Degradation of a-Factor by a *Saccharomyces cerevisiae* α -Mating-Type-Specific Endopeptidase: Evidence for a Role in Recovery of Cells from G₁ Arrest

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Mating response between opposite mating types of *Saccharomyces cerevisiae* is dependent upon α factor, a tridecapeptide, and a-factor, an isoprenylated, methyl esterified dodecapeptide whose interaction with the α target cell has not been characterized. We report on the first biochemical and physiological evidence of an α -mating-type-specific a-factor-degrading activity. Radioiodinated a-factor was used to identify the a-factor-degrading activity, which is cell associated, endoproteolytic, and not required for response to pheromone. a-factor degradation was not energy dependent, nor did it require pheromone internalization or interaction with its receptor. Phenylmethylsulfonyl fluoride and tosyl-L-arginyl-methyl ester inhibited degradation of a-factor and increased the time required by α cells to recover from a-factor-induced growth arrest and morphological alteration, providing evidence that a-factor degradation plays a role in the recovery of α cells from the pheromone response.

Conjugation between *Saccharomyces cerevisiae* a and α haploid cells to form a/ α diploids requires the reciprocal action of peptide pheromones termed a-factor (YIIKGVFW DPAC[S-farnesyl]-OCH₃; Fig. 1), produced by a cells, and α factor (WHWLQLKPGQPMY), which is secreted by α cells (reviewed in references 14, 24, 25, and 40). These pheromones exert on their target cells similar effects which serve to prepare the cells for mating. Growth arrest occurs at G₁ of the cell cycle (7, 22), cell surface agglutinins are produced (20), transcription of several genes is induced (1, 28, 53), and the cells become pear shaped, a morphological change termed shmooing (31, 51). Genetic analysis and nucleotide sequencing studies have led to the conclusion that the receptors which mediate the pheromone response pathway, a- and α -factor receptors, belong to the rhodopsin-adrenergic receptor-muscarinic acetylcholine receptor family (8) and interact with signal-transducing, membrane-associated GTP-binding protein(s) (G proteins) (15, 37). Thus, study of the yeast pheromone response is providing important insights into the signal transduction mechanisms common to many eucaryotic cells.

Desensitization, or the ability to adapt to or recover from a biological stimulus, is a characteristic found in organisms ranging from bacteria to mammals. Results from studies of the adrenergic and acetylcholine hormone receptor families have demonstrated that several general mechanisms are utilized by eucaryotic cells to desensitize from hormone or pheromone action (29, 35, 46). These include receptor down regulation, structural modification of the receptor (e.g., phosphorylation), and destruction of the hormone or pheromone itself. In recent years, studies from several laboratories have revealed that *S. cerevisiae* a haploid cells utilize these same mechanisms to recover from mating pheromone-induced G₁ arrest. The α -factor receptor is subject to down regulation after the binding of α factor, although new recep-

tors which are capable of binding pheromone appear on the cell surface (11, 26). Despite the appearance of new receptors at the cell surface, a cells remain unresponsive to α factor. This characteristic indicates that there is an uncoupling of these new receptors from the pheromone response pathway. The α -factor receptor is also subject to phosphorylation of its C-terminal domain after the binding of α -factor (42). Moreover, mutant receptors lacking this C-terminal domain appear to block recovery from pheromone-induced growth arrest, suggesting that modification of the C-terminal domain of the α -factor receptor plays a role in the process of desensitization to pheromone (27, 42).

Studies of the a-factor receptor similar to those mentioned above have only recently been initiated, but present evidence suggests that a and α cells share common signal transduction machinery (5, 41). These studies demonstrated that the a- and α -factor receptors (*STE3* and *STE2* gene products, respectively) are the only a- or α -specific gene products required for a *S. cerevisiae* haploid cell response to mating pheromone. Furthermore, in both a and α cells, the *SST2* gene product has been shown to play a role in desensitization to pheromone, possibly by blocking a component of the pheromone response signaling pathway (9, 10, 16). The specific function of the *SST2* gene product has not yet been determined, although it has been speculated that it plays a role in modifying the mating pheromone receptors (16). *KSS1*, a recently characterized gene encoding a putative protein kinase, also plays a role in recovery of both a and α cells from the pheromone response (13). Overexpression of *KSS1* allows *sst2* mutants to overcome pheromone-induced growth arrest. Several other gene products are also common to the signal transduction pathways of both a and α cells, including G protein subunits and products required for cell and nuclear fusion (for review, see references 14, 24, and 25).

Many mammalian hormones, neurotransmitters, and other bioactive molecules are known to be inactivated by enzymatic degradation (33), and in many cases, such inactivation

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FIG. 1. Structures of *S. cerevisiae* α -factor (2) and various analogs used in this study.

plays a role in the recovery of target cells from biological stimuli. Examples include the hydrolysis of acetylcholine by acetylcholine esterase (35) and the proteolysis of enkephalin by enkephalinase (6). Most pertinent to the present study, it has been demonstrated that *S. cerevisiae* α cells produce a secreted endopeptidase (BAR protease) that cleaves α -factor, thus inactivating this peptide pheromone and aiding in the recovery of these cells from α -factor-induced growth arrest (12). Mutants that do not produce this protease, termed *bar1* (*ssl1*) mutants, are supersensitive to α -factor (9, 10, 47). *MAT α ssl1* cells are not supersensitive to α -factor, indicating that *SST1* is an α -cell-specific gene.

The α -factor pheromone is an isoprenylated, methyl esterified lipopeptide whose structure has only recently been determined (2) (Fig. 1). Like α -factor, α -factor is essential for mating in *S. cerevisiae* (36). Although it had been speculated that α cells degrade α -factor, such an activity had not previously been identified. In this paper, we report direct biochemical and physiological evidence of an α -cell-specific α -factor-degrading activity, which we call α -factorase.

MATERIALS AND METHODS

Materials. Yeast extract and Bacto peptone were purchased from Difco, Detroit, Mich.; 3,4-dichloroisocoumarin, phenylmethylsulfonyl fluoride (PMSF), *L-trans*-epoxysuccinyl-leucylamide-(4-guanidino)-butane, pepstatin A, 1,10-phenanthroline, tosyl-L-arginyl methyl ester (TAME), EDTA, ethylene glycol-bis(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), potassium fluoride, sodium azide, and chloroquine were purchased from Sigma, St. Louis, Mo.; antipain was purchased from Boehringer Mannheim, Indianapolis, Ind.; Merck Silica gel (0.25 mm thick)

thin-layer chromatography (TLC) plates were purchased from Bodman Chemicals, Stone Mountain, Ga.; and glucose and solvents for high-pressure liquid chromatography (HPLC) and TLC were purchased from Mallinckrodt, St. Louis, Mo.

Strains. The following *S. cerevisiae* strains were used in degradation, halo, and/or shmoo assays: X2180-1B (*MAT α SUC2 mal mel gal2 CUP1*) (Yeast Genetics Stock Center), X2180-1A (*MAT α SUC2 mal mel gal2 CUP1*) (Yeast Genetics Stock Center), and 2180a/ α (from mating of X2180-1A and X2180-1B). XUM-20D (*MAT α ssl1-7 cry1-11 ura3-52 leu2-3 trp1*) and CR11 (*MAT α cry1-11 thr4 his6 lys1 gal12*) were the generous gift of Wolfgang Duntze. SY1187 (*MAT α ste2-10::LEU2 leu2 ura3 met14 trp1_{am} his6 adel ade2-1_{oc}*) and SY1372 (isogenic to SY1187, but *ste3 Δ*) were the kind gift of George Sprague. SM1229 (*MAT α mfa1 Δ ::LEU2 mfa2 Δ ::URA3 trp1 his4 leu2 ura3 can1*) was the generous gift of Susan Michaelis.

Synthesis and characterization of a factor and a factor analogs. The various α -factor analogs used in this study are shown in Fig. 1. The α factor (NH₂-YIIKGVFWDPAC[S-farnesyl]-OCH₃) and α -factor pentadecapeptide (NH₂-YIIKGVFWDPACVIA-COOH) were synthesized as previously reported (4, 53). All other analogs were synthesized by either solution-phase peptide synthesis or a combination of solid-phase and solution-phase peptide synthesis. All peptides were purified to greater than 98% homogeneity by reversed-phase HPLC and were characterized by amino acid analysis, 400-MHz ¹H-nuclear magnetic resonance spectroscopy and fast atom bombardment mass spectrometry. Details of the synthesis and characterization of these compounds will be published separately.

Radioiodination of a-factor. a-factor was radiolabeled with ^{125}I (Amersham) by using the Iodo-Gen reagent (Pierce) according to the manufacturers' instructions. Iodinated a-factor was purified by HPLC, dried by rotary evaporation, and dissolved in methanol. Subsequent HPLC analysis demonstrated that there was no noniodinated a-factor in the [^{125}I] a-factor preparation. [^{125}I]a-factor had biological activity comparable to that of a factor. Specific activities of 1×10^5 to 5×10^5 cpm/ μg were typically obtained.

Preparation of *S. cerevisiae* particulate fraction of cell lysate. *S. cerevisiae* X2180-1B was grown at 30°C to mid-log phase in YEPD (1% yeast extract, 2% peptone, 2% glucose). Cells were harvested by centrifugation, washed once with sterile distilled H_2O , and resuspended to 0.3 g/ml (wet weight/volume) in lysis buffer (0.1 M K_2HPO_4 , 2 mM EDTA, 20 mM β -mercaptoethanol, 20 mM MgCl_2 , pH 6.45). Cells were lysed with glass beads (0.45 to 0.50 mm; B. Braun, Melsungen, Germany) and vortexing for 60-s intervals, being placed in ice for 2 min between each interval. Unbroken cells were removed by centrifugation for 10 min at $6000 \times g$, and the supernatant was centrifuged for 45 min at $30,000 \times g$. The resulting pellet was washed once with lysis buffer and resuspended in a small volume of lysis buffer containing 50% glycerol. Protein estimation was carried out with Bio-Rad protein assay concentrate. Extracts were stored at -20°C .

Assay for a-factor degradation. *S. cerevisiae* strains were grown to late log phase in YEPD at 30°C with shaking. Cells were harvested by centrifugation, washed once with YEPD, and suspended at 10^8 cells per ml. Approximately 50,000 cpm of [^{125}I]a-factor was then added to 490 μl of cell suspension in siliconized glass tubes (13 by 100 mm). Particulate fractions of cell lysates were assayed by adding [^{125}I] a-factor stock to 50 μl of particulate fraction and 440 μl of YEPD. Proteolytic reactions were stopped at various times after a-factor addition by removing portions of the cell suspension, pelleting the cells by centrifugation, and then spotting 10 μl of the culture medium onto TLC plates, followed by rapid air drying of the spotted reaction mixture. TLC plates were developed with *n*-butanol-acetate- H_2O (4:1:1, by volume) and exposed to Kodak X-Omat AR film for 2 to 5 days. Where indicated, a-factor degradation was quantitated densitometrically by the use of a Visage 60 Image Analysis System (Bio Image, Ann Arbor, Mich.). Various physiological inhibitors were added to the proteolysis assay suspension from 100 $\times$ stocks as indicated below. Also, where indicated, proteolysis assay media were analyzed by HPLC.

HPLC analysis. All HPLC analyses used a Beckman instrument and System Gold computer software with the following methodology: column, Dupont Zorbax Protein Plus C_3 Reversed Phase; flow rate, 1.5 ml/min; gradient, 15% acetonitrile from 0 to 10 min and 15 to 80% acetonitrile from 10 to 75 min (1%/min); detection, UV at 220 and 280 nm, γ emissions from 1-min fractions (Beckman Gamma 4000 Counter).

a-factor recovery assays. *S. cerevisiae* X2180-1B was grown at 30°C with shaking to early log phase in YEPD. The cells were harvested by centrifugation at $1000 \times g$, washed twice with YEPD, and resuspended to 4.0×10^6 cells per ml in YEPD or YEPD with protease inhibitor (as indicated below). Dilution series (1:2) of a-factor samples were prepared in borosilicate glass tubes with YEPD as the diluent. Five hundred microliters of cell suspension was added to 500 μl of each of the a-factor dilutions, and the resulting cell suspensions were incubated at 30°C with shaking. Aliquots were removed hourly, placed on ice, and observed micro-

scopically to quantitate the total number of cells, percentage of shmoo, and percentage of unbudded cells (100 to 200 cells counted per sample).

RESULTS

a-factor is degraded by α cells but not by a cells or a/ α diploids. In order to carry out various studies of the interaction of a-factor with α target cells, we radioiodinated synthetic a-factor. The procedure employed iodination of the single tyrosine residue at the N terminus of synthetic a-factor (Fig. 1) (see reference 53 for synthesis of a-factor). Purified [^{125}I]a-factor had biological activity comparable to that of noniodinated a-factor, being only about fivefold less active than the noniodinated pheromone, as determined by the shmoo assay, a standard biological assay used to measure the activity of *S. cerevisiae* mating pheromones (data not shown). Although it had been speculated that α cells degrade a-factor in a manner analogous to the degradation of α -factor by a cells (48), direct evidence of an a-factor-degrading activity had not yet been obtained. a-factor was indeed degraded by α cells but was not degraded by a cells or a/ α diploid cells (Fig. 2). Only a single degradation product was observed.

It was conceivable that the reason we did not detect [^{125}I]a-factor degradation by a cells was that these cells were in fact producing a-factor which competed with [^{125}I]a-factor for the degrading activity (see below and Table 2). To address this possibility, we determined that a-cell mutant SM1229, which does not produce a-factor because of the deletion of the a-factor structural genes, *MFa1* and *MFa2*, did not degrade [^{125}I]a-factor (data not shown). This result supports the above observation that a-factor degradation is α cell specific.

A cell-associated endopeptidase is responsible for the degradation of a-factor. The α -factor-degrading protease (BAR1) produced by a cells is a secreted enzyme (33). To determine whether the a-factor-degrading activity is also secreted, we analyzed the α -cell culture medium and the particulate fraction of cell lysates in the a-factor degradation assay. Cell-free spent α culture medium from a late-log-phase culture did not degrade [^{125}I]a-factor (Fig. 2). Medium from α cells treated with a concentration of a-factor (25 ng/ml) sufficient for inducing a biological response exhibited a similar lack of a-factor-degrading activity (data not shown). However, the particulate fraction of cell lysates degraded [^{125}I]a-factor, producing the same ^{125}I -labeled breakdown product that whole cells did (Fig. 3B and C). These results indicate that the α -cell-specific a-factor-degrading activity is cell associated and not secreted. Preliminary experiments also suggest that pretreatment of wild-type cells with a-factor results in an increased level of particulate-associated a-factor-degrading activity (data not shown), indicating that the expression of the degrading activity is increased upon exposure of α cells to a-factor. The a-cell-specific BAR protease activity had previously been shown to increase substantially upon exposure of a cells to α -factor (34).

HPLC analysis of the α -cell degradation of [^{125}I]a-factor showed that the primary iodinated breakdown product had a retention time (RT) of approximately 20 min (Fig. 3B). The additional minor degradation product(s) (RT = 3 min, excluded volume) detected by HPLC (Fig. 3B and C) was attributed to a further hydrolysis of the peak at 20 min by cellular peptidases. Secondary proteolysis of α -factor by a cells was seen in another study (12). We had previously synthesized several different Cys-12 a-factor analogs in our

FIG. 2. **a**-factor is degraded by a cell-associated α -mating-type-specific activity. Radioiodinated **a**-factor was incubated with *S. cerevisiae* X2180-1B (*MAT α*) cells (α), X2180-1A (*MAT a*) cells (**a**), X2180 **a**/ α diploid cells (**a**/ α), or cell-free spent X2180-1B culture medium (α sup). **a**-factor degradation assays were carried out as described in Materials and Methods. At the times indicated above the lanes, cells were removed by centrifugation, and then 10 μ l of each supernatant was spotted onto TLC plates. Plates were developed with *n*-butanol-acetate- H_2O (4:1:1, by volume) and exposed to film for 2 days. The R_f of **a**-factor was 0.71, while the degradation product had an R_f of 0.60. No other spots were detected.

laboratory, including the unmodified **a**-factor dodecapeptide (NH_2 -YIIKGVFWDPAC-COOH), nonmethylated **a**-factor (NH_2 -YIIKGVFWDPAC[S-farnesyl]-COOH), nonfarnesylated **a**-factor (NH_2 -YIIKGVFWDPAC-COOCH₃), and a C-terminal truncated **a**-factor decapeptide (NH_2 -YIIKGVFWDPAC-COOH) (Fig. 1) (53; unpublished data). In the HPLC system used for the analysis of **a**-factor degradation by α cells, these peptides had RTs as follows: NH_2 -YIIKGVFWDPAC-COOH, 30 min; NH_2 -YIIKGVFWDPAC[S-farnesyl]-COOH, 44 min; NH_2 -YIIKGVFWDPAC-COOCH₃, 32 min; and NH_2 -YIIKGVFWDPAC-COOH, 29 min. Since the primary ¹²⁵I breakdown product resulting from α -cell degradation of [¹²⁵I]**a**-factor had an RT of 20 min, it was less hydrophobic than any of the above-mentioned **a**-factor analogs. Thus, the degradation of **a**-factor was due not to defarnesylation or demethylation but rather to proteolysis at a bond to the N-terminal side of the Pro-10-Ala-11 peptide bond. Furthermore, **a**-factor degradation to generate the primary product (RT = 20 min) was not the result of the action of an aminopeptidase, since iodotyrosine and diiodotyrosine, the only amino acid residues iodinated by the method used, had RTs of 3.2 and 4.6 min, respectively. Additional chromatographic comigration experiments using iodinated **a**-factor analogs suggest that **a**-factor may be cleaved at the Val-6-Phe-7 peptide bond by an endopeptidase (data not shown). However, definitive determination of the site of cleavage of **a**-factor by α cells cannot be made without structural characterization of the actual breakdown products (i.e., amino acid analysis and mass spectrometry). Such analytical procedures require quantities of breakdown products which will be most effectively obtained by using purified or partially purified enzyme.

Degradation of **a-factor plays a role in α -cell recovery from the pheromone response.** To further characterize the α -cell-specific **a**-factor-degrading activity, degradation assays were carried out in the presence of various protease inhibitors. PMSF, 1,10-phenanthroline, and TAME were found to inhibit α -cell degradation of [¹²⁵I]**a**-factor, whereas antipain, pepstatin, aprotinin, and several other inhibitors had no detectable effect on **a**-factor degradation (Table 1).

To investigate the functional role of α -cell proteolysis of **a**-factor, we determined the effect of PMSF and TAME on both α -cell growth and response to **a**-factor (1,10-phenanthroline was extremely toxic to cells and was not tested). If

the proteolysis of **a**-factor is a mechanism of desensitization, we expect α cells to exhibit a prolonged response to **a**-factor in the presence of these inhibitors. Conversely, if **a**-factor proteolysis is required for biological activity, we expected to see a significantly lowered response to **a**-factor in the presence of PMSF and TAME. The former proved to be the case (Fig. 4). PMSF (2 mM) had only a slight effect on cell growth, while TAME (1 mM) had no detectable effect. However, both inhibitors markedly prolonged the **a**-factor response of α cells. Two protease inhibitors, aprotinin (0.01 mM) and pepstatin A (0.01 mM), which did not inhibit the degradation of [¹²⁵I]**a**-factor (Table 1), were also found to neither prolong nor enhance the α -cell response to **a**-factor (Fig. 4D). Ciejek and Thorner (12) previously showed that TAME prolongs the **a**-cell response to **a**-factor. However, they also found, and we have verified (Fig. 4D, inset), that PMSF has no effect on the recovery of **a** cells from the α -factor response, indicating that this inhibitor does not affect the part(s) of the desensitization process common to both **a** and α cells. These results provide physiological evidence that the degradation of **a**-factor plays a role in the desensitization of α cells from the **a**-factor pheromone response.

α -cell degradation of **a-factor is not energy dependent and does not require receptor-mediated endocytosis of the pheromone.** To determine whether **a**-factor degradation is energy dependent, assays were carried out in the presence of energy inhibitors. Sodium azide (10 mM) and potassium fluoride (10 mM) did not inhibit **a**-factor proteolysis when added together or separately to the assay. Thus, **a**-factor degradation does not appear to be an energy-dependent process.

To determine whether the degradation of **a**-factor is dependent in some way on the **a**-factor receptor (*STE3* gene product), we analyzed the ability of a *ste3* deletion mutant (SM1372) to proteolyze **a**-factor (Fig. 5). This receptorless mutant was in fact able to degrade **a**-factor. This result indicates that (i) **a**-factor proteolysis cannot be attributed to a function of the **a**-factor receptor, (ii) degradation is not dependent on receptor-mediated endocytosis, and (iii) the expression of the endopeptidase responsible for **a**-factor proteolysis is not dependent on pheromone induction, since *ste3 Δ* mutants are incapable of producing this response. However, as discussed above, pretreatment of wild-type α cells with **a**-factor appeared to result in an increased level of

FIG. 3. HPLC analysis of α -factor degradation by α cells and particulate fraction of cell lysate. Radioiodinated α -factor was incubated with *S. cerevisiae* α cells (X2180-1B) or the particulate fraction of the cell lysate, portions of the suspensions were subjected to centrifugation, and supernatants were analyzed by HPLC as described in Materials and Methods. (A) [125 I] α -factor; (B) degradation of [125 I] α -factor after incubation with α cells for 2 h; (C) degradation of [125 I] α -factor after incubation with the particulate fraction of α -cell lysate for 4 h.

TABLE 1. Effect of protease inhibitors on **a**-factor degradation

Inhibitor ^a	Protease class affected	Concn (mM) in degradation assay ^b	a -factor degradation ^c
Antipain	Serine/cysteine	1	++++
Aprotinin	Serine	0.01	++++
3,4-DCI	Serine	0.1	++++
E-64	Cysteine	0.01	++++
EDTA	Metallo	10	++++
EGTA	Metallo	10	++++
Pepstatin	Aspartic	0.01	++++
PMSF	Serine	2	++
1,10-Phenanthroline	Metallo	2	—
TAME	Serine	10	+

^a 3,4-DCI, 3,4-Dichloroisocoumarin; E-64, L-trans-epoxysuccinyl-leucylamide-(4-guanido)-butane.

^b Degradation assays were performed as described in Materials and Methods. Inhibitors were used at the concentrations shown to be maximally effective for a number of proteases (43).

^c The degree of [¹²⁵I]**a**-factor degradation was determined by densitometric quantitation of the degradation products seen in autoradiograms with a Visage 60 Image Analysis System (Bio Image). Scale of degradation of [¹²⁵I]**a**-factor after 6 h: + + + +, 75 to 100%; + +, 10 to 25%; +, 5 to 10%; —, no degradation.

[¹²⁵I]**a**-factor degradation, suggesting that exposure of cells to pheromone induces enhanced expression of the **a**-factor-degrading activity.

The **a-factor-degrading protease shows substrate specificity.** Several nonradiolabeled **a**-factor analogs were tested for their ability to act as competitors of **a**-factor degradation. Incubation of [¹²⁵I]**a**-factor with α cells in the presence of a 100-fold molar excess of nonradiolabeled **a**-factor resulted in complete inhibition of proteolysis of the radiolabeled pheromone (Table 2). In the presence of either a 100-fold excess of nonradiolabeled, nonfarnesylated **a**-factor or a 100-fold excess of nonradiolabeled pentadecapeptide (Fig. 1), degradation of [¹²⁵I]**a**-factor was measurably inhibited but not abolished. A 100-fold excess of the truncated **a**-factor analog, NH₂-YIIKGVFWDP-COOH, did not inhibit the proteolysis of **a**-factor. Likewise, a 25-fold molar excess of bovine serum albumin (BSA) had no effect on **a**-factor degradation. In a separate experiment, α cells did not exhibit any detectable degradation of [¹²⁵I]*S*-hexadecanoyl **a**-factor, which is biologically active, even in autoradiograms greatly overexposed (data not shown). These results suggest that the **a**-factor-degrading activity exhibits substrate specificity.

***ssl1* mutants degrade **a**-factor.** *S. cerevisiae* mutants supersensitive to **a**-factor were recently isolated by Steden et al. (48). One complementation group of mutants (*ssl1*) displayed an inability to remove the biological activity of **a**-factor added to cell suspensions, as compared with wild-type α cells. In addition, *ssl1* was found to be an α -cell-specific mutation. These investigators suggested that *SSL1* might encode the α -cell equivalent of the **a**-cell-specific BAR protease. However, we determined that the *ssl1-7* mutant is not defective in the ability to degrade **a**-factor (Fig. 5). Steden et al. (48) had found that *ssl1* mutants revert to *SSL1*⁺ at a high frequency, although the *ssl1-7* mutation appeared to be somewhat stable (18). To ensure that we were not dealing with a revertant, we determined by use of the halo assay that the *ssl1-7* mutant (XUM-20D) was approximately eightfold more sensitive to **a**-factor than its *SSL1*⁺ parent strain (CR11). Thus, our results suggest that *SSL1* is not the α -cell equivalent of the **a**-cell-specific *BAR1* gene but rather encodes a product whose mode of action is not yet known.

DISCUSSION

Destruction of ligands by their target cells is thought to be an important mechanism for desensitization from pheromone or hormone responses in eucaryotic organisms. *S. cerevisiae* α cells had been previously shown to secrete an endopeptidase that specifically cleaves the α -factor peptide, thus effectively inactivating this mating pheromone and assisting in the recovery of the cells from G₁ arrest (12, 33). We have reported here the first biochemical and physiological evidence of an α -cell-specific **a**-factor-degrading activity, which we call **a**-factorase. In contrast to the **a**-cell-specific BAR protease, which is secreted, **a**-factorase is a cell-associated enzyme. Our results suggest that **a**-factorase is an endopeptidase which most likely cleaves **a**-factor at the Val-6-Phe-7 peptide bond.

Mating type *a* cells of the heterobasidiomycetous yeast *Rhodospiridium toruloides* have also been shown to produce a cell-associated endopeptidase that cleaves rhodotorucine A, the farnesylated peptide pheromone produced by cells of the *A* mating type (38, 39). However, in contrast to the degradation of α -factor by *S. cerevisiae* α cells, proteolysis of rhodotorucine A is required for biological activity. Since **a**-factor, like rhodotorucine A, is a farnesylated peptide, and since **a**-factorase is cell associated, we asked whether the α -cell-specific proteolysis of **a**-factor was required for biological activity or whether it played a role in the recovery of α cells from the pheromone response. Several protease inhibitors were tested for their ability to inhibit **a**-factor proteolysis by α cells. Two inhibitors, PMSF and TAME, were found to significantly inhibit the proteolysis of **a**-factor and markedly prolong the α -cell response to **a**-factor but did not alter the onset of the response. These results suggest that the α -cell proteolysis of **a**-factor plays a role in desensitization from the pheromone response in a manner analogous to that of the degradation of α -factor by the **a**-cell-specific BAR protease. Further evidence for the role of **a**-factor proteolysis in desensitization, but not in biological activity, was provided by showing that α cells are incapable of degrading [¹²⁵I]*S*-hexadecanoyl **a**-factor, a structural analog of **a**-factor that is biologically active (unpublished results) (Fig. 1). This result, however, must be interpreted cautiously, as *S*-hexadecanoyl **a**-factor is about 30-fold less active than **a**-factor. Thus, if *S*-hexadecanoyl **a**-factor is proteolyzed at levels not detectable by our assay, a correlation might be made between activity and proteolysis. Furthermore, while our results suggest that **a**-factor degradation is not required for biological activity, they do not rule out the possibility that **a**-factor degradation products enhance the biological activity of the pheromone or that the degradation products themselves are biologically active. Previously, biologically inactive, N-terminal fragments of α -factor were shown to prolong the time needed for a cells to recover from the action of the native pheromone (49). Conclusive determination of the physiological role of **a**-factorase will require the isolation of mutants defective in **a**-factorase activity and/or the cloning and subsequent disruption or deletion of the gene encoding this enzyme.

The observation that PMSF and TAME inhibited **a**-factor proteolysis suggests that **a**-factorase may be a serine protease. Furthermore, the enzyme may be a metal-dependent or metal-activated, since α cells were incapable of proteolyzing **a**-factor in the presence of 1,10-phenanthroline. However, the lack of effects of other metal chelators (EDTA and EGTA) and serine protease inhibitors (aprotinin and 3,4-dichloroisocoumarin) make it difficult to make a definite

FIG. 5. *ste3Δ* mutants and *ssl1* mutants degrade α -factor. Radioiodinated α -factor was incubated with *S. cerevisiae* CR11 (*SSL1*), XUM-20D (*ssl1*), SY1187 (*STE3*), or SY1372 (*ste3Δ*). α -factor degradation assays were carried out as described in Materials and Methods. At the times indicated above the lanes, cells were removed by centrifugation, and then 10 μ l of each supernatant was spotted onto TLC plates. Plates were developed with *n*-butanol-acetate- H_2O (4:1:1, by volume) and exposed to film for 2 days.

conclusion about the protease class of α -factorase. Such a determination, as well as analysis of the enzyme's substrate specificity, will require characterization of the purified enzyme.

Experiments showed that α -factor proteolysis is not energy dependent, since the degradation of α -factor was not affected by energy inhibitors and does not require receptor-mediated internalization of the ligand, as evidenced by the fact that a receptor-deleted mutant (*ste3Δ*) was not defective in degradation. In addition, the α -factor receptor itself does not contain the endopeptidase activity responsible for the degradation of α -factor, since the *ste3Δ* mutant retains α -factorase activity. These results also indicate that α -factor degradation is not dependent on either pheromone response, since *ste3Δ* mutants are incapable of producing this response, or receptor-mediated endocytosis. Although pheromone induction is not required for expression of α -factorase, preliminary experiments suggest that pretreatment of α cells with α -factor prior to the proteolysis assay results in an increased level of α -factor degradation. Thus, pheromone exposure may result in increased expression of α -factorase. The α -cell-specific BAR protease is under similar expressional control (34).

A report on the isolation of *S. cerevisiae* mutants supersensitive to α -factor was published recently (48). Two complementation groups of mutants, *ssl1* and *ssl2* were isolated. *ssl2* was shown to be allelic to *ssl1*, a mutation conferring pheromone supersensitivity to both α and α cells. However, *ssl1* was shown to be an α -cell-specific mutation in that *MAT α ssl1* cells were supersensitive to α -factor but *MAT α ssl1* cells were not supersensitive to α -factor. In addition, *MAT α ssl1* mutants were found to be incapable of inactivating externally added α -factor, in contrast to *SSL1⁺* cells. Contrary to the expectation that *ssl1* mutants might be incapable of degrading α -factor, we found that the *ssl1-7*

mutant does degrade α -factor. Thus, our results suggest that the *SSL1* gene is not the α -cell equivalent of the α -cell-specific *BAR1* gene. However, it is possible that in addition to proteolyzing α -factor, α cells also produce a moiety which binds to the pheromone, rendering it inactive. Thus, *SSL1* may encode an α -factor-blocking or -binding product. Alternatively, *ssl1* mutants may be defective in receptor-mediated endocytosis of α -factor. If this is found to be the case, then it would suggest that α and α cells have differences in specific recovery mechanisms of the pheromone response pathway. Further studies are clearly required to determine the function of the *SSL1* gene product.

The possibility has been proposed that α -factor, because of its modification by the addition of a farnesyl lipid tail, is anchored to α cells and that its primary effect on α cells results from cell-cell contact rather than the activity of secreted pheromone (25). However, it is also conceivable that secreted hydrophobic α -factor enters the lipid bilayer of the plasma membrane of α cells and then flows through the membrane to reach its receptor. Such a process, proposed for neuropeptide-membrane interactions (3, 44, 45), would possibly make a membrane-bound degrading protease more effective than a secreted protease for serving a role in the process of desensitization. Preliminary experiments suggest that radiolabeled α -factor associates rapidly with the particulate fraction of cell lysates, suggesting that α -factor does readily associate with membrane lipids.

Mammalian hormones and neurotransmitters are also known to be degraded as a mechanism of inactivation (17, 32, 35). However, the proteases responsible specifically for the inactivation of most peptide hormones have not been identified. One of the best-characterized peptide hormone-degrading proteases is neutral endopeptidase, also called enkephalinase. Neutral endopeptidase is a membrane-bound zinc metalloendopeptidase that hydrolyzes a variety of bio-

FIG. 4. PMSF and TAME enhanced and prolonged the α -cell response to α -factor. Recovery assays were performed as described in Materials and Methods, with *S. cerevisiae* X2180-1B at a starting concentration of 2.5×10^6 cells per ml. (A) Effect of PMSF and TAME on cell growth in the absence (closed symbols) and presence (open symbols) of α -factor (80 ng/ml). ● and ○, Growth in YEPD; ■ and □, growth in YEPD with 2 mM PMSF; ▲ and △, growth in YEPD with 1 mM TAME. (B and C) Recovery of cells as measured by shmoos observed (percentage of total cells) in YEPD containing α -factor at 80 and 40 ng/ml, respectively. ○, Recovery in the absence of protease inhibitor; □, recovery in the presence of 2 mM PMSF; △, recovery in the presence of 1 mM TAME. (D) Same as in panel B, except different protease inhibitors were tested. ○, Recovery in the absence of protease inhibitor; ▽, recovery in the presence of 0.01 mM aprotinin; ◇, recovery in presence of 0.01 mM pepstatin A. (Inset) X2180-1A (*MAT α*) cells were monitored for recovery from α -factor (1 μ g/ml of YEPD) in the absence (○) or presence (□) of 2 mM PMSF.

TABLE 2. Competition of [¹²⁵I]a-factor degradation by nonradiolabeled a-factor, various structural analogs, and BSA

Competitor ^a	Molar excess (fold) to [¹²⁵ I]a-factor	a-factor degradation ^b
a-factor	100	—
Nonfarnesylated a-factor	100	+
a-factor pentadecapeptide	100	+
a-factor decapeptide	100	++++
BSA	25	++++

^a Structures of competitors are shown in Fig. 1.

^b Degradation assays were performed as described in Materials and Methods. Scale of degradation of [¹²⁵I]a-factor after 6 h: + + + +, 75 to 100%; +, 5 to 10%; —, no degradation.

active peptides, including enkephalins (23), bradykinin (21), substance P (51), and the atrial natriuretic peptide (19). Moreover, the common acute lymphocytic leukemia antigen, which is found on leukemic but not normal cells, has been shown to be identical to neutral endopeptidase (30). Thus, it is possible that the improper expression of peptide hormone-degrading proteases can, in some cases, lead to carcinogenesis. It will be of great interest to purify a-factorase, clone the gene encoding this lipopeptide-degrading endopeptidase to better understand its modes of action and regulation, and determine whether related proteases are found in mammalian cells.

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