Grönberg, L., & Slotte, J. P. (1990) *Biochemistry 29*, 3173-3178.
Grunberger, D., Haimovitz, R., & Shinitzky, M. (1982) *Biochim. Biophys. Acta 688*, 764-774.
Haase, W., Schafer, A., Murer, H., & Kinne, R. (1978) *Biochem. J. 172*, 57-62.
Lange, Y., & Ramos, B. (1983) *J. Biol. Chem. 258*, 15130-15134.
Lange, Y., Cutler, H. B., & Steck, T. L. (1980) *J. Biol. Chem. 255*, 9331-9337.
Lange, Y., Matthies, H., & Steck, T. L. (1984) *Biochim. Biophys. Acta 769*, 551-562.
Le Grimellec, C., Giocondi, M. C., Carrière, B., Carrière, S., & Cardinal, J. (1982) *Am. J. Physiol. 242*, F246-F253.
Le Grimellec, C., Friedlander, G., & Giocondi, M. C. (1988) *Am. J. Physiol. 255*, F22-F32.
Levi, M., Baird, B. M., & Wilson, P. V. (1990) *J. Clin. Invest. 85*, 231-237.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265-275.
Molitoris, B., Alfrey, A. C., Harris, R. A., & Simon, F. R. (1985) *Am. J. Physiol. 249*, F12-F19.
Moore, N. F., Patzer, E. J., Barenholz, Y., & Wagner, R. R. (1977) *Biochemistry 16*, 4708-4715.
Mrsny, R. J., Volwerk, J. J., & Griffith, O. H. (1986) *Chem. Phys. Lipids 39*, 185-191.
Omodeo-Salé, F., Marchesini, S., Fishman, P. H., & Berra, B. (1984) *Anal. Biochem. 142*, 347-350.
Pal, R., Barenholz, Y., & Wagner, R. R. (1980) *J. Biol. Chem. 255*, 5802-5806.
Patzer, E. J., Wagner, R. R., & Barenholz, Y. (1978) *Nature 274*, 394-395.
Schroeder, F. (1988) in *Advances in Membrane Fluidity* (Aloia, R. C., Curtain, C. C., & Gordon, L. M., Eds.) Vol. 1, pp 193-217, Alan R. Liss, New York.
Slotte, J. P., Hedström, G., Rannström, S., & Ekman, S. (1989) *Biochim. Biophys. Acta 985*, 90-96.
Slotte, J. P., Härmälä, A. S., Jansson, C., & Pörn, M. I. (1990) *Biochim. Biophys. Acta 1030*, 251-257.
Spiegel, S., Matyas, G. R., Cheng, L., & Sacktor, B. (1988) *Biochim. Biophys. Acta 983*, 270-278.
Thurnhofer, H., Gains, N., Mutsch, B., & Hauser, H. (1986) *Biochim. Biophys. Acta 856*, 174-181.
van Dijck, P. W. M. (1979) *Biochim. Biophys. Acta 555*, 89-101.
Van Meer, G. (1987) *Trends Biochem. Sci. 12*, 375-376.
Van Meer, G. (1988) *Trends Biochem. Sci. 13*, 242-243.
Vênien, C., & Le Grimellec, C. (1988) *Biochim. Biophys. Acta 942*, 159-168.
Vênien, C., Aubry, M., Crine, P., & Le Grimellec, C. (1988) *Anal. Biochem.* 174, 325-330.
Waite, M. (1987) in *Handbook of Lipid Research* (Hanahan, D. J., Ed.) Vol. 5, pp 191-241, Plenum Press, New York.
Yeagle, P. L., & Young, J. E. (1986) *J. Biol. Chem. 261*, 8175-8181.

Antagonistic and Synergistic Peptide Analogues of the Tridecapeptide Mating Pheromone of *Saccharomyces cerevisiae*†

Effimia Eriotou-Bargiota,‡ Chu-Biao Xue,§ Fred Naider,§ and Jeffrey M. Becker*,‡
Department of Microbiology, University of Tennessee, Knoxville, Tennessee 37996-0845, and the Department of Chemistry, College of Staten Island, Staten Island, New York 10301
Received July 23, 1991; Revised Manuscript Received October 2, 1991

ABSTRACT: Biologically inactive, truncated analogues of the *Saccharomyces cerevisiae* α-mating factor (WHWLQLKPGQPMY) either antagonized or synergized the activity of the native pheromone. An amino-terminal truncated pheromone [WLQLKPGQP(Nle)Y] had no activity by itself, but the analogue acted as an antagonist by competing with binding and activity of the mating factor. In contrast, a carboxyl-terminal truncated pheromone [WHWLQLKPGQP] was not active by itself nor did the peptide compete with α-factor for binding to the α-factor receptor, but it acted as a synergist by causing a marked increase in the activity of α-factor. The observation that residues near the amino terminus may be involved in signal transduction whereas those near the carboxyl terminus influence binding allows us to separate binding and signal transduction in the yeast pheromone response pathway. If found for other hormone–receptor systems, synergists may have potential as therapeutic compounds.

Communication between cells is mediated by an elegant information system which involves the specific recognition of effector molecules by membrane-bound receptors and subsequent signal transduction to the intracellular machinery. The molecular interactions between messenger molecules and their receptors and the pathway of signal transduction are two of the most intensively studied areas of cellular biology. Such phenomena occur throughout nature and can be examined in lower eukaryotes such as *Saccharomyces cerevisiae*.

Sexual conjugation in *S. cerevisiae* is dependent upon diffusible peptide pheromones, the a-factor and the α-factor [for recent reviews, see Cross et al. (1988) and Herskowitz (1988)], which are recognized by membrane-bound receptors (Burkholder & Hartwell, 1985; Hagen et al., 1986; Nakayama et al., 1985). On the basis of the sequence of the genes encoding receptors, significant structural similarity is predicted among the yeast α-factor receptor (*STE2* gene product) and a-factor receptor (*STE3* gene product) and receptors as diverse as rhodopsin and those for β-adrenergic and cholinergic agents

† This work was supported by Grants GM-22086 and GM-22087 from the National Institutes of General Medical Sciences.
‡ University of Tennessee.
§ College of Staten Island.

0006-2960/92/0431-551$03.00/0

(Lefkowitz & Caron, 1988). The yeast signaling pathway manifests remarkable similarities to hormonal systems of higher eukaryotes not only in the receptor structural motif but also in the signal transduction through GTP-binding proteins (Blumer & Thorner, 1990, 1991; Dietzel & Kurjan, 1987; Marsh & Herskowitz, 1988; Miyajima et al., 1987; Whiteway et al., 1989).

NMR studies in solution (Jelicks et al., 1988) and in lipids (Naider et al., 1989), and analysis of the biological activity of conformationally restricted (Shenbagamurthi et al., 1985) and conformationally constrained analogues (Xue et al., 1989), have led to the conclusion that α-factor (WHWLQLKPGQPMY) may assume a type II β-turn involving residues $Lys^7Pro^8Gly^9Gln^{10}$ when bound to its receptor. If this is correct, it is possible that this bend directly interacts with the receptor or that the bend allows residues which are far away in the primary structure to fold into the biologically active conformation. Although this is a rather simplistic view of pheromone–receptor interaction considering that both pheromone termini and bend regions may be interacting simultaneously with the receptor, the model does lead to straightforward testing. For example, if the bend occupies the receptor binding site and the termini do not interact with the receptor, the truncation of the pheromone might not alter biological activity. In order to probe this possibility, we decided to prepare and evaluate analogues of α-factor truncated at either the amino or carboxyl terminus. We now report on the discovery of a new α-factor antagonist and of analogues of this pheromone which synergize its activity.

Materials and Methods

Organisms, Media, and Growth Conditions. The *S. cerevisiae* strains used were *S. cerevisiae* 2180-1A *MAT*a from the Yeast Genetics Stock Center, Berkeley, CA, *S. cerevisiae* RC629 *MAT*a *sst1-2* [supersensitive to α-factor by virtue of a mutation in the BAR1 protease (allelic to *sst1*) which hydrolyzes α-factor] from R. Chan (Chan & Otte, 1982), and *S. cerevisiae* RC631 *MAT*a *sst2-1* (supersensitive to α-factor by an unknown mechanism) from R. Chan (Chan & Otte, 1982). A *MAT*a *ste2*^{ts} *sst2* strain was constructed by crossing strains 50B^{ts} (*MAT*a *ste2*^{ts}) and RC757 (*MAT*α *sst2*). The resulting diploid cells were sporulated, and the desired haploid genotype was obtained through random spore selection. The strains were maintained and grown on YEPD medium (1% yeast extract; 2% peptone; 2% dextrose; and 2% agar). All incubations took place at 30 °C.

Fast Atom Bombardment–Mass Spectrometry. The FAB–mass spectrum was obtained with a VG ZAB EQ mass spectrometer equipped with a flow FAB ion source of an Ion Tech fast atom gun. Approximately 2 μL of a peptide solution (1.0 nmol/μL) dissolved in acetonitrile/water (2:1) with 0.1% trifluoroacetic acid was dispersed on the stainless steel target in a matrix of monothioglycerol. The accelerating voltage of the mass spectrometer was maintained in 8 kV, whereas 8-keV xenon atoms at a discharge current of 1 mA were used to bombard the sample. Spectra were recorded in the "multichannel acquisition" mode from m/z 800 to 1800 at 20 s/decade by the VG 11-250 J data system. Instrument resolution ($m/\Delta m$) was set at 2000. All the truncated peptides exhibited the calculated molecular ions as determined by FAB–mass spectrometry.

Generation of the Truncated Peptides. Natural α-factor [WHWLQLKPGQPMY, designated α-factor (1–13)-OH throughout this paper] and [Nle^{12}]α-factor(1–13)-OH are equally biologically active (Raths et al., 1988). [Nle^{12}]α-factor(1–13)-OH was utilized as the reference and starting pheromone because the isosteric replacement of Met^{12} by Nle^{12} results in a pheromone less prone to oxidative decay. To digest the peptide, 20 units of carboxypeptidase A (Sigma) was incubated with 100 μL of α-factor(1–13)-OH (1 mg/mL in distilled water) for 2.5 h at 25 °C with shaking. In a separate experiment, 0.01 unit of aminopeptidase M (Pierce), cross-linked to 4% beaded agarose, was incubated with 200 μL of α-factor(1–13)-OH (1 mg/mL in 0.05 M phosphate buffer, pH 7.5) for 3 h at 35 °C. After incubation, the mixtures were centrifuged through a Centricon 10 filter column (Amicon, with 10 000 molecular weight cut-off) to remove the carboxypeptidase A (molecular weight of 36 000) or the aminopeptidase M and left at −20 °C overnight to destroy any remaining enzyme activity. Following removal of enzyme activity, the mixture was fractionated and purified using HPLC. For this purpose, a Waters μBondapak C_{18} column was employed using a water/CH_3CN/TFA gradient from 90:10:0.025 to 40:60:0.025 over 50 min. The flow rate was 1.4 mL/min, and detection was at 220 nm.

Peptide Synthesis. The [Nle^{12}]α-factor(1–13)-OH and cyclo^{7,10}[Nle^{12}]α-factor(1–13)-OH were synthesized by solid phase methodology using the procedures described previously (Xue et al., 1989). In order to ensure that the truncated peptides generated by enzyme digestion were not contaminated by trace amounts of the [Nle^{12}]α-factor(1–13)-OH, we also synthesized α-factor(1–11)-OH (WHWLQLKPGQP), and α-factor(2–11)-OH (HWLQLKPGQP) on a (phenylacetamido)methyl (PAM) resin. The Boc group was used for all *N*-α-protection; formyl, tosyl, and (2-chlorobenzyl)oxycarbonyl were used for the side-chain protection of Trp, His, and Lys, respectively. The Boc group was deprotected with 45% trifluoroacetic acid/2% dimethylsulfide/CH_2Cl_2 except for Gln where the Boc group was deprotected with 4 N HCl in dioxane. Coupling was carried out twice for each residue using diisopropylcarbodiimide in CH_2Cl_2 for the first coupling and diisopropylcarbodiimide/HOBt in CH_2Cl_2/DMF for the second coupling. BocGln was coupled via its HOBt ester preformed in DMF at 0 °C. After HF cleavage, the product was treated with piperidine in DMF to remove the formyl group and with HOBt in water/acetonitrile to remove the incompletely deprotected tosyl group. The crude peptide was purified using reverse-phase HPLC to over 98% homogeneity. The structure of the synthetic peptides were confirmed by amino acid analysis and fast atom bombardment–mass spectrometry. The synthetic WHWLQLKPGQP was identical to the fragment generated enzymatically.

Growth Arrest (Halo Assay). YEPD plates were overlayered with 4 mL of *S. cerevisiae MAT*a cells (2.5×10^5 cells per mL) in Noble agar. Filter disks (sterile blanks from Difco), 8 mm in diameter, were placed on the overlay, and 10-μL portions of peptide solutions at various concentrations were pipetted onto the disks. The plates were incubated at 30 °C for 24 h and then observed for clear zones (halos) around the disks. The data were expressed as the diameter of the halo including the diameter of the disk. Therefore, a minimum value for growth arrest is 9 mm, which represents the disk diameter (8 mm) and a small zone of inhibition.

Effects of α-Factor(1–13)-OH on Cellular Morphology of S. cerevisiae RC629 in the Presence or Absence of α-Factor(1–11)-OH. A culture of *S. cerevisiae* RC629 was grown at 30 °C with shaking to log phase (1×10^7 cells/mL) in YEPD. A portion of these cells and α-factor(1–13)-OH or analogue were added to fresh YEPD so that the cell concentration was 1×10^6 cells/mL. The suspensions were incubated at 30 °C and after a 4-h incubation a portion was removed

Table I: Nomenclature of α-Factor(1–13)-OH Analogues

amino acid sequence	designation
WHWLQLKPGQPMY[a]	α-factor(1–13)-OH
WHWLQLKPGQP[Nle]Y[b]	[Nle[12]]α-factor(1–13)-OH
HWLQLKPGQP[Nle]Y	[Nle[12]]α-factor(2–13)-OH
WLQLKPGQP[Nle]Y	[Nle[12]]α-factor(3–13)-OH
WHWLQLKPGQP[Nle]	[Nle[12]]α-factor(1–12)-OH
WHWLQLKPGQP	α-factor(1–11)-OH
HWLQLKPGQP	α-factor(2–11)-OH

[a] Standard one-letter code for all amino acids is used. [b] Nle is norleucine. α-Factor(1–13)-OH and [Nle[12]]α-factor(1–13)-OH have equal biological activity. The isosteric replacement of Met[12] by Nle[12] results in a pheromone less prone to oxidative degradation.

for microscopic evaluation of the number of morphologically aberrant cells (shmoos) and the number of non-shmooing cells. From these results the proportion of shmoos was calculated for each concentration of peptide tested.

HPLC Analysis of α-Factor(1–13)-OH Degradation. *S. cerevisiae* 2180-1A or RC629 (1×10^6 cells/mL) were incubated with α-factor(1–13)-OH (5 μg/mL) at 30 °C in YEPD for various time periods. Portions of the culture were removed and centrifuged (3000 rpm, 10 min) to remove cells. The supernatant was analyzed by HPLC using a Waters μBondapak C_{18} column with a gradient phase of 10–60% acetonitrile with 0.025% trifluoroacetic acid. Under these conditions, α-factor(1–13)-OH and the major degradation peak exhibited retention times of 25.5 and 27.5 min, respectively. The amount of peptide at various time points was quantitated by determination of the area under the peaks.

Competition of Binding of [^{3}H]α-Factor(1–13)-OH by Unlabeled [Nle[12]]α-Factor(3–13)-OH and α-Factor(1–11)-OH. Competition of bound [^{3}H]α-factor(1–13)-OH [[^{3}H],[Nle[12]]α-factor(1–13)-OH] by unlabeled α-factor(1–13)-OH analogues was measured as described previously (Raths et al., 1988) with the exception that the *S. cerevisiae* strain used in the assay was *S. cerevisiae* RC629 (*sst1*).

Steady-State Saturation Binding of [^{3}H]α-Factor(1–13)-OH to RC629 Cells in the Presence or Absence of α-Factor(1–11)-OH. The procedure followed to obtain steady-state saturation binding was the same as described previously (Raths et al., 1988) with the exception that the *S. cerevisiae* strain used in the assay was *S. cerevisiae* RC629 (*sst1*).

Results

Chemical Characterization of α-Factor(1–13)-OH Analogues. The truncated analogs [Nle[12]]α-factor(2–13)-OH [HWLQLKPGQP(Nle)Y] (see Table I), [Nle[12]]α-factor(3–13)-OH [WLQLKPGQP(Nle)Y], [Nle[12]]α-factor(1–12)-OH [WHWLQLKPGQP(Nle)], and α-factor(1–11)-OH [WHWLQLKPGQP] were generated by treating synthetic [Nle[12]]α-factor(1–13)-OH [WHWLQLKPGQP(Nle)Y] with either aminopeptidase M or carboxypeptidase A. The shortened fragments were separated, isolated, and purified using reverse-phase high performance liquid chromatography (HPLC). Truncated pheromones were >99% homogeneous and were characterized by both amino acid analysis and fast atom bombardment–mass spectrometry (Table II). The complete absence of amino acids such as Tyr, Met, and His in the various analogues demonstrated that the analogues were not contaminated with residual α-factor. α-Factor(1–11)-OH synthesized by solid phase procedures (see Materials and Methods) had chemical properties identical to the same peptide generated by the enzymatic cleavage of [Nle[12]]α-factor(1–13)-OH.

Biological Activity of Amino- and Carboxyl-Truncated Peptides. The activity of the truncated analogues was tested with a growth arrest assay against *MAT*a haploid strains which represent both wild-type (2180-1A) and supersensitive (RC629 and RC631) phenotypes. The truncated peptides HWLQLKPGQP(Nle)Y and WHWLQLKPGQP(Nle), each lacking one amino acid, were approximately 10-fold and >40-fold less active, respectively, than the native α-factor(1–13)-OH against strain 2180-1A or RC629 (Table III). In strain RC631, [Nle[12]]α-factor(2–13)-OH was about half as active as native α-factor(1–13)-OH whereas [Nle[12]]α-factor(1–12)-OH was 100-fold less active than α-factor(1–13)-OH. WHWLQL, the amino-terminal hexapeptide of α-factor(1–13)-OH, and HWLQLKPGQP, an analogue with Trp[1], Met[12], and Tyr[13] removed, had no activity in any tester strain (data not shown).

Removal of two amino acids from either the amino or carboxyl terminus of the pheromone resulted in peptides which were virtually devoid of activity (at least 10^3-fold less active) against strain 2180-1A or RC629 (data not shown). Identical conclusions could be drawn using assay of cell shape as the criterion for biological activity (data not shown). However, in strain RC631 both [Nle[12]]α-factor(3–13)-OH and α-factor(1–11)-OH were active, although about 10-fold and 100-fold less active, respectively, than [Nle[12]]α-factor(1–13)-OH (Table IV). To further characterize the effect of α-factor-(1–11)-OH in a *sst2* background, we generated a *sst2 ste2*^{ts} mutant. This strain gave results identical with those for RC631 for the permissive temperature and did not respond

Table II: Chemical Properties of Truncated α-Factor(1–13)-OH Analogues[a]

		α-factor(1–13)-OH derived peptides			
analysis	[Nle[12]]α-factor(1–13)-OH	[Nle[12]]α-factor(1–12)-OH	[Nle[12]]α-factor(1–11)-OH	[Nle[12]]α-factor(2–13)-OH	[Nle[12]]α-factor(3–13)-OH
AA[b]					
Glu	2.10	1.91	2.00	1.97	1.94
Gly	1.14	1.16	1.02	1.11	1.00
His	0.79	0.97	0.89	0.96	0.00
Leu	1.98	2.08	2.04	1.89	1.87
Lys	0.88	1.05	0.93	0.88	0.92
Nle	0.99	0.92	0.00	1.16	1.12
Pro	2.01	2.01	2.12	2.14	2.03
Tyr	1.06	0.00	0.00	0.91	0.91
FAB–MS[c]					
found		1503	1389	1480	1343
calcd [M + H$^+$]		1503	1389	1480	1342

[a] The peptides were [Nle[12]]α-factor(1–13)-OH = WHWLQLKPGQP[Nle[12]]Y; [Nle[12]]α-factor(1–12)-OH = WHWLQLKPGQP[Nle[12]]; [Nle[12]]α-factor(1–11)-OH = WHWLQLKPGQP; [Nle[12]]α-factor(2–13)-OH = HWLQLKPGQP[Nle[12]]Y; [Nle[12]]α-factor(3–13)-OH = WLQLKPGQP[Nle[12]]Y. Peptides were purified by HPLC from carboxypeptidase A or aminopeptidase M digested [Nle[12]]α-factor(1–13)-OH as described in detail under Materials and Methods. [b] AA = amino acid analysis. [c] FAB–MS = fast atom bombardment–mass spectroscopy.

Table III: Halo Assay on [Nle[12]]α-Factor(2–13)-OH and [Nle[12]]α-factor(1–12)-OH[a]

peptide (μg/disc)	2180-1A [Nle[12]]-α-factor-(1–13)-OH	2180-1A [Nle[12]]-α-factor-(2–13)-OH	2180-1A [Nle[12]]-α-factor-(1–12)-OH	RC629 (*sst1*) [Nle[12]]-α-factor-(1–13)-OH	RC629 (*sst1*) [Nle[12]]-α-factor-(2–13)-OH	RC629 (*sst1*) [Nle[12]]-α-factor-(1–12)-OH	RC631 (*sst2*) [Nle[12]]-α-factor-(1–13)-OH	RC631 (*sst2*) [Nle[12]]-α-factor-(2–13)-OH	RC631 (*sst2*) [Nle[12]]-α-factor-(1–12)-OH
20	ND[b]	ND	0	ND	ND	12	ND	ND	26
15	20	ND	0	ND	ND	10	ND	ND	24
10	19	12	0	32	21	0	36	35	21
5	17	9	0	31	18	0	35	33	17
1	10	0	0	23	12	0	29	28	11
0.5	9	0	0	21	10	ND	27	24	0
0.1	0	0	0	14	0	0	21	17	0
0.05	0	ND	ND	11	0	ND	14	10	0
0.01	0	ND	ND	0	0	ND	0	0	0

[a] The values represent the mean of at least three experiments. In all cases, the three values averaged were within 2 mm of each other. [b] ND, not determined.

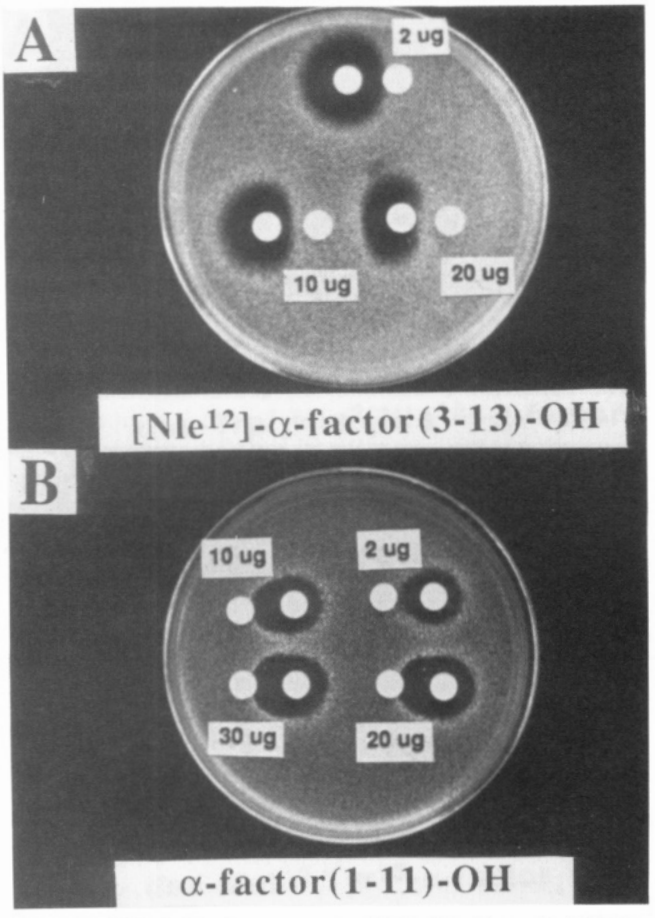

FIGURE 1: Effect of various amounts of [Nle[12]]α-factor(3–13)-OH (A) and α-factor(1–11)-OH (B) on [Nle[12]]α-factor activity. [Nle[12]]α-factor(1–13)-OH (1 μg) was placed on the disk which appears at the center of growth arrest. The amounts of peptides on disks at the halo periphery are indicated in the figure. Antagonism was shown by reversal of growth arrest, whereas synergism was shown by stimulation of growth arrest.

Table IV: Halo Assay on [Nle[12]]α-Factor(3–13)-OH and α-Factor(1–11)-OH Using RC631 (*sst2*) Strain[a]

peptide (μg/disk)	[Nle[12]]-α-factor-(1–13)-OH	[Nle[12]]-α-factor-(3–13)-OH	α-factor-(1–11)-OH
20	ND[b]	ND	19
15	ND	ND	17
10	36	28	15
5	35	25	12
1	29	19	0
0.5	27	16	0
0.1	21	10	0
0.05	14	0	0
0.01	0	0	0

[a] The values represent the mean of at least three experiments. In all cases, the three values averaged were within 2 mm of each other. [b] Not determined.

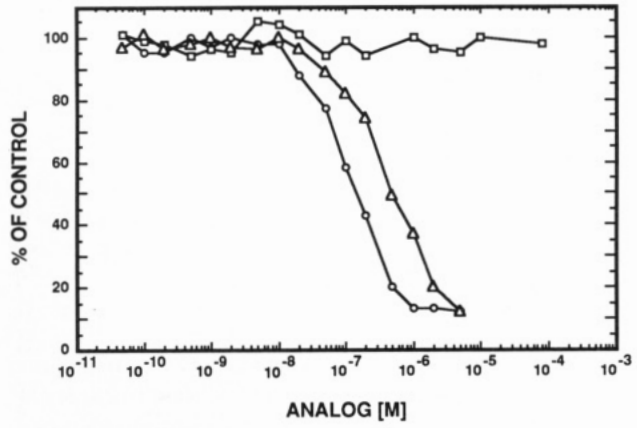

FIGURE 2: Competition of binding of [[3]H]α-factor(1–13)-OH by unlabeled [Nle[12]]α-factor(3–13)-OH and α-factor(1–11)-OH. The final assay concentrations of the competing peptides are indicated in the figure. The curves represent [[3]H]α-factor(1–13)-OH plus (○) [Nle[12]]α-factor(1–13)-OH, (△) [Nle[12]]α-factor(3–13)-OH, or (□) α-factor(1–11)-OH.

to the peptide at the restrictive temperature (data not shown).

We investigated the effect of the truncated peptides on the activity of [Nle[12]]α-factor(1–13)-OH using a growth arrest assay (Figure 1). Although [Nle[12]]α-factor(3–13)-OH did not inhibit growth of *S. cerevisiae* RC629, it reversed growth arrest by [Nle[12]]α-factor(1–13)-OH. This is clearly indicated by the bean-shaped halos elicited by [Nle[12]]α-factor(1–13)-OH proximal to WLQLKPGQP(Nle)Y on the agar plate. The conclusion that WLQLKPGQP(Nle)Y is an antagonist is supported by the fact that this analogue effectively competed with radioactively labeled α-factor(1–13)-OH for receptor binding (Figure 2).

In the presence of WHWLQLKPGQP, the halo due to α-factor(1–13)-OH was elongated toward the undecapeptide-containing disc (Figure 1). This elongation indicates that the analogue lacking the two carboxyl-terminal residues increased α-factor(1–13)-OH activity. This increase in activity was pheromone specific as no effect, either antagonism or synergism, was seen with α-factor(1–11)-OH on a-factor activity on a *MATα* target cell. The potentiation of pheromonal activity is also seen in the shmoo assay (Figure 3). In the presence of 10 μg/mL WHWLQLKPGQP, a concentration at which the undecapeptide had no effect on cellular morphology, the dose response of strain RC629 to α-factor(1–

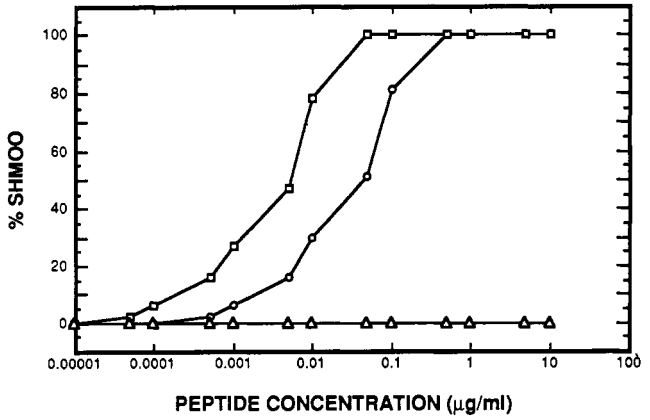

FIGURE 3: Effects of [Nle[12]]α-factor(1–13)-OH on cellular morphology of *S. cerevisiae* RC629 in the presence or absence of α-factor(1–11)-OH. The symbols represent [Nle[12]]α-factor(1–13)-OH only (○) at the indicated concentrations, [Nle[12]]α-factor(1–13)-OH at the indicated concentrations and α-factor(1–11)-OH at 10 μg/mL (□), and α-factor(1–11)-OH alone (△) at the indicated concentrations.

Table V: α-Factor(1–13)-OH Hydrolysis by Wild-Type (2180-1A) and *sst1* (RC629) Strains of *S. cerevisiae*[a]

	% of peak area (2180-1A)		% of peak area [RC629 (*sst1*)]	
time (h)	α-factor-(1–13)-OH	degradation product	α-factor-(1–13)-OH	degradation product
0	100	0	100	0
2	53	47	100	0
4	0	100	100	0
8	0	100	100	0

[a] α-Factor(1–13)-OH was incubated with *S. cerevisiae* 2180-1A or RC629 for various times as indicated. Portions of the culture were centrifuged, and the supernatant was analyzed by HPLC.

13)-OH shifts by nearly one order of magnitude. A similar potentiation of α-factor(1–13)-OH activity was seen when 1 μg/mL of WHWLQLKPGQP was used (data not shown). Synergistic activity was also observed using a truncated version [cyclo[7,10]α-factor(1–11)-OH] derived from cyclo[7,10][Nle[12]]α-factor(1–13)-OH, which we have previously shown to be an α-factor(1–13)-OH agonist (Xue et al., 1989). Furthermore, in contrast to the antagonist[WLQLKPGQP(Nle)Y, up to 10^{-4} M of the synergist(WHWLQLKPGQP) had no effect on α-factor(1–13)-OH binding (Figure 2). Neither HWLQLKPGQP nor WHWLQL had any antagonistic or synergistic activity with any strain in the halo or shmoo assay.

Effect of Synergist on α-Factor(1–13)-OH Degradation. The potentiation we observed could result from a variety of molecular interactions. The synergist could compete with degradation of α-factor(1–13)-OH, leading to an apparent increase in α-factor(1–13)-OH activity. Indeed, α-factor(1–13)-OH is cleaved by a secreted protease which is the product of the *BAR1* gene expressed in *MAT*a cells (Mackay et al., 1988; Sprague & Herskowitz, 1981). However, strain RC629, in which potentiation is exhibited, is deficient in this protease (Chan & Otte, 1982). To quantitate the protease deficiency in strain RC629, direct peptidase assays were carried out using HPLC analysis of α-factor(1–13)-OH and its degradation products. In a 4-h time period using 10^6 cells/mL, no α-factor(1–13)-OH was cleaved by RC629 whereas 100% of the pheromone was cleaved by an identical amount of 2180-1A cells (Table V).

Effect of Synergist on α-Factor(1–13)-OH Steady-State Saturation Binding. To determine whether the affinity of α-factor(1–13)-OH for its receptor was affected by the synergist(WHWLQLKPGQP), we performed a steady-state saturation binding assay. The binding of [^{3}H]α-factor(1–13)-OH was assayed either alone or in the presence of two different (but constant) concentrations of the WHWLQLKPGQP undecapeptide. As can be seen in Figure 4, the three curves showing the specific binding of [^{3}H]α-factor(1–13)-OH to its receptor are identical within experimental error. Thus, these results indicate that the affinity of the receptor for α-factor(1–13)-OH is not influenced by the undecapeptide under the conditions of this experiment.

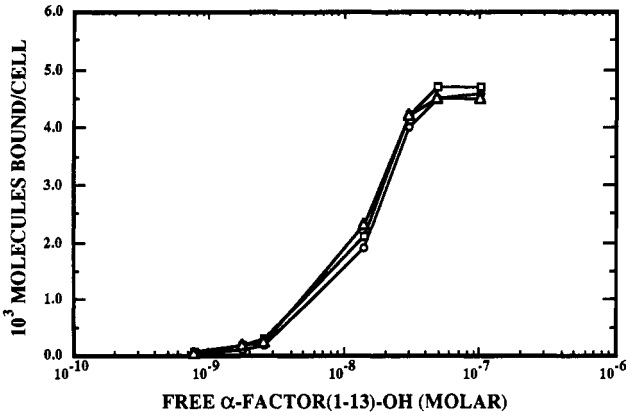

FIGURE 4: Steady-state saturation binding of [^{3}H]α-factor(1–13)-OH to RC629 cells in the presence or absence of α-factor(1–11)-OH. [^{3}H]α-Factor(1–13)-OH was used at the concentrations indicated without (○) α-factor(1–11)-OH or with 7×10^{-6} M analogue (△) or 1.4×10^{-6} M analogue (□).

Discussion

The activities found for the truncated analogues are consistent with results previously reported by Masui et al. (1977). However, a more detailed analysis of molecules in which two amino acid residues were removed from either end of α-factor has led to interesting and unexpected results. The amino-terminal truncated peptide was an antagonist of α-factor activity. The carboxyl-terminal truncated peptide, on the other hand, potentiated α-factor activity. It is interesting that residues whose removal leads to antagonism are separable from those residues whose removal results in synergism. It appears that binding and signal transduction are separable processes in the interaction between pheromone and receptor. A similar separation between binding and signal transduction has been noted recently using various analogues of glucagon (Unson et al., 1991).

Previous structure–activity studies showed that in des-Trp[1] analogues [α-factor(2–13)-OH] replacement of Trp[3] with Ala or Phe resulted in dodecapeptide antagonists (Shenbagamurthi et al., 1983). We have observed that position 2 analogues, and in particular the stereochemistry of His, plays a role in antagonism (unpublished results). Thus, residues in positions 1, 2, and 3 seem to influence signal transduction and removal of both residues 1 and 2 leads to loss of activity. Nevertheless the amino-truncated pheromone still competes with binding and therefore appears to be recognized by the α-factor receptor binding site. The binding of WLQLKPGQP(Nle)Y indicates that the His[2] residue is not necessary for binding of the pheromone to its receptor, conflicting with the suggestion of Blumer and Thorner (1990) that ligand recognition by the receptor involves a histidine residue.

In contrast, removal of residues from the C terminus leads to an analogue in which the binding is not measurable under the conditions used in our experiments. Nevertheless, this analogue (WHWLQLKPGQP) potentiates the activity of

α-factor(1–13)-OH. Similar potentiation was found with the cyclic undecapeptide (cyclo^{7,10}[WHWLQLKPGQP]) and the synthetic dodecapeptide YWHWLQLKPGQP (unpublished results). To our knowledge these compounds represent the first examples of peptide hormone analogues which act as synergists. The specificity of this phenomenon is demonstrated by the failure of closely related peptides, e.g., HWLQLKPGQP and WHWLQL, to elicit increased α-factor(1–13)-OH potency.

In a previous study, Stotzler et al. (1977) demonstrated that HWLQL and WLQL derived from α-factor(1–13)-OH did not increase α-factor(1–13)-OH activity but increased the amount of time taken by wild-type cells to recover from α-factor(1–13)-OH addition. It appeared that these peptides interferred with the hydrolysis of α-factor(1–13)-OH by the BAR1 protease which led to an increase in the lifetime of α-factor(1–13)-OH. We used the amino-terminal hexapeptide (WHWLQL), one of the two proteolytic products of BAR1 protease action on α-factor(1–13)-OH, and showed no activity in the antagonist/synergist plate assay using strain RC629 (data not shown). Furthermore, the results shown in Table V clearly indicate no cell-mediated degradation of α-factor-(1–13)-OH by strain RC629 (BAR1 protease deficient). These data lead us to conclude that the explanation for potentiation does not involve α-factor(1–13)-OH degradation or the BAR1 protease.

The binding competition studies suggest that the synergist does not bind to the α-factor(1–13)-OH binding site on the receptor. However, it is possible that the synergist binds to the same receptor site with low affinity ($K_D > 10^{-4}$ M); thus, at the concentrations of the synergist examined, we did not detect competition. The possibility that the synergist interacts with the receptor is supported by studies using an *sst2* mutant (RC631). This strain is highly sensitive to α-factor(1–13)-OH. Interestingly, the synergist is a weak agonist when tested against RC631 (Table IV). However, using a strain with a temperature-sensitive receptor and the *sst2* mutation, we found that WHWLQLKPGQ is an agonist at the permissive temperature but has no activity at the restrictive temperature. These results suggest that the synergist interacts with the α-factor receptor of strain RC631.

Several mechanisms have been proposed to explain the signaling that occurs after binding of ligand to receptor. Limbird and Lefkowitz (1977) have spectulated that, in the presence of agonist, the conformation of the β-adrenergic receptor changes. Weiland et al. (1988) added that this conformationally altered form of the receptor is what interacts directly or indirectly with an intracellular messenger to cause activation of the hormone response pathway. In contrast, Schlessinger (1988) postulated that the epidermal growth factor (EGF) receptor forms oligomeric structures in the presence of agonist with a resulting higher affinity for ligand of oligomerized receptor than that of the monomeric receptor. Recently, EGF-induced receptor dimerization was demonstrated in cell-free systems (Kashles et al., 1991). A very similar example to that of the EGF receptor is the platelet-derived growth factor (PDGF) receptor (Kelly et al., 1991). It is possible that the synergist binds to the same site as α-factor(1–13)-OH (with very low affinity) or to some other site on the receptor, causing the formation of oligomers. Such changes in receptor state alone do not trigger any response, and so the synergist does not induce a biological response by itself. However, when α-factor(1–13)-OH is also present, the "activated receptor" leads to a greatly enhanced response. Indeed, in a study where α-factor(1–13)-OH was cross-linked to the receptor, there was some evidence for multimeric receptor on acrylamide gels (Blumer et al., 1988; Reneke et al., 1988).

Other explanations for the observed synergy are (1) post-translational processing of the *STE2* gene product resulting in more than one receptor protein subtype with different binding domains; (2) activation of cryptic receptors or binding sites in the plasma membrane, in a manner analogous to EGF receptor (Lokeshwar et al., 1989); (3) different but overlapping binding sites for synergist and α-factor(1–13)-OH as is the case of agonists and antagonists of A_1 adenosine receptors (Olah & Stiles, 1990). In any of these mechanisms, the synergist could exert its effect with the possibility of not detecting its binding to the α-factor receptor binding site.

If the observation of synergy is extended to mammalian peptide hormones, it would provide an alternate and novel approach to prepare compositions with increased therapeutic efficacy. Clearly, further studies on a variety of peptide hormones and target cells are necessary to extend these findings, to further clarify the molecular interaction between the synergist and the target cells, and to determine the therapeutic potential of synergists.

ACKNOWLEDGMENTS

We thank David Miller and Forrest Sowell for technical assistance and John Koontz for helpful discussions. FAB–mass spectrometry was carried out by Kelsey Cook and Al Tuinman of the University of Tennessee. The UTK Chemistry Mass Spectrometry Center is funded by the Science Alliance, a State of Tennessee Center of Excellence. The NSF Chemical Instrumentation Program also contributed to the acquisition of the ZAB-EQ (Grant No. CHE-86-09251). We are grateful to the Molecular Biology Resources Facility of the University of Tennessee for the amino acid analyses.

Registry No. [Nle^{12}]α-Factor(1–13)-OH, 70204-23-8; [Nle^{12}]α-factor(2–13)-OH, 137918-37-7; [Nle^{12}]α-factor(3–13)-OH, 137918-39-9; [Nle^{12}]α-factor(1–12)-OH, 137918-38-8; α-factor(1–11)-OH, 64655-25-0.

REFERENCES

Blumer, K. J., & Thorner, J. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 4363–4367.

Blumer, K. J., & Thorner, J. (1991) *Annu. Rev. Physiol. 53*, 37–57.

Blumer, K. J., Reneke, J. E., & Thorner, J. (1988) *J. Biol. Chem. 263*, 10836–10842.

Burkholder, A. C., & Hartwell, L. H. (1985) *Nucleic Acids Res. 13*, 8463–8475.

Chan, R. K., & Otte, C. A. (1982) *Mol. Cell. Biol. 2*, 11–20.

Cross, F., Hartwell, L. H., Jackson, C., & Konopka, J. B. (1988) *Annu. Rev. Cell Biol. 4*, 429–457.

Dietzel, C., & Kurjan, J. (1987) *Cell 50*, 1001–1010.

Hagen, D. C., McCaffrey, C., & Sprague, G. F., Jr. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 1418–1422.

Herskowitz, I. (1988) *Microbiol. Rev. 52*, 536–553.

Jelicks, L. A., Naider, F. R., Shenbagamurthi, P., Becker, J. M., & Broido, M. S. (1988) *Biopolymers 27*, 431–449.

Kashles, O., Yarden, Y., Fisheer, R., Ullrich, A., & Schlessinger, J. (1991) *Mol. Cell. Biol. 11*, 1454–1463.

Kelly, J. D., Haldeman, B. A., Grant, F. J., Murray, M. J., Seifert, R. A., Bowen-Pope, D. F., Cooper, J. A., & Kazlauskas, A. (1991) *J. Biol. Chem. 266*, 8987–8992.

Lefkowitz, R. J., & Caron, M. G. (1988) *J. Biol. Chem. 263*, 4993–4996.

Limbird, L. E., & Lefkowitz, R. J. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 228–232.

Lokeshwar, V. B., Huang, S. S., & Huang, J. S. (1989) *J. Biol. Chem. 264*, 19318-19326.
Marsh, L., & Herskowitz, I. (1988) in *Cold Spring Harbor Symposia on Quantitative Biology*, Vol. LIII, pp 557-565, Cold Spring Harbor, NY.
Masui, Y., Chino, N., Sakakibara, S., Tanaka, T., Murakami, T., & Kita, H. (1977) *Biochem. Biophys. Res. Commun. 78*, 534-538.
Mayo, K. H., Nunez, M., Burke, C., Starbuck, C., Lauffenburger, D., & Savage, C. C. (1989) *J. Biol. Chem. 264*, 17838-17844.
Miyajima, I., Nakafuku, M., Nakayama, N., Brenner, C., & Miyajima, A. (1987) *Cell 50*, 1011-1019.
Naider, F. R., Jelicks, L. A., Becker, J. M., & Broido, M. S. (1989) *Biopolymers 28*, 487-497.
Nakayama, N., Miyajima, A., & Arai, K. (1985) *EMBO J. 4*, 2643-2648.
Olah, M., & Stiles, G. L. (1990) *J. Neurochem. 55*, 1432-1438.
Raths, S. K., Naider, F. R., & Becker, J. M. (1988) *J. Biol. Chem. 263*, 17333-17341.
Reneke, J. E., Blumer, K. J., Courchesne, W. E., & Thorner, J. (1988) *Cell 55*, 221-234.
Schlessinger, J. (1988) *Biochemistry 27*, 3119-3123.
Shenbagamurthi, P., Baffi, R., Khan, S. A., Becker, J. M., & Naider, F. (1983) *Biochemistry 22*, 1298-1304.
Shenbagamurthi, P., Kundu, B., Raths, S., Becker, J. M., & Naider, F. (1985) *Biochemistry 24*, 7070-7076.
Sprague, G. F., & Herskowitz, I. (1981) *J. Mol. Biol. 153*, 305-321.
Stotzler, D., Betz, R., & Duntze, W. (1977) *J. Bacteriol. 132*, 28-35.
Unson, C. G., MacDonald, D., Ray, K., Durrah, T. L., & Merrifield, R. B. (1991) *J. Biol. Chem. 266*, 2763-2766.
Weiland, G. A., Minneman, K. P., & Molinoff, P. B. (1988) *Nature 281*, 114-117.
Whiteway, M., Hougan, L., Dignard, D., Bell, L., Saari, G., Grant, F., O'Hara, P., & MacKay, V. L. (1989) *Cell 56*, 467-477.
Xue, C.-B., Bargiota, E. E., Miller, D., Becker, J. M., & Naider, F. (1989) *J. Biol. Chem. 264*, 19161-19168.

Interactions of Acyl-Coenzyme A with Phosphatidylcholine Bilayers and Serum Albumin†

John G. Boylan* and James A. Hamilton
Biophysics Department, Housman Medical Research Center, Boston University School of Medicine, 80 East Concord Street, Boston, Massachusetts 02118-2394

Received June 28, 1991; Revised Manuscript Received October 2, 1991

ABSTRACT: Interactions of oleoyl- and octanoyl-coenzyme A (CoA) with phosphatidylcholine (PC) vesicles and bovine serum albumin (BSA) were investigated by NMR spectroscopy. Binding of acyl-CoA to small unilamellar PC vesicles and to BSA was detected by changes in ^{13}C and ^{31}P chemical shifts relative to the chemical shifts for aqueous acyl-CoA. When oleoyl-CoA (≤15 mol %) was added to preformed vesicles, the ^{13}C thioester signal (200.1 ppm) was upfield from the signal for micellar oleoyl-CoA (201.7 ppm), suggesting decreased H-bonding (partial dehydration) at the carbonyl group upon binding to the bilayer. When vesicles were prepared by cosonication of oleoyl-CoA and PC, a second peak (199.8 ppm) was seen. The major peak at 200.1 ppm broadened and shifted after addition of $Dy(NO_3)_3$ and was not seen after addition of BSA, while the peak at 199.8 ppm was unaffected by either perturbation. Thus, oleoyl-CoA in each bilayer leaflet was distinguished, and transbilayer movement was shown to be slow ($t_{1/2} \geq$ hours). PC vesicles remained intact with ≤15 mol % oleoyl-CoA, while higher oleoyl-CoA proportions produced mixed micelles. In contrast, ^{13}C spectra revealed rapid exchange (ms) of octanoyl-CoA between the aqueous phase and PC vesicles and a low affinity for the bilayer. Thus, the binding affinity of acyl-CoA for PC bilayers is dependent on the acyl chain length. Oleoyl-CoA in the presence of BSA (1 mol/mol) gave rise to three carbonyl signals at 197.2-203.6 ppm. With 2-5 mol of oleoyl-CoA/BSA, 1-2 additional signals were observed. None of the signals corresponded to unbound oleoyl-CoA. Addition of [^{13}C]carboxyl-enriched oleic acid to oleoyl-CoA/BSA mixtures revealed simultaneous binding of oleic acid and oleoyl-CoA to BSA, with some perturbation of binding interactions. Thus, BSA contains multiple binding sites for oleoyl-CoA and can bind fatty acid and acyl-CoA simultaneously.

Fatty acid thioesters of coenzyme A (acyl-CoA)[1] are important intermediates in fatty acid metabolism and lipid biosynthesis. They are powerful inhibitors of several enzymes (Bortz & Lynen, 1963a; Caggiano & Powell, 1979) and may play an important regulatory role in a variety of biochemical processes, such as adenine nucleotide transport across mitochondrial membranes (Shug & Shrago, 1973). Their regulatory effects appear to be independent of their detergent-like properties, since many enzymes are inhibited by acyl-CoA below the critical micelle concentration (CMC). Furthermore,

† Supported by Grants HL26335 and HL07224 from National Institutes of Health.
* To whom correspondence should be addressed.

[1] Abbreviations: BSA, bovine serum albumin; CMC, critical micelle concentration; CoA, coenzyme A; EM, electron microscopy; NMR, nuclear magnetic resonance (spectroscopy); NOE, nuclear Overhauser enhancement; PC, phosphatidylcholine; THF, tetrahydrofuran; TLC, thin layer chromatography.

0006-2960/92/0431-557$03.00/0