold for response and the stringency of response in each assay could be expected to vary. Indeed, previous studies have demonstrated that various pheromone responses require markedly different minimum doses of α-factor, from 10^{-11} M for agglutination to 10^{-8} M for shmooing (for review, see reference 12). Each of the four assays used in this study measures the end result of a complex series of intracellular and/or intercellular events. While great strides have been made in characterizing specific aspects of the pheromone response pathway, it is on the whole still poorly understood. However, there is some evidence that the biochemical events subsequent to pheromone induction follow a branching pathway, with physiological responses as the end result of different branches (9, 32). Therefore, some of the various responses measured by different bioassays may reflect the different thresholds for different branches of the pathway.

While it is clear from our analyses of the biological activity of **a**-factor and various Cys-12 analogs that hydrophobic modification of the C-terminal cysteine of this pheromone is required for high biological activity, it is equally evident that activity is dependent on more subtle factors than just the overall hydrophobicity of the C terminus of **a**-factor. Characterization of the **a**-factor analogs by HPLC (data not shown) demonstrated that the *S*-hexadecanyl **a**-factor is the most hydrophobic analog and that both the 3-methyl butyl and the 3-methyl-2-butenyl ester analogs are more hydrophobic than the **a**-factor. It is possible that optimal interaction of the pheromone with the receptor requires a long branched hydrocarbon on the cysteine sulfur and a smaller organic group on the cysteine carboxyl. Such a requirement would be similar to those models used to explain the interaction of aspartamelike sweeteners with their receptor (20). The possibility must also be considered that the requirement of hydrophobic modification of **a**-factor for biological activity reflects not only the specificity of the **a**-factor receptor of *MAT*α cells, but also the ability of hydrophobically modified peptides to enter the plasma membranes of these cells. It is conceivable that the hydrophobic **a**-factor might enter the lipid bilayer of *MAT*α cells and then diffuse through the membrane to bind its receptor. A similar process has been proposed for the interaction of mammalian peptide hormones with the receptors of their target cells (3, 53).

An additional factor which might have an effect on the apparent activities of **a**-factor analogs is *MAT*α cell degradation of **a**-factor. Previous studies on rhodotorucine *A*, the pheromone produced by mating type *A* cells of *R. toruloides*, have demonstrated that proteolysis of this farnesylated peptide by the mating type *a* target cell is a requirement for induction of the pheromone response (46). In contrast, *S. cerevisiae MAT***a** cells proteolyze α-factor as one mechanism of recovery from pheromone-induced growth arrest (10). Mutants which are unable to proteolyze this mating peptide (*sst1* or *bar1* mutants) still respond and are, in fact, supersensitive to α-factor (7, 8). These and other studies have demonstrated that hydrolysis of α-factor by *MAT***a** cells is not required for a pheromone response. We have recently identified a *MAT*α cell-specific **a**-factor-degrading activity (40). As is the case for α-factor proteolysis by *MAT***a** cells, proteolysis of **a**-factor seems to be a mechanism of *MAT*α cell desensitization from the pheromone response. The substrate specificity of the **a**-factor-degrading protease appears to depend on both the peptide sequence and the cysteine-modifying group. For example, *S*-hexadecanyl **a**-factor is not proteolyzed by *MAT*α cells. We have not determined whether all the analogs tested in this study are proteolyzed by *MAT*α cells, but the preferential degradation of **a**-factor analogs may influence their relative bioactivities.

Because of its lipophilic nature and results showing that *MAT***a** mutants defective in **a**-factor production were incapable of mating with wild-type (*SST2*$^+$) *MAT*α cells even when **a**-factor was provided exogenously, some speculation had been raised that **a**-factor may be tethered to *MAT***a** cells and that its primary action on *MAT*α cells occurs via cell-cell contact (26, 44). However, since **a**-factor is found in the culture supernatant of *MAT***a** cells, the pheromone is clearly released or secreted by these cells. Moreover, as demonstrated by numerous studies, the presence of *MAT***a** cells is not required for *MAT*α cells to respond to **a**-factor supplied as unpurified or partially purified preparations from spent medium of *MAT***a** cells. Our results demonstrate that synthetic **a**-factor can function to restore the ability of *MAT***a** mutants defective in **a**-factor production to mate with either *sst2* or wild-type *MAT*α cells (Table 5), although mating was not restored to a wild-type level in either case. We found that the relative rank order of activities of the various **a**-factor analogs tested in this mating restoration assay was similar to the rank order of activities obtained in the halo assay (Table 6), which is also done in agar. The mating restoration experiments indicate that (i) *MAT***a** cells do not have to actively produce **a**-factor in order to mate with *MAT*α cells, although **a**-factor production might be required for mating at a wild-type level and (ii) the cysteine modifications of **a**-factor are no more or less significant for inducing a *MAT*α cell response to pheromone than for allowing *MAT***a** and *MAT*α cells to conjugate. A possible explanation for the inability of concentrated *MAT***a** culture supernatant fractions to restore mating of *mf***a***1 mf***a***2* mutants to wild-type *MAT*α cells in an earlier study (44) is that the preparation did not contain a sufficient amount of **a**-factor to restore mating or contained a substance that inhibited mating.

As indicated above, exogenous **a**-factor could not restore

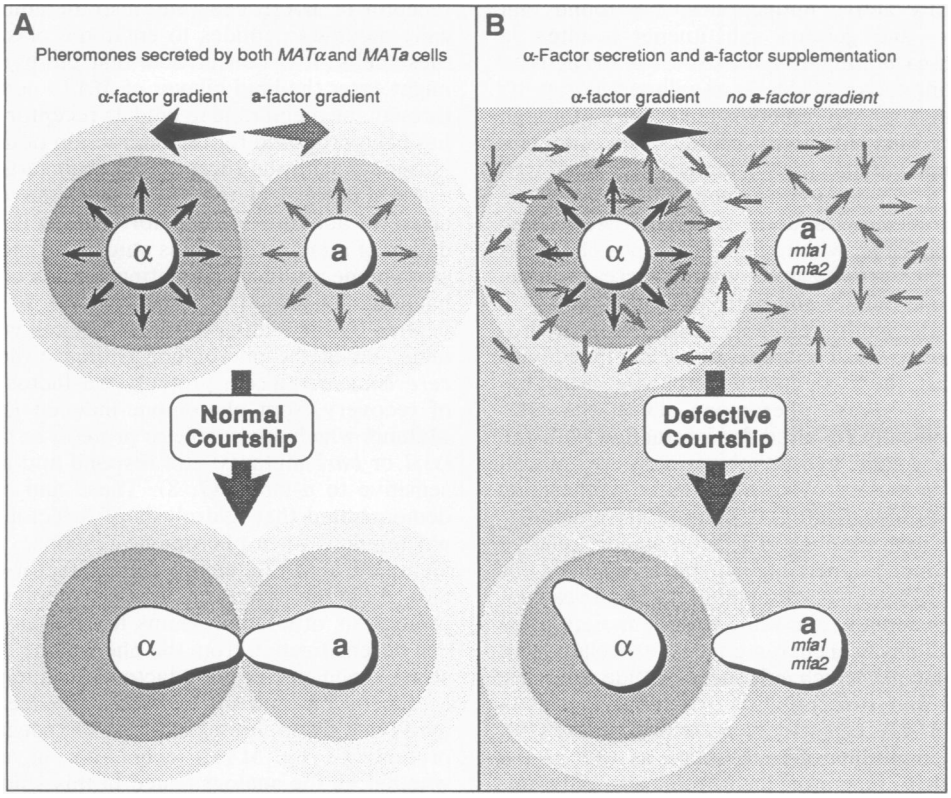

FIG. 4. Model to explain why exogenous a-factor does not restore mating of *mfa1 mfa2* mutants to a wild-type level. (A) Under normal conditions, apposed *MAT*α and *MAT***a** cells produce α-factor and **a**-factor, respectively, resulting in pheromone gradients which are highest in concentration at the surface of the secreting cells. Shmoo tip projections are formed in the direction of the gradients, resulting in successful courtship. (B) When an *mfa1 mfa2* mutant is in apposition to a wild-type *MAT*α cell, the *mfa1 mfa2* mutant is unable to court the *MAT*α cell. Instead, since **a**-factor is supplied exogenously, the *MAT*α cell forms its projection randomly.

the mating efficiency of *mfa1 mfa2* mutants to a wild-type level (Table 5). However, it is important to note that in previous studies (35), addition of exogenous α-factor also did not restore mating to wild-type levels in *mfα1 mfα2 MAT*α cells. A possible explanation for the inability of exogenous pheromones to restore mating of pheromone-defective *MAT***a** or *MAT*α cells to a wild-type level is that **a**- and α-factor play an intracellular role that cannot be complemented by exogenous pheromone. To examine this possibility, we tested a *ste6* mutant in the mating restoration assay. The *STE6* gene product has a high degree of homology to mammalian multidrug resistance transporters (34, 43) and is thought to act as a transporter which secretes **a**-factor. No other role for *STE6* has been proposed, and it has been shown that *ste6* mutants produce mature **a**-factor intracellularly (34). We found that exogenous **a**-factor did not restore mating at all when the Δ*ste6* mutant was crossed with wild-type *MAT*α cells (Table 7). A full interpretation of this result awaits better characterization of the *STE6* gene product, although our results may indicate roles in addition to **a**-factor secretion for this protein in the mating process. Perhaps the *STE6* gene product is necessary to correctly orient internally produced or exogenously supplied **a**-factor in order to facilitate mating.

An alternative explanation for our observation that exogenous **a**-factor does not restore mating efficiency of *mfa1 mfa2* mutants to a wild-type level can be made by considering the results of Jackson and Hartwell (29, 30) on the courtship response of *S. cerevisiae MAT***a** and *MAT*α haploids. These investigators showed that when *MAT***a** and *MAT*α cells are given a choice of several different potential mating partners, the cell type(s) which produces the most pheromone is preferentially selected. The authors concluded that mating partner selection occurs via a response to pheromone gradients. Our results are, in fact, predictable from Jackson and Hartwell's model for courtship (29, 30). Normal courtship occurs when *MAT***a** and *MAT*α cells are each producing normal levels of pheromone, which results in recognizable gradients of the respective pheromones toward which projections are directed (Fig. 4A). However, when the *MAT***a** cell does not produce **a**-factor (*mfa1 mfa2* mutant) and the pheromone is instead provided exogenously, no **a**-factor gradient is established (Fig. 4B). Thus, while the *MAT*α cell, which is producing α-factor and therefore establishing a pheromone gradient, can effectively court the *mfa1 mfa2* mutant, the *mfa1 mfa2* mutant cannot court the *MAT*α cell, even though the **a**-factor is provided exogenously. Instead, since there is no **a**-factor gradient, the *MAT*α cell forms its projection randomly. Our observation that the mating efficiency of wild-type *MAT***a** and *MAT*α cells in the presence of exogenous **a**-factor is close to that of *mfa1 mfa2* mutants and wild-type *MAT*α cells (Table 5) is consistent with the model shown in Fig. 4. Thus, this model provides a likely explanation of why exogenous **a**-factor does not restore mating efficiency of *mfa1 mfa2* mutants to a wild-type level.

Our results also show that in the presence of exogenous

a-factor, *sst2 MATα* mutants mate with *mfa1 mfa2* mutants with about 60-fold greater efficiency than do wild-type (*SST2*$^+$) *MATα* cells. However, this result must be interpreted cautiously. We were not able to test the effect of higher doses of a-factor on the mating efficiency of wild-type *MATα* cells to *mfa1 mfa2* mutants because of the extremely limited solubility of the pheromone. Thus, it is possible that higher doses of a-factor might have increased the degree of mating restoration of wild-type *MATα* cells to a level closer to that of *sst2 MATα* cells.

The results reported herein reveal several important relationships between the structure of a-factor and its biological activity and provide evidence that the pheromone works exogenously. Recently, studies have appeared which delineate the steps occurring during the posttranslational processing of this farnesylated, carboxy-terminal methyl-esterified peptide (28, 39, 41, 55). We are presently investigating in detail the specific interaction of the lipopeptide a-factor with its receptor and are searching for antagonists and superactive analogs. It is evident that, together with the α-factor, the a-factor represents a powerful paradigm for studying cell-cell communication and for learning about peptide hormone action. We anticipate that this system will provide further insights, some possibly unforeseen, into the role of prenylation and carboxy-terminal methyl esterification in the biochemistry of eukaryotic organisms.

ACKNOWLEDGMENTS

We thank Wolfgang Duntze, Lorraine Marsh, Susan Michaelis, and John McGrath for *S. cerevisiae* strains and Greg Abel for technical assistance.

This work was supported by Public Health Service grants GM-22086 and GM-22087 from the National Institutes of Health.

REFERENCES

1. **Achstetter, T.** 1989. Regulation of α-factor production in *Saccharomyces cerevisiae*: a-factor pheromone-induced expression of the *MFα1* and *STE13* genes. Mol. Cell. Biol. **9**:4507–4514.
2. **Anderegg, R. J., R. Betz, S. A. Carr, J. W. Crabb, and W. Duntze.** 1988. Structure of the *Saccharomyces cerevisiae* mating hormone a-factor: identification of *S*-farnesyl cysteine as a structural component. J. Biol. Chem. **263**:18236–18240.
3. **Behling, R. W., and L. W. Jelinski.** 1990. Importance of the membrane in ligand-receptor interactions. Biochem. Pharmacol. **40**:49–54.
4. **Brake, A., C. Brenner, R. Najarian, P. Laybourne, and J. Merryweather.** 1985. Structure of genes encoding precursors of the yeast peptide mating pheromone a-factor, p. 103–108. *In* M. J. Gething (ed.), Protein transport and secretion. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
5. **Bucking-Throm, E., W. Duntze, L. H. Hartwell, and T. R. Manney.** 1973. Reversible arrest of haploid yeast cells at the initiation of DNA synthesis by a diffusible sex factor. Exp. Cell Res. **76**:99–110.
6. **Casey, P. J., P. A. Solski, C. J. Der, and J. E. Buss.** 1989. p21ras is modified by a farnesyl isoprenoid. Proc. Natl. Acad. Sci. USA **86**:8323–8327.
7. **Chan, R. K., and C. A. Otte.** 1982. Isolation and genetic analysis of *Saccharomyces cerevisiae* mutants supersensitive to G_1 arrest by a factor and α factor pheromones. Mol. Cell. Biol. **2**:11–20.
8. **Chan, R. K., and C. A. Otte.** 1982. Physiological characterization of *Saccharomyces cerevisiae* mutants supersensitive to G_1 arrest by a-factor and α-factor pheromones. Mol. Cell. Biol. **2**:21–29.
9. **Chang, F., and I. Herskowitz.** 1990. Identification of a gene necessary for cell cycle arrest by a negative growth factor of yeast: FAR1 is an inhibitor of a G_1 cyclin, CLN2. Cell **63**:999–1011.
10. **Ciejek, E., and J. Thorner.** 1979. Recovery of *S. cerevisiae* a-cells from G_1 arrest by alpha-factor pheromone requires endopeptidase action. Cell **18**:623–635.
11. **Clarke, S. J., J. P. Vogel, R. J. Deschenes, and J. Stock.** 1988. Posttranslational modification of the Ha-*ras* oncogene protein: evidence for a third class of protein carboxyl methyltransferase. Proc. Natl. Acad. Sci. USA **85**:4643–4647.
12. **Cross, F., L. H. Hartwell, C. Jackson, and J. B. Konopka.** 1988. Conjugation in *Saccharomyces cerevisiae*. Annu. Rev. Cell Biol. **4**:429–457.
13. **Deschenes, R. J., J. B. Stimmel, S. Clarke, J. Stock, and J. R. Broach.** 1989. *RAS2* protein of *Saccharomyces cerevisiae* is methyl-esterified at its carboxyl terminus. J. Biol. Chem. **264**: 11865–11873.
14. **Farnsworth, C. C., M. H. Gelb, and J. A. Glomset.** 1990. Identification of geranylgeranyl-modified proteins in HeLa cells. Science **247**:320–322.
15. **Fehrenbacher, G., P. Perry, and J. Thorner.** 1978. Cell-cell recognition in *Saccharomyces cerevisiae*: regulation of mating-specific adhesion. J. Bacteriol. **134**:893–901.
16. **Finegold, A. A., W. R. Schafer, J. Rine, M. Whiteway, and F. Tamanoi.** 1990. Common modification of trimeric G-proteins and ras protein: involvement of polyisoprenylation. Science **249**:165–169.
17. **Fugino, M., C. Kitada, Y. Sakagami, A. Isogai, S. Tamura, and A. Suzuki.** 1980. Biological activity of synthetic analogs of tremerogen *A*-10. Naturwissenschaften **67**:406–408.
18. **Glomset, J. A., M. H. Gelb, and C. C. Farnsworth.** 1990. Prenyl proteins in eukaryotic cells: a new type of membrane anchor. Trends Biochem. Sci. **15**:139–142.
19. **Goldstein, J. L., and M. S. Brown.** 1990. Regulation of the mevalonate pathway. Nature (London) **343**:425–430.
20. **Goodman, M., J. Coddington, D. F. Mierke, and W. D. Fuller.** 1987. A model for the sweet taste of sterioisomeric retro-inverso and dipeptide amides. J. Am. Chem. Soc. **109**:4712–4714.
21. **Hagen, D. C., and G. F. Sprague.** 1984. Induction of the yeast α-specific *STE3* gene by the peptide pheromone a-factor. J. Mol. Biol. **178**:835–852.
22. **Hancock, J. F., A. I. Magee, J. E. Childs, and C. J. Marsh.** 1989. All *ras* proteins are polyisoprenylated but only some are palmitoylated. Cell **57**:1167–1177.
23. **Hartwell, L. H.** 1973. Synchronization of haploid yeast cell cycles, a prelude to conjugation. Exp. Cell Res. **76**:111–117.
24. **Hartwell, L. H.** 1980. Mutants of *Saccharomyces cerevisiae* unresponsive to cell division control by polypeptide mating hormone. J. Cell Biol. **85**:811–822.
25. **Herskowitz, I.** 1988. Life cycle of the budding yeast *Saccharomyces cerevisiae*. Microbiol. Rev. **52**:536–553.
26. **Herskowitz, I.** 1989. A regulatory hierarchy for cell specialization in yeast. Nature (London) **342**:749–757.
27. **Horecka, J., and G. F. Sprague (University of Oregon).** 1990. Personal communication.
28. **Hrycyna, C. A., and S. Clarke.** 1990. Farnesyl cysteine C-terminal methyltransferase activity is dependent upon the *STE14* gene product in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **10**:5071–5076.
29. **Jackson, C. L., and L. H. Hartwell.** 1990. Courtship in *Saccharomyces cerevisiae*: an early cell-cell interaction during mating. Mol. Cell. Biol. **10**:2202–2213.
30. **Jackson, C. L., and L. H. Hartwell.** 1990. Courtship in *Saccharomyces cerevisiae*: both cell types choose mating partners by responding to the strongest pheromone signal. Cell **63**:1039–1051.
31. **Kamiya, Y., A. Sakurai, S. Tamura, and N. Takahashi.** 1978. Structure of rhodotorucine *A*, a novel lipopeptide, inducing mating tube formation in *Rhodosporidium toruloides*. Biochem. Biophys. Res. Commun. **83**:1077–1083.
32. **King, K., H. G. Dohlman, J. Thorner, M. G. Caron, and R. J. Lefkowitz.** 1990. Control of yeast mating signal transduction by a mammalian β_2-adrenergic receptor and G_s α subunit. Science **250**:121–123.
33. **Kronstad, J. W., J. A. Holly, and V. L. MacKay.** 1987. A yeast operator overlaps an upstream activation site. Cell **50**:369–377.

34. **Kuchler, K., R. E. Sterne, and J. Thorner.** 1989. *Saccharomyces cerevisiae STE6* gene product: a novel pathway for protein export in eukaryotic cells. EMBO J. **8**:3973–3984.
35. **Kurjan, J.** 1985. α-Factor structural gene mutations in *Saccharomyces cerevisiae*: effects on α-factor production and mating. Mol. Cell. Biol. **5**:787–796.
36. **Lipke, P. N., A. Taylor, and C. E. Ballou.** 1976. Morphogenetic effect of α-factor on *Saccharomyces cerevisiae*. J. Bacteriol. **127**:610–618.
37. **Maltese, W. A., and R. A. Erdman.** 1989. Characterization of isoprenoid involved in the post-translational modification of mammalian cell proteins. J. Biol. Chem. **264**:18168–18172.
38. **Maltese, W. A., and J. D. Robishaw.** 1990. Isoprenylation of C-terminal cysteine in a G-protein γ subunit. J. Biol. Chem. **265**:18071–18074.
39. **Marcus, S., G. A. Caldwell, C.-B. Xue, F. Naider, and J. M. Becker.** 1990. Total *in vitro* maturation of the *Saccharomyces cerevisiae* a-factor lipopeptide mating pheromone. Biochem. Biophys. Res. Commun. **172**:1310–1316.
40. **Marcus, S., C.-B. Xue, F. Naider, and J. M. Becker.** 1991. Degradation of a-factor by a *Saccharomyces cerevisiae* α mating type-specific endopeptidase: evidence for a role in recovery of cells from G_1 arrest. Mol. Cell. Biol. **11**:1030–1039.
41. **Marr, R. S., L. C. Blair, and J. Thorner.** 1990. *Saccharomyces cerevisiae STE14* gene is required for COOH-terminal methylation of a-factor mating pheromone. J. Biol. Chem. **265**:20057–20060.
42. **McCaffrey, G., F. J. Clay, K. Kelsay, and G. F. Sprague, Jr.** 1987. Identification and regulation of a gene required for cell fusion during mating of the yeast *Saccharomyces cerevisiae*. Mol. Cell. Biol. **7**:2680–2690.
43. **McGrath, J. P., and A. Varshavsky.** 1989. The yeast *STE6* gene encodes a homologue of the mammalian multidrug resistance P-glycoprotein. Nature (London) **340**:400–404.
44. **Michaelis, S., and I. Herskowitz.** 1988. The a-factor pheromone of *Saccharomyces cerevisiae* is essential for mating. Mol. Cell. Biol. **8**:1309–1318.
45. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
46. **Miyakawa, T., M. Nishihara, E. Tsuchiya, and S. Fukui.** 1982. Role of metabolism of the mating pheromone in the sexual differentiation of the heterobasidiomycete *Rhodosporidium toruloides*. J. Bacteriol. **151**:1184–1194.
47. **Naider, F., and J. M. Becker.** 1986. Structure-activity relationships of the yeast alpha-factor. Crit. Rev. Biochem. **21**:225–248.
48. **Powers, S., S. Michaelis, D. Broek, S. S. Anna-A., J. Field, I. Herskowitz, and M. Wigler.** 1986. *RAM*, a gene of yeast required for a functional modification of *RAS* proteins and for production of mating pheromone a-factor. Cell **47**:413–422.
49. **Reynolds, A., and V. Lundblad.** 1989. Assay for β-galactosidase in liquid cultures, p. 13.6.1–13.6.4. *In* Ausubel et al. (ed.), Current protocols in molecular biology. Wiley Interscience, New York.
50. **Rilling, H. C., E. Breunger, W. W. Epstein, and P. F. Crain.** 1990. Prenylated proteins: the structure of the isoprenoid group. Science **247**:318–320.
51. **Sakagami, Y., A. Isogal, A. Suzuki, S. Tamura, C. Kitada, and M. Fujino.** 1979. Structure of tremerogen *A*-10, a peptidyl hormone inducing conjugation tube formation in *Tremella mesenterica*. Agric. Biol. Chem. **43**:2643–2645.
52. **Sakagami, Y., M. Yoshida, A. Isogal, and A. Suzuki.** 1981. Structure of tremerogen a-13, a peptidyl hormone of *Tremella mesenterica*. Agric. Biol. Chem. **45**:1045–1047.
53. **Sargent, D. F., and R. Schwyzer.** 1986. Membrane lipid phase as catalyst for peptide-receptor interactions. Proc. Natl. Acad. Sci. USA **83**:5774–5778.
54. **Schafer, W. R., R. Kim, R. Sterne, J. Thorner, S.-H. Kim, and J. Rine.** 1989. Genetic and pharmacological suppressors of oncogenic mutations in *RAS* genes of yeast and humans. Science **245**:379–385.
55. **Schafer, W. R., C. E. Trueblood, C.-C. Yang, M. P. Mayer, S. Rosenberg, C. D. Poulter, S.-H. Kim, and J. Rine.** 1990. Enzymatic coupling of cholesterol intermediates to a mating pheromone precursor and to the Ras protein. Science **249**:1133–1139.
56. **Steden, M., R. Betz, and W. Duntze.** 1989. Isolation and characterization of *Saccharomyces cerevisiae* mutants supersensitive to G1 arrest by the mating hormone a-factor. Mol. Gen. Genet. **219**:439–444.
57. **Tkacz, J. S., and V. L. MacKay.** 1979. Sexual conjugation in yeast. Cell surface changes in response to the action of mating pheromones. J. Cell Biol. **80**:326–333.
58. **Trueheart, J., J. D. Boeke, and J. R. Fink.** 1987. Two genes required for cell fusion during yeast conjugation: evidence for a pheromone-induced surface protein. Mol. Cell. Biol. **7**:2316–2328.
59. **Tsuchiya, E., S. Fukui, Y. Kamiya, Y. Sakagami, and M. Fugino.** 1978. Requirement of chemical structure of lipopeptidyl factors inducing sexual differentiation on vegetative cells of heterobasidiomycetous yeasts. Biochem. Biophys. Res. Commun. **85**:459–463.
60. **Van Ardsdell, S. W., G. L. Stetler, and J. Thorner.** 1987. The yeast repeated element sigma contains a hormone-inducible promoter. Mol. Cell. Biol. **7**:749–759.
61. **Vorburger, K., G. T. Kitten, and E. A. Nigg.** 1989. Modification of nuclear lamin proteins by a mevalonic acid derivative occurs in reticulocyte lysates and requires the cysteine residue of the C-terminal CXXM motif. EMBO J. **8**:4007–4013.
62. **Xue, C.-B., G. A. Caldwell, J. M. Becker, and F. Naider.** 1989. Total synthesis of the lipopeptide a-mating factor of *Saccharomyces cerevisiae*. Biochem. Biophys. Res. Commun. **162**:253–257.
63. **Xue, C.-B., A. Ewenson, J. M. Becker, and F. Naider.** 1990. Solution phase synthesis of *Saccharomyces cerevisiae* a-mating factor and its analogs. Int. J. Peptide Protein Res. **36**:362–373.