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Cell–cell recognition in yeast: Purification of *Hansenula wingei* 21-cell sexual agglutination factor and comparison of the factors from three genera

(*Saccharomyces/Pichia*/5-agglutinin/receptor–ligand/cognor–cognon)

DAVID BURKE, LUCIA MENDONÇA-PREVIATO*, AND CLINTON E. BALLOU†

Department of Biochemistry, University of California, Berkeley, California 94720

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ABSTRACT **Trypsin digestion of *Hansenula wingei* 21-cells releases a protein (21-factor-T) that inhibits the agglutination of 21-cells by purified 5-agglutinin obtained from 5-cells by subtilisin digestion [Crandall, M. A. & Brock, T. D. (1968) *Bacteriol. Rev.* 32, 139–163]. We have purified this inhibitor 415-fold by ion-exchange chromatography, affinity adsorption to 5-cells, and gel permeation chromatography. The material shows a diffuse band, on polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, with an apparent M_r of 27,000. It has a pI of 3.8, is rich in acidic amino acids, contains 5% mannose and a trace of glucosamine, and is stable to reducing agents but is inactivated by heat. Zymolyase (β1→3-glucanase) digestion of 21-cells releases a similar inhibitor that, after purification, has a larger size than 21-factor-T. This 21-factor-Z appears to contain an additional portion that may serve to anchor 21-factor in the cell wall. Haploid cells of the yeasts *Pichia amethionina* and *Saccharomyces kluyveri* also show a constitutive sexual agglutination, and little or no crossreactivity is observed in heterologous mixtures. The agglutination factors in all three genera, however, have parallel properties; one cell type of each pair is heat stable and is inactivated by reducing agents (*H. wingei* 5-cells, *P. amethionina* α-cells, and *S. kluyveri* 16-cells), and the other is heat labile and is unaffected by reducing agents (*H. wingei* 21-cells, *P. amethionina a*-cells, and *S. kluyveri* 17-cells). Because *S. kluyveri* 16-cells respond to *Saccharomyces cerevisiae* α-factor with the typical morphogenetic change of a mating half-reaction, the heat-stable agglutinin appears related to the *S. cerevisiae a* mating type and the heat-labile factor to the *S. cerevisiae* α mating type.**

Sexual mating in some yeasts is facilitated by cell surface macromolecules that mediate a specific recognition and agglutination between the haploid cells of opposite mating type. The prototype of this interaction is that between *Hansenula wingei* 5-cells and 21-cells, which show a strong, constitutive sexual agglutination (1). From the 5-cell can be isolated a large heat-stable mannoprotein that agglutinates 21-cells and is known to consist of a glycoprotein core to which several small glycopeptide recognition sites are attached by disulfide linkage (2, 3). This 5-agglutinin is inactivated by reducing agents, which release the recognition sites in a monovalent form that will bind to 21-cells but not agglutinate them. Trypsinization of 21-cells releases a small heat-labile protein (21-factor) that will bind to 5-agglutinin and neutralize it, but this monovalent 21-factor does not agglutinate 5-cells (1). In this report we describe some additional properties of purified 21-factor.

Saccharomyces cerevisiae X2180 does not show a strong constitutive sexual agglutination, although a weak agglutination is induced in the haploid cells by the exchange of pheromones (4). This enhanced agglutination is associated with the appearance of a fuzzy coat on the tip of the extensions of *a*-cells exposed to α-factor (5). We have now compared *H. wingei* with two other yeast strains that show a strong constitutive agglutination, *Pichia amethionina* (6) and *Saccharomyces kluyveri* (7), and we have related the two haploid cells of *S. kluyveri* to the mating types of *S. cerevisiae*. The agglutinative property of one haploid cell type of each of the three strains (*H. wingei* 5-cell, *P. amethionina* α-cell, and *S. kluyveri* 16-cell) is heat-stable but is inactivated by reducing agents, whereas the other haploid cell of each pair (*H. wingei* 21-cell, *P. amethionina a*-cell, and *S. kluyveri* 17-cell) is heat-labile but is unaffected by reducing agents. Because *S. kluyveri* 16-cells respond to *S. cerevisiae* α-factor sex pheromone, we conclude they have the *a* mating type and that, therefore, the α mating type may control the 21-factor-like recognition component of each strain. Whether the weak sexual agglutination of *S. cerevisiae* is mediated by similar factors is unknown.

MATERIALS AND METHODS

Materials. *H. wingei* NRRL Y-2340 cultures of mating types 5 and 21 were obtained from C. P. Kurtzman, Northern Utilization Research and Development Division, Peoria, IL; *P. amethionina* cultures of mating type *a* and α, and *S. kluyveri* cultures of mating type 61-16 (type 3) and 61-17 (type 26) were provided by H. J. Phaff, University of California, Davis, CA. α-Factor was purified from the cultural filtrate of *S. cerevisiae* X2180-1B cells according to Duntze *et al.* (8), and 5-agglutinin was isolated from *H. wingei* 5-cells as described by Yen and Ballou (3). Chromatographic and ion-exchange resins were from Bio-Rad. Zymolyase 60,000 (β1→3-glucanase) was from the Kirin Brewery, Tokyo, subtilisin BPN′ and chymotrypsin were from Sigma, pancreatic trypsin (grade A) and Pronase were from Calbiochem, and endo-β-*N*-acetylglucosaminidase H (9) and bacterial α-mannosidase (10) were purified by published procedures.

General Methods. *H. wingei* and *P. amethionina* cells were grown on a medium consisting of 0.7% yeast extract, 0.5% KH_2PO_4, and 3% glucose (1). *S. kluyveri* cells were grown on a medium (YEPD) containing 1% yeast extract, 2% peptone, and 2% glucose. Cultures were maintained on media containing 2% agar. *S. kluyveri* 16 and 17 and *S. cerevisiae a*- and α-cells were tested for their response to *S. cerevisiae* α-factor in a minimal medium composed of 2% glucose and 0.67% Difco yeast nitrogen base without amino acids, after they had been grown on liquid YEPD for 8 hr. A positive response resulted in a typical 2-fold elongation of the cells after about 4 hr (5).

Cell agglutination was assayed (3) by mixing together, in a

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Abbreviation: YEPD, 1% yeast extract/2% peptone/2% glucose.

* On leave from the Universidade Federal do Rio de Janeiro, Brazil.
† To whom reprint requests should be addressed.

well of a microtiter plate, equal amounts of the two cell types suspended in 50 μl of saline or buffer. A positive reaction resulted in formation of an aggregated mass, whereas nonagglutinating cells settled to the bottom of the well in a diffuse layer. When it occurred, interspecies agglutination was observed under a microscope as clumps of 10–50 cells after the cells were incubated together on a YEPD plate for several hours and then suspended in saline. Heat stability was determined by heating the cells at 100°C for 40 min in saline or buffer, whereas stability to reducing agents was determined by incubating the cells in 50 mM mercaptoethanol at 25°C for 30 min. After either treatment, the cells were washed in fresh buffer and then tested for agglutinability. 21-Factor was assayed in microtiter plates by its ability to reverse the agglutination of 21-cells by purified 5-agglutinin.

Sodium dodecyl sulfate/polyacrylamide gel electrophoresis was performed according to Laemmli (11) on a 10-cm slab gel containing 10% polyacrylamide, native gels according to Orstein (12), and gel electrofocusing as described by Wrigley (13). Carbohydrate was determined by the phenol/sulfuric acid assay (14) and protein by absorbance at 220 nm or by the Lowry procedure (15).

RESULTS AND DISCUSSION

Purification and Properties of 21-Factor. The 21-factor, released from *H. wingei* 21-cells by trypsin treatment (1), was purified 415-fold in a yield of 10% (Table 1). A typical assay of the material is illustrated in Fig. 1. On gel electrophoresis in sodium dodecyl sulfate, the purest preparations showed a single somewhat diffuse band (Fig. 2, lane F). The apparent molecular weight of this 21-factor-T is 27,000 (Fig. 3). Nondenaturing gels also gave a single band (not shown). All biological activity coelectrophoresed with the one band, although recovery of activity from the nondenaturing gel was low. Isoelectric focusing gels showed a single band with a pI of 3.8.

From the size of 5-agglutinin and the number of binding sites (2), it should have an agglutination equivalent weight of 160,000. With a M_r of 27,000, 21-factor-T would be expected to combine with 5-agglutinin in a weight ratio of 1:6. From Fig. 1, it is apparent that a weight ratio of 1:3 to 1:6 is required for reversal of the agglutination reaction. Because this assay involves the interaction of two multivalent components, 5-agglutinin and 21-cells, and its reversal by a monovalent 21-factor in what is expected to be an unfavorable competition, we interpret the apparent combining ratios to mean that the isolated 21-factor-T is quite pure and has retained full biological activity.

The release of 21-factor by trypsinization probably results from proteolysis of the factor itself. In support of this, we observe that the 21-factor released by digestion of the cell with Zymolyase, which solubilizes the wall, has a larger molecular weight (not shown). In Ouchterlony double diffusion experiments using antiserum to 21-factor-T, immunological identity was observed between the two 21-factor preparations, which shows that 21-factor-Z contained all of the determinants present in the smaller form (Fig. 4). Controlled trypsin digestion of 21-factor-Z produced the smaller form, whereas extensive trypsin treatment destroyed the molecule. The factor was also destroyed by Pronase, chymotrypsin, and subtilisin, but it was not affected by bacterial α-mannosidase under conditions that remove most of the carbohydrate (not shown).

The unusual composition of 5-agglutinin has been noted (3),

Table 1. Purification of *H. wingei* 21-factor

Step	Total protein, mg	Total activity, units*	Purification, -fold
Trypsin digest†	30,000	250	1
DEAE-cellulose chromatography	2,300	220	12
Affinity adsorption to 5-cells	507	125	30
DEAE-cellulose chromatography	34	36	127
Bio-Gel P-150 chromatography	7	25	415

* One unit inhibits agglutination of 5×10^6 21-cells in 50 μl of buffer by 25 μg of pure 5-agglutinin.
† From 500 g of wet cell paste.

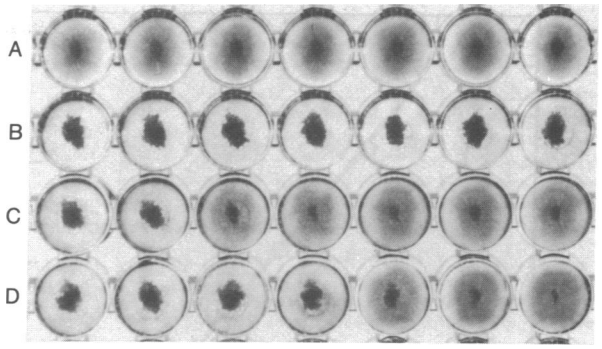

FIG. 1. Assay of *H. wingei* 21-factor-T by inhibition of 21-cell agglutination by purified 5-agglutinin. Row A contains 5×10^6 21-cells in 50 μl of buffer; row B contains 5×10^6 21-cells per well and serial dilutions of 5-agglutinin from 1280 ng per well on the left to 20 ng per well on the right in a total volume of 50 μl; row C is the same as B except it also contains 100 ng per well of 21-factor; and row D is the same as B except it also contains 25 ng per well of 21-factor. The added 21-factor reverses agglutination at well 3 in row C and at well 5 in row D.

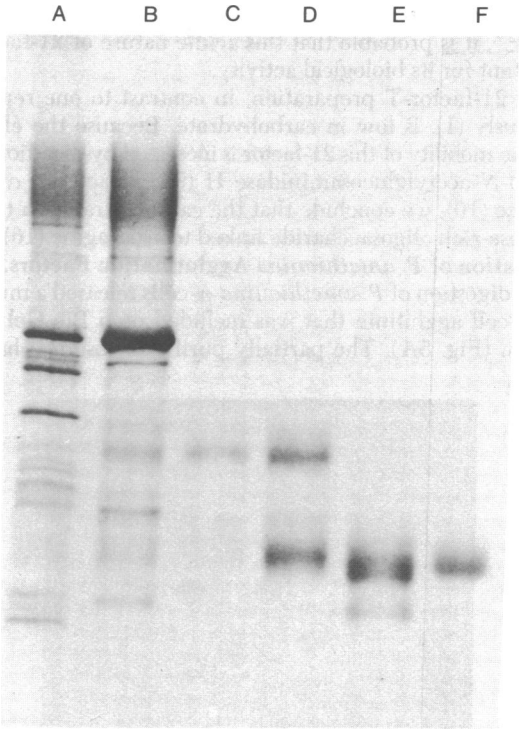

FIG. 2. Gel electrophoresis in sodium dodecyl sulfate of trypsin-released 21-factor-T preparations. Lane A shows the initial trypsin digest, B the first DEAE-cellulose fraction, C the material from the 5-cell affinity step, D the second DEAE-cellulose fraction, and E the Bio-Gel P-150 fraction. Lane F is a gel of an independent preparation of 21-factor-T that lacked the lower molecular weight contaminant in E.

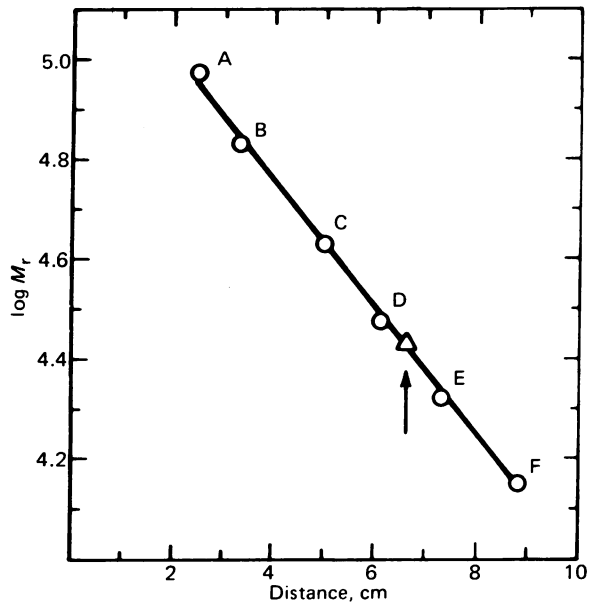

FIG. 3. Molecular weight determination of 21-factor-T by gel electrophoresis in sodium dodecyl sulfate. The references indicated by Os are: A, phosphorylase B (94,000); B, bovine serum albumin (68,000); C, ovalbumin (43,000); D, carbonic anhydrase (30,000); E, soybean trypsin inhibitor (21,000); and F, lysozyme (14,300). The 21-factor is indicated by Δ and an arrow.

particularly the high content of hydroxy amino acids, which total 65% of the protein. The two 21-factor preparations also have unusual compositions, with a high content of acidic amino acids (Table 2). This composition is associated with a pI of 3.8, which indicates that many of these amino acids occur in the free carboxyl form rather than the amide. Because the interaction of 5-cells with 21-cells is reversed at low pH and is dependent on Mg^{2+}, it is probable that this acidic nature of 21-factor is important for its biological activity.

Our 21-factor-T preparation, in contrast to one reported previously (1), is low in carbohydrate. Because the electrophoretic mobility of this 21-factor is increased by digestion with endo-β-*N*-acetylglucosaminidase H (9) or bacterial α-mannosidase (10), we conclude that the carbohydrate is a typical mannose-rich oligosaccharide linked to asparagine (16).

Isolation of *P. amethionina* Agglutination Factors. Zymolyase digestion of *P. amethionina* α-cells released a multivalent *a*-cell agglutinin that was included on a Bio-Gel A-5m column (Fig. 5*A*). The partially purified substance had the properties of a large glycoprotein, and it was stable to heat but lost its activity on treatment with mercaptoethanol. Subtilisin digestion of *P. amethionina a*-cells released a small molecular

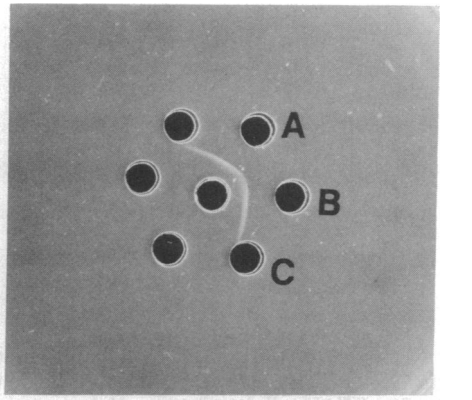

FIG. 4. Comparison of 21-factor preparations by immunodiffusion. The center well contains rabbit antiserum against 21-factor-T, whereas well A contains 21-factor-T, well B contains 21-factor-Z, and well C contains buffer.

Table 2. Amino acid compositions of 21-factor preparations

Amino acid	Residues per 100 amino acids	
	21-Factor-T*	21-Factor-Z†
Lysine	1.68	2.77
Histidine	0.53	0.77
Arginine	0.85	1.03
Aspartate/asparagine	15.91	15.18
Threonine	12.89	11.76
Serine	8.33	10.24
Glutamate/glutamine	12.66	9.44
Proline	4.33	3.21
Glycine	7.89	9.86
Alanine	6.13	8.78
Valine	7.69	6.47
Methionine	<0.1	<0.1
Isoleucine	6.26	4.85
Leucine	7.49	6.88
Tyrosine	4.32	4.24
Phenylalanine	3.06	4.51

* Also contained about 5% mannose by weight and a trace of glucosamine.

† About 95% of this material was a protein with M_r of 30,000.

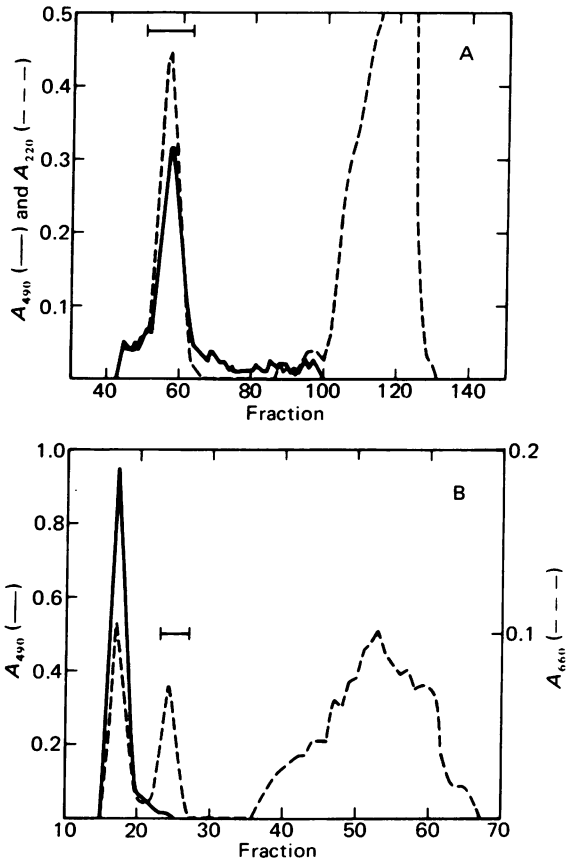

FIG. 5. Isolation of *P. amethionina* sexual agglutination factors. In *A*, the Zymolyase digest of α-cells was fractionated on a Bio-Gel A-5m column (1 × 100 cm) in 0.1 M KH_2PO_4. Carbohydrate, A_{490}, and protein, A_{220}, are indicated. Fractions that agglutinated *a*-cells are indicated by the bar. In *B*, the subtilisin digest of *a*-cells was fractionated on a Bio-Gel P-60 column (1 × 50 cm) in 0.1 M KH_2PO_4. Carbohydrate, A_{490}, and protein, A_{660}, are indicated. Fractions that inhibited the agglutination of *a*-cells by the α-agglutinin are indicated by the bar.

weight protein that was included on a Bio-Gel P-60 column and appeared to contain little if any carbohydrate (Fig. 5*B*). The substance did not agglutinate α-cells, but it inhibited the agglutination of *a*-cells by the α-agglutinin. This *a*-factor, which parallels *H. wingei* 21-factor in its properties, and is probably released by proteolysis of intact *a*-factor, was inactivated by heat but was stable to reducing agents.

Crossreactivity Between the Agglutination Systems. Compared to the massive agglutination observed between the homologous haploid cells, there is little or no crossreactivity between the strains we have studied here. Clumps of 10–50 cells were observed, however, when *H. wingei* 5-cells were mixed with *P. amethionina a*-cells. Taylor (17) reported that *H. wingei* 21-cells crossreact strongly with *S. kluyveri* 16-cells (type 3), but we failed to confirm this observation with our strains. Both of these crossreactivities, however, are consistent with the type specificity assigned on the basis of chemical properties (see below). Although the crossreactions seem related to sexual agglutination, nonspecific factors such as surface charge may play a role in such weak associations, so their significance is unclear. Although the *P. amethionina* α-agglutinin and *a*-factor have obvious similarities to the 5-agglutinin and 21-factor of *H. wingei*, none of the isolated molecules showed any activity in heterologous systems, nor did *a*-factor crossreact with antiserum to 21-factor-T.

Inactivation of the Agglutination Factors on Intact Cells. The homologous agglutination activities of *H. wingei* 21-cells, *P. amethionina a*-cells, and *S. kluyveri* 17-cells were all destroyed by heat (Table 3). In contrast, *H. wingei* 5-cells, *P. amethionina* α-cells, and *S. kluyveri* 16-cells were stable to this treatment, although they were inactivated by mercaptoethanol, a treatment that did not affect the first three cell types. Taylor (17) had already noted this effect of reducing agents on *H. wingei* 5-cells and *S. kluyveri* 16-cells. Although he reported that the agglutinative activity of *S. kluyveri* 17-cells was also sensitive to mercaptoethanol, we found it to survive this treatment. Thus, our observations are consistent with the notion that the agglutination factors in all three pairs have parallel properties, one being heat stable and sensitive to reducing agents and the other being heat labile and insensitive to reducing agents.

Response of *S. kluyveri* 16-Cells to *S. cerevisiae* α-Factor. *S. kluyveri* 16-cells, grown to early log phase on YEPD medium, were suspended in minimal medium that contained 40 units of α-factor per ml. After 6 hr, many cells showed the morphological change characteristic of *S. cerevisiae a*-cells exposed to the pheromone. *S. kluyveri* type 17-cells were unaffected.

Table 3. Comparison of sexual agglutination properties

Yeast strain	Inactivated by heat	Inactivated by reduction
H. wingei		
5-Cell	No	Yes
21-Cell	Yes	No
*P. amethionina**		
α-Cell	No	Yes
a-Cell	Yes	No
S. kluyveri		
16-Cell†	No	Yes
17-Cell	Yes	No

* The mating type designations of this yeast were originally assigned arbitrarily (6) and have no relation to those of *S. cerevisiae*. To be consistent, however, our results suggest they should be reversed.

† This haploid responds to *S. cerevisiae* α-factor and, therefore, is analogous to the *S. cerevisiae a* mating type.

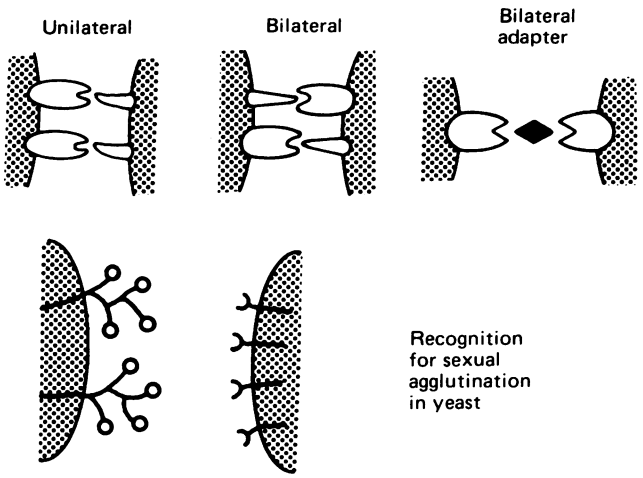

FIG. 6. Models for cellular recognition and adhesion. Three types of cell–cell interacting systems shown at the top are redrawn from Greaves (19). The model for sexual agglutination in yeast, deduced from this and other work, is analogous to the unilateral model (20).

A similar observation was reported recently by McCullough and Herskowitz (18).

Agglutination Factor Classification and Terminology. It is clear that the yeast sexual agglutination factors described here are of two distinct types that function unilaterally (Fig. 6): a heat-labile, active, recognizer molecule having the properties of an enzyme, if not a catalytic activity; and a heat-stable, passive, recognition site. Similar properties are possessed by the interacting species in other cell–cell recognition systems (21, 22), and it would be useful to have simple descriptive terms for such two-component systems that imply structural type as well as function and that parallel those of enzyme–substrate, antibody–antigen, and receptor–ligand. For the former, active, recognizer part of such systems, we propose the term "cognor"; and for the latter, passive, component that provides the site that is recognized, the term "cognon."‡ Thus, lectins and antibody-like molecules that have a recognition function would, by this definition, be cognors; and the carbohydrate chains of glycoproteins and glycolipids, or other passive sites of recognition, would be cognons. We emphasize that in this system of nomenclature the location of the recognition factors is irrelevant. In the sexual agglutination of yeasts, both components are found on cell surfaces, whereas soluble lectins that mediate the interaction of a root hair and its infecting rhizobial strain would be cognors and the cell surface molecules that they recognized and bound would be cognons.

A distinctive feature of cognors is their heat-sensitive proteinaceous nature, which implies that they have an organized

‡ From the root "cognition—to come to know," proposed by one of us (C.E.B.) at the North Atlantic Treaty Organization Conference on Surface Membrane Receptors in Bellagio, Italy, September 1975. In a discussion of cell surface receptors, Greaves (19) refers to "membrane associated 'cognitive' elements." The terms "receptor" and "ligand," which might serve to describe such cognitive elements, have through wide and variable use developed meanings that carry no structural implication. Thus, a recent major review (23) refers to lectins as "ligands," and the glycoproteins to which they bind on lymphocyte surfaces as "lectin receptors." In the same vein, the erythrocyte cholera toxin recognition site, ganglioside GM_1, has been called "the toxin receptor" (24). It is apparent that a receptor on a cell surface can be any entity to which something smaller than the cell (a ligand) binds, the binding of which initiates a physiological response mediated by the receptor (25). We feel that such terms are not adaptable to a description of the biochemistry of cell–cell interactions.

structure with the ability to interact cooperatively with cognons. Although some recognition systems appear to be inhibited by small hapten-like molecules, we expect that the physiologically important cognons will have to be more complex in order to provide the exquisite specificity that is evident in differentiating multicellular systems (26). In this respect, the sexual mating factors of yeasts are no exception. Whether this terminology, which is based on structural parallels, will prove adaptable to all cell–cell recognition systems remains to be seen.

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