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Binding of Rhodotorucine A, a Lipopeptidyl Mating Hormone, to a Cells of Rhodosporidium toruloides for Induction of Sexual Differentiation

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Rhodotorucine A, a lipopeptidyl mating hormone, selectively bound to the cells of Rhodosporidium toruloides M-1057, mating type a, having a large number of hormone-binding site, approximately 5 X 10^7 molecules per cell. The binding is temperature-independent. The bound hormone is sensitive to pronase, while the binding site is resistant to the protease. The cells,which were preincubated with the hormone at 25°C for 10 min (10-min hormone-preincubation), completely lost the mating tube-forming ability by pronase treatment, while the cells of 15-min hormone-preincubation remained the ability after the treatment. Thus, it is concluded that the establishment of intracellular information inducing sexual differentiation depends on the term of preincubation, such as 15 min, with the hormone at 25°C.

Our previous papers (1-12) described the biological and chemical aspects on induction of sexual differentiation in life cycle of heterobasidiomycetous yeasts; Rhodosporidium toruloides and Tremella mesenterica. For example, we discovered two mating hormones, rhodotorucine A and a, which are essentially required for transition from haplophase to diplophase in life cycle of Rh. toruloides (dipolar in compatibility; mating type A or a in haplophase). Rhodotorucine A and a are secreted in culture media of haploid cells of mating types A and a (A and a cells), and arrest a and A cells in G_1 phase of cell division cycle, respectively, followed by mating tube formation, a parameter of sexual differentiation (1,2). The transition of ploidy is achieved by conjugation leading to the sequential events for karyogamy (2,11,12). Recently, rhodotorucine A was isolated from culture medium of A cells (Rh. toruloides M-919) and its chemical structure was confirmed to be H-Tyr-Pro-Glu-

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Ile-Ser-Trp-Thr-Arg-Asn-Gly-Cys(S-farnesyl)-OH, a novel lipopeptide (3-7). Furthermore, tremerogen A-10, which was produced by the cells of Tremella mesenterica IFO-9310 (mating type AB, tetrapolar in compatibility), was also isolated and confirmed to be another novel lipopeptide having a chemical structure, H-Glu-His-Asp-Pro-Ser-Ala-Pro-Gly-Asn-Gly-Tyr-Cys(S-alkyl)-OR. The structure of lipophilic (alkyl) moiety of tremerogen A-10 is still unknown.(8-10).

On the other hand, we are interested in the primary reaction or intracellular information for induction of sexual differentiation caused by the mating hormone. In this paper, we describe the biological significance of rhodotorucine A-binding to a cells.

Materials and Methods

Microorganisms, media and cultivation. Microorganisms used were Rhodosporidium toruloides M-919 (A cells) and M-1057 (a cells) (13). These were obtained from Institute of Fermentation, Osaka. GYP medium had a composition, yeast extracts (Daigo Eiyo, Osaka) 0.4%, peptone (Daigo Eiyo, Osaka) 0.5%, glucose 2.0%, KH_2PO_4 0.1% and $MgSO_4 7H_2O$ 0.05%. GP medium was composed of glycerol 2.0%, peptone 0.7%, KH_2PO_4 0.1%, $MgSO_4 7H_2O$ 0.05% and vitamin mixture 0.1 ml per 100 ml. Vitamin mixture contained biotin 2 mg, Ca-pantothenate 400 mg, inositol 2 g, thiamine-HCl 400 mg, pyridoxine-HCl 400 mg, nicotinic acid 400 mg, p-aminobenzoic acid 200 mg, and riboflavin 200 mg in 1 liter. Cultivation was carried out on rotary shaker at 28°C.

Preparation of rhodotorucine A. In this paper, partially purified rhodotorucine A was prepared and used. To the 19-hr culture filtrate (20 l) of A cells grown in GP medium was added solid ammonium sulfate to give an 0.8 saturation, followed by standing over night at 4°C. The precipitate thus formed was collected by filtration with celite and dissolved in 20 mM Na,K-phosphate buffer (400 ml), pH 6.0. Rhodotorucine A was extracted twice with each 100 ml of n-butanol at room temperature. The n-butanol fractions were combined and washed twice with each 100 ml of distilled water. n-Butanol was removed by evaporation under reduced pressure at 40°C from the n-butanol fraction. Before dryness, rhodotorucine A was dissolved in 20 mM Na,K-p-osphate buffer (approximately 10 ml), pH 7.0, and used.

Assay of rhodotorucine A. Determination of the amount of rhodotorucine A was carried out with serialy diluted samples by biological method described previously (3) with some modification. To 1 ml of GYP medium containing 0.1 ml of the sample, should be assayed, were immersed two pieces of agar film (1x5x5 mm^3), in which approximately 10^3 of fresh a cells (mid-log growth phase in YPG medium) were immobilized, followed by incubation at 25°C for 5 hr. Then, alteration in cell morphology (bud or mating tube formation) of a cells in the film was observed under microscope. One unit of the mating hormone is defined the amount capable of the induction of mating tube formation in 50% of assay cells in the film.

Results and Discussion

Binding of rhodotorucine A to a cells

A 1.0 ml amount of cell suspension (20 mM Na,K-phosphate buffer, pH 7.0) containing 5 X 10^6 a cells was incubated with 2,600 units of rhodotorucine A at indicated temperatures for 10 min, then the suspension was immediately chilled in ice water. Separation of cell and supernatant fraction was performed by centrifugation at 2°C. The incubation described above and the cells thus obtained were designated as hormone(or rhodotorucine A)-preincubation and hormone(or rhodotorucine A)-preincubated cells, respectively, in this paper. Hormone-preincubated cells were washed twice with 5 ml of cold distilled water and resuspended in 1 ml of fresh GYP medium (hormone-free), followed by incubation at 30°C to assay their ability to form mating tube. After 6-hr assay incubation, number of mating tube-formed cells was counted under microscope. With the supernatant fraction, the amount of the mating hormone was determined.

As seen in Fig. 1, by hormone-preincubation, a cells gained the mating tube-forming activity in hormone-free medium, and the supernatant fraction lost remarkable amount of hormonal activity. The temperature of hormone-preincubation did not affect on these alterations in biological activity. Therefore, it is concluded that the binding of the hormone to a cells occurred during hormone-preincubation. A cells of Rh. toruloides, AB and ab cells of Tremella mesenterica and a and α cells of Saccharomyces cerevisiae did not decrease the hormonal activity in the medium for hormone-preincubation.

From the results in Fig. 1 and other experiments (did not present here), hormone-binding capacity of a cells was calculated to be approximately 0.5 unit rhodotorucine A per 10^3 cells; equivalent to 5 X 10^7 molecules per cell. This value is extremely higher than the value, 10^4 molecules of α-factor per a cell, obtained with Sacch. cerevisiase (14). However,

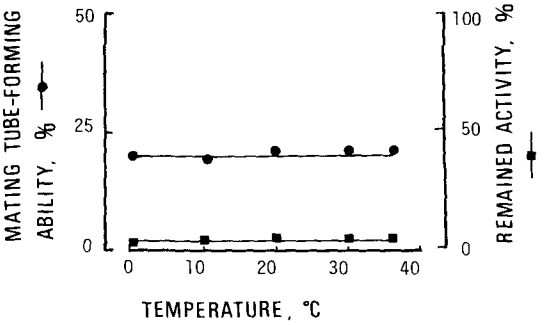

Fig. 1 Temperature Independency of Rhodotorucine A-Binding to a cells
Mating tube forming Ability: percentage of mating tube-formed cells.
Remained Activity: percentage of remained hormonal activity in the supernatant fraction after preincubation.

we could not clarify that this high value of hormone binding is essential or not for induction of sexual differetiation. Previously (11,12), a mutant strain defective in rhodotorucine A-binding ability was isolated as one of mating-less mutants from a cells used here. We consider the revertant mutants derived from this hormone-binding less mutant might be useful tool for characterization of the functional hormone-binding site. Saturation of binding site with the hormone took place within 1-min hormone-preincubation at 0°C, and the optimal pH for the binding was 5-8 in a buffer system of phosphate.

As described above, the functionally bound hormone to a cells was not removed by washing with cold distilled water. However, when a 1.0 ml amount of the cell suspension (20 mM Na,K-phosphate buffer, pH 7.0) containing 5 X 10^6 of hormone-bound cells, which were prepared by the preincubation at 0°C for 5 min, was treated with pronase (Kaken Kagaku Chemicals, Tokyo) under the conditions: 150 tyrosine-units of pronase, 30°C and 10 min, the cells completely lost their activity to form mating tube (details are in next section in this paper). Rhodotorucine A, a lipopeptide, is sensitive to pronase, so that the bound hormone might

Table I Effect of Pronase Treatment on Mating Tube-Forming Ability

Pronase Treatment		Mating Tube-Forming Ability %
Before Hormone-Preincubation	After Hormone-Preincubation	
Without	Without	9.2
Without	With	0
With	Without	11.0

Conditions of hormone-preincubation are as follows: 3,200 units of rhodotorucine A per 5 X 10^6 cells, at 0°C for 5 min.
Mating tube-forming ability: percentage of mating tube-formed cells per total cells after assay incubation.

be degraded to inactive form by the treament. These findings indicate that the biologically functional bound-hormone locates in a pronase-permeable space of the yeast cell. On the other hand, the pronase treatment prior to hormone-preincubation gave no effect on the functional structure of binding site; the binding site is resistant to pronase (Table I).

Rhodotorucine A-preincubation term for establishment of sexual differentiation

A 1.0 ml amount of cell suspension (20 mM Na,K-phosphate buffer, pH 7.0) containing 10^6 a cells was preincubated with 3,200 units of the hormone at 25°C. The cells were as possible as quickly collected on membrane filter at every 5-min preincubation and washed with cold water to remove unbound hormone. Then the hormone-bound cells were resuspended in 1.0 ml of 20 mM Na,K-phosphate buffer, pH 7.0, containing 105 tyrosine-units of pronase, followed by incubation at 30°C for 10 min. The pronase-treated cells were washed with cold distilled water to remove pronase, suspended in 1.0 ml of the same phosphate buffer and spread onto GYP agar plate (hormone-free). Then incubation at 30°C was carried out to assay the mating tube-forming activity. After 6-hr incubation,

Table II Time Dependency of Establishment of Intracellular Information for Sexual Differetiation During Hormone-Preincubation

Time Length of Hormone-Preincubation min	Mating Tube Forming Ability: Pronase Treatment After Hormone-Preincubation	
	Without	With
1	+	-
5	+	-
10	+	-
15	+	±
20	+	+
25	+	+
30	+	+

Mating tube-forming ability: -, 0%; ±,approximately 1%; +, more than 5%

number of mating tube-formed cells was counted under microscope. By the pronase treatment, the 10-min cells of hormone-preincubation completely lost the ability to form mating tube, whereas the 15-min cells of the preincubation remained the ability in a significant extent as seen in Table II. Then it might be stated that rhodotorucine A-preincubation term required for establishment of intracellular information inducing sexual differetiation is approximately 15 min at 25°C. As the mode of hormone action, two basic principles had been emerged. The first is that lipid-soluble hormones, such as steroid hormones, pass through cell membrane and bind to the primary receptor located in cytosol, then the hormone-bound receptor complex itself becomes the intracellular messenger. The second is that water-soluble hormones, such as epinephirine and glucagon, bind to the receptor located in cell surface and do not pass through cell membrane readily, however, the binding of the hormone to the receptor induces the formation of intracellular messenger (second messenger), such as cyclic nucleotides, which stimulates or derepresses some biochemical activity. Rhodotorucine A is a chemically unique sex hormone, a lipopeptide which consists

of polar (peptidyl) and non-polar (farnesyl) parts in a molecule, therefore, we wonder which basic principle is applicable to this hormone. Since the bound mating hormone was altered by pronase treatment to inactive form, the primary receptor for the hormone should be located in cell surface. Furthermore, a cells required at least 15 min of hormone-preincubation term at 25°C for establishment of intracellular information inducing sexual differentiation. These findings suggest the occurrence of second messenger in cytosol. Addition of cAMP, cGMP and their analogues, however, to a cells did not show any effect (stimulation or repression) on induction of mating tube formation. Survey experiments for second messenger are in progress. In order to follow the fate of rhodotorucine A during 15-min hormone-preincubation, preparation of ^{125}I-labeled hormone is also in progress.

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