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Different Structure-Function Relationships for α-Factor-Induced Morphogenesis and Agglutination in *Saccharomyces cerevisiae*

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Eight synthetic analogs of the mating pheromone α-factor-induced morphogenesis and increased agglutinability in **a** cells. Most analogs induced increased agglutinability at lower concentrations than those at which they induced morphogenesis, but the ratio of the potencies for the two effects varied 140-fold among different analogs. Morphological response to pheromone required exposure for at least 90 min, but increased agglutinability followed exposures of 20 s. Two synthetic analogs induced neither response. In competition experiments, both of these analogs prevented induction of increased agglutinability and morphogenesis by active α factor. The inactive peptides blocked increased agglutinability at lower concentrations than those at which they blocked morphogenesis. α factors exhibited different structure-function relationships for morphogenesis as compared with agglutinability. Thus, response of *Saccharomyces cerevisiae* to α factor is complex and may be mediated by more than one receptor.

In *Saccharomyces cerevisiae*, the transition from haploid to diploid occurs through cell fusion between individual cells of opposite mating type (reviewed in reference 22). The mating types are called **a** and α, and cells of each mating type secrete peptide pheromones which induce pre-mating events in cells of the opposite mating type. α factor is the pheromone secreted by α cells. **a** cells treated with α factor show increased sexual agglutinability (2, 7, 21), inhibition of initiation of DNA synthesis (23), and an altered morphology (9, 10). We will use the term "morphogenesis" as a succinct description of the pheromonal induction of altered morphology. The morphogenesis appears to be a result of continued cell growth and synthesis of a cell wall primed for mating (10, 24). Although the physiological role of morphogenesis in mating is not known, the response is a consistent result of challenge by pheromone. Morphogenesis therefore has been used as a common assay for the effects of α factor (6, 9–13, 16, 17, 19, 22, 24). The pheromone-induced increase in agglutinability is apparent within 15 min of pheromone treatment and is due to greater cell surface expression of the agglutinating glycoproteins (15, 21). Conversely, **a** cells secrete **a** factor, which induces similar changes in α cells (1, 3).

α factor can be isolated from culture medium in which α cells have been grown, and consists of a mixture of four peptides based on the tridecapeptide sequence Trp-His-Trp-Leu-Gln-Leu-Lys-Pro-Gly-Gln-Pro-Met-Tyr (19). The four forms differ in the presence of the N-terminal Trp and in the oxidation state of the Met residue. Stotzler and Duntze (19) reported that the unoxidized form of the tridecapeptide was twice as potent as the corresponding dodecapeptide in inducing morphogenesis. The oxidized forms were less active.

Four groups have synthesized α factors. Ciejek et al. (6) reported that synthetic tridecapeptide and dodecapeptide were active in the range of 500 to 1,000 ng/ml. However, they noted that this activity was ca. 10% of that of the natural products and suggested that synthetic contaminants were acting as antagonists. Samokhin et al. (16) synthesized tridecapeptide active at 85 ng/ml. Masui et al. (12, 13) claimed activity in the picogram-per-milliliter range, but the claim remains unsubstantiated. Recently, we reported synthesis and activity of a number of analogs (8, 17). We have found that the tridecapeptide and dodecapeptide are not equipotent, but are active around 50 and 800 ng/ml, respectively (17).

The availability of homogeneous synthetic peptides and of semiquantitative bioassays allowed us to carry out a detailed analysis of response of **a** cells to these pheromones. In this article, we report the effect of amino acid substitution on both agglutination and morphological responses. Our results are consistent with multiple receptors for the α-factor peptide.

MATERIALS AND METHODS

Strains. Haploid strains X2180-1A (*MAT***a**) and X2180-1B (*MAT*α) were grown in minimal medium containing (per liter) 2.2 g of yeast nitrogen base (Difco Laboratories), 4.5 g of $(NH_4)_2SO_4$, and 20 g of glucose. All cells were grown at 25°C to mid-log phase.

α factors. Natural α factor was isolated by the method of Strazdis and MacKay (20) from 100-liter cultures of X2180-1B. Activity was determined by the morphogenesis assay as previously described (17). The lowest concentration inducing morphogenesis was defined as 1 U/ml (21). Preparation of the synthetic analogs has been detailed previously (8, 17). All synthetic peptides had the expected amino acid analysis and were chromatographically pure by high-pressure liquid chromatography in two systems (17). Concentrations were based on dry weight after lyophilization.

The analogs were named according to IUPAC conventions and are denoted by the substitutions in the natural sequence. Residue positions are numbered according to the tridecapeptide sequence. Nonstandard abbreviations are: Dns, diaminonaphythyl-sulfonyl; Nle, norleucyl; Cha, cyclohexylalanyl;

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TABLE 1. Potency of α-factors

α factor	No.	Potency[a]		
		Morphogenesis (mg/ml)	Agglutination (ng/ml)	Ratio
Natural sequence tridecapeptide[b]	1	70 (21)	0.5 (2)	140
des Trp^1	2	720 (12)	8 (2)	90
des Trp^1,Cha^3	3	270 (12)	35 (2)	8
des Trp^1,α-DnsHis^2,Cha^3	4	700 (5)	120 (4)	6
des Trp^1,Cha^3,Lys^7 (Bio)	5	3,100 (5)	1,300 (2)	2
des Trp^1,Cha^3,Nle^7	6	2,700 (5)	1,500 (2)	2
des Trp^1,Cha^3,Orn^7	7	2,800 (4)	90 (3)	30
des Trp^1,Cha^3,Lys^7 (Ac)	8	900 (4)	1,300 (3)	1
des Trp^1,Lys^7 (α-DnsGly)	9	800 (5)	300 (3)	3
Isolated from X2180-1B		1 U/ml[c]	0.015 U/ml (9)	65

[a] Shown as the minimum concentration for induction of morphogenesis and the concentration inducing a half-maximal increase in agglutinability. Each number in parentheses is the number of independent determinations. Each determination included duplicate samples for morphogenesis and quadruplicate samples for agglutination.

[b] The natural sequence tridecapeptide is Trp^1-His^2-Trp^3-Leu^4-Gln^5-Leu^6-Lys^7-Pro^8-Gly^9-Gln^{10}-Pro^{11}-Met^{12}-Try^{13}. des Trp^1,Cha^3 denotes His^2-Cha^3-Leu^4-Gln^5-Leu^6-Lys^7-Pro^8-Gly^9-Gln^{10}-Pro^{11}-Met^{12}-Tyr^{13}.

[c] This concentration (1 U/ml) is the minimum concentration inducing morphogenesis.

Orn, ornithyl; Bio, biotinyl; Dns-Gly, *N*-α-diaminonaphthyl sulfonyl-glycyl.

Bioassays. The morphogenesis assay was carried out in microtiter plates, each well containing 100 μl of medium, 3×10^2 **a** cells, and twofold serial dilutions of α-factor analog. The lowest concentration of α factor causing visible morphogenesis after a 4-h incubation was determined. Because the assay was a serial dilution assay, mean and standard deviation were determined for the logarithms of the lowest active concentrations. The reported values (see Table 1) are the antilog of the mean log concentration for 4 to 21 separate determinations on each peptide.

The agglutination potency was determined by the cocentrifugation assay of Terrance and Lipke, in which induction of agglutinability is separated from the agglutination assay itself (21). **a** cells were incubated with 10-fold serial dilutions of the α-factor analogs at a cell density of 2×10^7 per ml. After a 25-min incubation, the cells were washed into agglutination buffer (0.1 M sodium acetate [pH 5], 10 μg of cycloheximide per ml). This buffer inhibits induction of agglutinability by α factor and induction within agglutinated cell pellets (21; P. N. Lipke, unpublished data). The **a** cells were then mixed with α cells, and agglutination was determined in quadruplicate. The concentration inducing an increase in agglutinability half that induced by 1 U of biological α factor per ml was determined from interpolation of dose-response curves. Two to four separate experiments were performed for each analog, and the results were averaged as logarithms. In both morphogenesis and agglutination assays, standard errors of the means were calculated from the logarithms of the mean potencies. The standard errors (see Fig. 2) are plotted as logarithms.

RESULTS

Morphogenic activity of α-factor analogs. Morphogenic activities of various α-factor analogs are listed in Table 1. Although individual assays were accurate only to a factor of 2, activities were more reliably determined by estimating the mean and standard error from multiple assays. The synthetic peptides induced morphogenesis at minimum concentrations ranging from 70 ng/ml to 3.1 μg/ml. The synthetic tridecapeptide was the most potent pheromone by a factor of 4. Standard errors for the determinations are shown in Fig. 2.

Induction of agglutinability. All morphogenic analogs of α factor also induced increased agglutinability of the **a** cells. Representative dose-response curves are shown in Fig. 1. Standard errors and ranges are shown in Fig. 2.

The peptides induced half-maximal agglutinability over a wide range of concentrations (Table 1). The synthetic tridecapeptide was 16-fold more potent than the next most active peptide. The potencies were not correlated with morphogenic potencies. The ratio of morphogenic dose to the dose-inducing agglutinability varied by a factor of 140 among the tested analogs. The precision of the assays was such that ratio differences of a factor of 2 or less were probably not significant. Most analogs were more potent for agglutination, but the des Trp^1,Cha^3,Lys^7 (Ac)-peptide was approximately equipotent in the two assays. α factor isolated from culture medium had a potency ratio of 65, which was similar to those of synthetic tridecapeptide and dodecapeptide.

A logarithmic plot of the potencies (Fig. 2) showed that most of the modifications reduced potencies in both assays. Such reduction corresponds to movement up and to the right when a parent structure (such as des Trp^1,Cha^3 dodecapeptide, point 3) is compared with its analog (such as des Trp^1,Cha^3,Nle^7 dodecapeptide, point 6). However, substitution of Lys(Dns-Gly) (compound 9) for Lys^7 (compound 2) in the des Trp^1 analog decreased agglutination potency by 1.5 orders of magnitude without affecting potency for morphogenesis. In addition, substitution of Cha for Trp^3 in the des Trp^1 series resulted in a decrease in agglutination potency but an increase in morphogenic potency (compound 3 versus compound 2).

Competition by inactive analogs. The des Trp^1,Phe^3 and des Trp^1,Ala^3 peptides fail to induce morphogenesis at concentrations up to 500 μg/ml (17). However, both inactive analogs block induction of morphogenesis by natural α factor, the synthetic tridecapeptide, and the des Trp^1,Cha^3 analog (17). We believe that such blocking is caused by competition of the inactive analog for pheromone binding

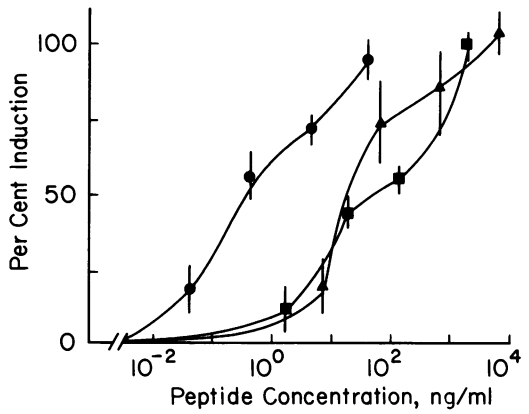

FIG. 1. Induction of increased agglutinability. **a** cells were treated with α factor for 30 min at 25°C in complete medium. Percent maximal induction was determined as the difference in optical density between α-factor-treated and -untreated cells divided by the difference in optical density between cells treated with 1 U of natural α factor per ml and untreated cells (21). Error bars represent standard error for quadruplicate tubes. Symbols: ●, tridecapeptide; ▲, des Trp^1 dodecapeptide; ■, des Trp^1,Cha^3 dodecapeptide.

sites on α-factor receptors. The des Trp[1],Phe[3] and des Trp[1],Ala[3] analogs also failed to induce increased agglutinability at concentrations up to 50 μg/ml. Both inactive analogs prevented induction of increased agglutinability by the des Trp[1],Cha[3] peptide (Table 2). Both analogs were able to block agglutinability increase at concentrations that were 5- to 15-fold lower than those blocking morphogenesis. The difference in blocking effect was observed at two concentrations of active pheromone.

Time of induction. Although induction of increased agglutinability requires 20 min (15, 21) and induction of morphogenesis requires 3 to 4 h (10, 22), it is not known whether continuous exposure to α factor is necessary to induce increased agglutination. We therefore determined the time of exposure to natural α factor required for induction of increased agglutinability and morphogenesis. a cells (X2180-1A) were treated with α factor for specified times, filtered, washed, suspended in fresh medium without α factor, and reincubated. Agglutinability was determined at 20 min, which is sufficient for full induction (21). Morphogenesis was observed at 4 h of incubation. The doubling time of untreated cells under the conditions of this experiment was 2.5 h. Although increased agglutinability followed exposures to α factor for 20 s or more, induction of morphogenesis required 120 min of exposure to α factor (Table 3). At each tested time, all cells of the population responded similarly. There was no visible morphogenesis of any cells at exposure times of 60 min or less. Exposures of 120 min or more resulted in morphogenesis of all cells. The morphology was dependent on the duration of exposure. Results were similar for natural α factor and the synthetic tridecapeptide.

DISCUSSION

The various mating factors exhibited markedly different structure-function relationships for morphogenesis as compared with agglutination. The effects of amino acid replacement on morphogenesis were discussed previously (17) and are summarized in Table 1. In every case we examined, substitution for a naturally occurring residue in the primary sequence of α factor resulted in a decrease in potency in the agglutination assay. The magnitude of the effect was dependent, however, on the nature of the replacement. Whereas replacing Trp[3] with Phe or Ala resulted in total loss of agglutination potency (>10^4-fold decrease), insertion of Cha in this position decreased activity by a factor of 4. This finding suggests that either a minimum hydrophobicity or steric bulk is required in the 3-position side chain. Moreover, the des Trp[1],Cha[3] dodecapeptide was inferior to the des Trp[1] peptide in induction of agglutinability, but was a threefold-better inducer of morphogenesis. Although the charge on Lys[7] was not essential for induction of agglutinability, it did influence the potency of the α factor, as the des Trp[1],Cha[3],Nle[7] and des Trp[1],Cha[3],Lys[7](Ac) dodecapeptides had a 40-fold-decreased potency. Shortening the side chain while retaining the charge (des Trp[1],Orn[7] analog) decreased potency 2.6-fold. Modification of the Lys side chain usually resulted in smaller effects on morphogenic potency. Finally, removal of the N-terminal Trp or derivitization of the α amine of His in the dodecapeptide had similar influences on induction of morphogenesis and agglutinability.

TABLE 2. Competition of des Trp[1],Cha[3] dodecapeptide by inactive analogs[a]

Competitor	Concn of des Trp[1],Cha[3] (ng/ml)	Minimum Concn (ng/ml) of competitor preventing induction	
		Morphogenesis	Agglutination
des Trp[1],Ala[3]	250	7,500	1,250
	2,500	60,000	7,500
des Trp[1],Phe[3]	250	3,750	250
	2,500	15,000	2,500

[a] a cells were incubated with des Trp[1],Cha[3] dodecapeptide at 250 or 2,500 ng/ml in the presence of twofold serial dilutions of the competitors. After incubation, the cells were assayed for morphogenesis and for increased agglutination, using procedures described in the text. The competitors had no effect on the constitutive agglutinability of a cells at concentrations up to 25 μg/ml (des Trp[1],Phe[3] peptide) and 75 μg/ml (des Trp[1],Ala[3] peptide). The des Trp[1],Phe[3] peptide (20 μg/ml) had no effect on agglutinability of a cells induced with natural α factor.

The different potency ratios reflected the different structure-function relationships for α-factor-induced morphogenesis compared with agglutination. If there were a single cellular response leading to diverse physiological changes in the cell, then all the potency ratios would be similar and all the points in Fig. 2 would lie on a straight line of slope 1. No line could be constructed that fell within three standard errors of more than 6 points in Fig. 2. The lack of correlation implied the action of two or more α-factor receptors, or at least two responses of a single receptor.

The different structure-function relationships have implications for the mechanisms of cellular response to α factor. There remains, however, the problem that the role of morphogenesis is unknown. Although inhibitors of cell wall synthesis also inhibit morphogenesis and mating (10, 18), it is not clear whether responses leading to morphogenesis are essential to mating. On the other hand, there is evidence that cell cycle arrest is physiologically important (22, 23), and that G1 arrest shares characteristics of induction of agglutinability (14). Until we can separate the various responses to α factor and test the role of each in sexual conjugation, we will not be able to determine how they contribute to mating in *S. cerevisiae*.

We observed conditions under which either morphogenesis or agglutination could be induced without the other. For many of the peptides, concentrations substantially less than morphogenic doses induced increased agglutinability. Brief exposure to α factor also resulted in increased agglutinability but not morphogenesis. Conversely, exposure to the des

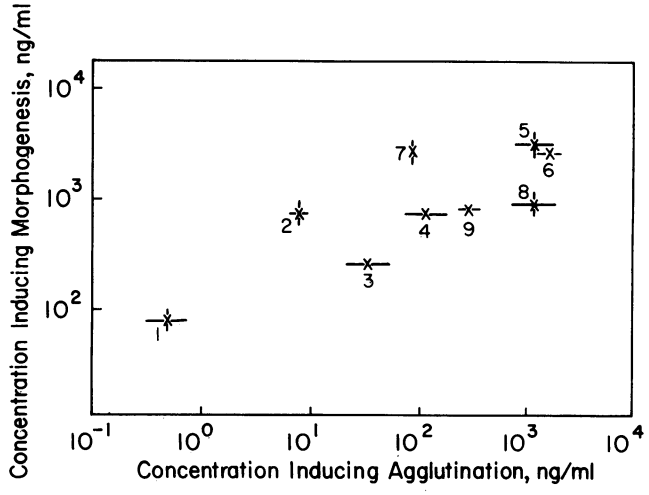

FIG. 2. Potencies of α factors. Each active analog is plotted with standard errors for each assay. When $n = 2$, range is plotted. Errors not shown are smaller than symbols. Numbers correspond to those of Table 1.

TABLE 3. Time of exposure to α factor necessary to induce responses

Time of exposure (min)	Agglutinability[a] (% induction)	Morphogenesis[b]
0	0	None
0.3	69	None
1	86	None
2	92	None
5	78	None
10	94	None
15	92	None
20	100	None
40	ND[c]	None
60	ND	None
90	ND	Poor
120	ND	Fair
180	ND	Good
210	ND	Good
240	ND	Very good

[a] a cells were incubated with 1 U of natural α factor per ml for the indicated time, washed into fresh medium, and incubated for 20 min. At 20 min, all cells were agglutinated with α cells. Percent induction was determined as described in the legend to Fig. 1.

[b] a cells were incubated with 20 U of α factor per ml for the indicated time, washed into fresh medium, and reincubated. Morphology was inspected at 240 min. Descriptions: "none," normal vegetative morphology; "poor," budding inhibition in all cells; "fair," budding inhibition and slight alteration of cell shape; "good," budding inhibition and gross distortion of some cells; "very good," most cells grossly distorted.

[c] ND, Not determined.

Trp^1,Cha^3 analog in the presence of suitable concentrations of either des Trp^1,Phe^3 or des Trp^1,Ala^3 dodecapeptide resulted in morphogenesis without increased agglutinability. For example, at a des Trp^1,Cha^3 dodecapeptide concentration of 250 ng/ml, 1,250 ng of the des Trp^1,Ala^3 analog per ml prevented increased agglutinability but did not affect morphogenesis. Several models for pheromone action are not supported by the observation of morphogenesis without increased agglutinability. Thorner (22) has proposed that the effects of α factor are mediated through a drop in the intracellular concentration of cyclic AMP. Such a model would predict that increased agglutinability would result from a change in cyclic AMP concentration or a short duration of the effect. Morphogenesis would result from more marked or longer changes in cyclic AMP concentrations. If both responses resulted from the same metabolic effector, there could be no morphogenesis without increased agglutinability. Our results are not consistent with any model in which a single cellular response results from binding of α factor to its receptor. It might be argued that the pheromone triggers a variety of secondary messengers upon interaction with a single receptor. For example, amino acid residues in different positions of the α factor could induce release of effectors that lead to various responses. It would appear, however, that the competition experiments also eliminate this hypothesis, unless one proposes that the competing peptides displace only part of the active pheromone from the receptor. We believe that such partial displacement is unlikely, given the similar structures of the active pheromone and the antagonists, and the fact that the antagonists are inactive in both the morphogenesis and agglutination assays.

Another model proposes that morphogenesis results from exposure of cells to pheromone at a specific point of the cell cycle. In an asynchronous population, some cells pass through each point of the cell cycle during any exposure to pheromone. Those cells passing through the critical point should then undergo morphogenesis. However, no cells responded morphologically after exposures of less than 120 min (Table 3). Since changes of shape were not observed in any cells, the proposal that morphogenesis results from pheromone exposure at a specific point of the cell cycle is not consistent with our findings.

Differential destruction of the analogs by the cells might mimic the effects of a second response mechanism. Since the agglutination response required very short exposure times, this response might be indicative of receptor affinity. Morphogenesis would then be sensitive to receptor affinity and pheromone longevity (4, 5). Such a model cannot easily accommodate the observation of induction of morphogenesis without increased agglutinability, since morphogenesis is induced after much longer exposures than those required for increased agglutinability. Moreover, our morphogenesis determinations were carried out at low cell density (3×10^3 cells per ml). Using the equation presented by Moore (14), we calculated that 99.5% of the natural sequence tridecapeptide α factor would remain in the medium after the 4-h incubation used in the morphogenesis assay. Furthermore, studies in our laboratories indicate that all dodecapeptides are degraded more slowly than the natural sequence tridecapeptide and that there is no correlation between the ratio of morphogenic to agglutination-induction potencies for an α-factor analog and its rate of degradation (J. Becker and F. Naider, unpublished data). Thus, there is no evidence that morphogenic potency is correlated with pheromone longevity under the assay conditions we used. Neither differential destruction of pheromone nor cellular desensitization (14, 22) can explain the differential potencies of the various α factors.

The metabolic response to α factor obviously diverges to lead to induction of the various cellular effects. Our results suggest that the divergence of the morphogenic and agglutination pathways is very early in the sequence, probably at the level of the α-factor receptors. We have been unable to accommodate our data within a model containing a single primary receptor response to the binding of α factor. Thus, two of the cellular effects of α factor appear to be mediated by different response mechanisms. Using an elegant and detailed kinetic assay, Moore (14) recently showed that the natural α factor causes half-maximal cell division arrest and induces increased agglutinability at similar concentrations (10^{-10} M), whereas morphogenesis is maximal at 10^{-8} M. She speculated that these findings are consistent either with different receptors for morphogenesis and agglutination induction or with a higher receptor occupancy required for morphogenesis. Our results extend the findings of Moore and support the hypothesis that more than one class of receptor for α factor is present in *S. cerevisiae* **a** cells. The observation that morphogenesis can be induced without increased agglutinability rules out the possibility that morphogenesis is due to increased receptor occupancy.

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