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Molecular Complementarity of Yeast Glycoprotein Mating Factors

(*Hansenula wingei*/cellular recognition/Sepharose gel filtration/complementary glycoprotein complex formation)

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ABSTRACT **Cell fusion between opposite mating types *5* and *21* of the yeast *Hansenula wingei* is initiated by a strong sexual agglutination reaction. The mating factors responsible for the specificity of cellular recognition are complementary glycoproteins which form a physical complex *in vitro*. The complex is assayed by recovery of agglutination activity of the multivalent 5-factor after the univalent 21-factor has been inactivated by treatment of the complex with alkali. The 5-factor·21-factor complex, purified on Sepharose 6B, is large (several million daltons) and heterogeneous. The three peaks of 5-factor activity contain a number of combining sites proportional to molecular size.**

In this paper, evidence is presented for complex formation *in vitro* between yeast glycoprotein mating factors. These cell-wall factors determine the specificity of cellular recognition as the first step in mating. Thus, these factors may be considered "complementary" in the sense proposed by Emil Fisher (1) in his analogy:... *"dass Enzym und Glucosid wie Schloss und Schlussel"*.... Shortly later, in 1913, Lillie (2) described an activity extracted from sea-urchin eggs that specifically agglutinated sperm cells and was therefore complementary to a sperm-surface component. These early ideas concerning preformed combining sites on macromolecules formed the basis of models proposed by Tyler (3) and Weiss (4) in which the specificity of cellular adhesion in tissue development was explained by the presence of complementary molecules on opposing cell surfaces which combined in a manner analogous to antigen–antibody complex formation. Subsequent studies of cell contact have been reviewed extensively (see refs. 5–16 and other papers in these volumes). In systems involving adhesion of identical cells, additional theoretical considerations and biophysical parameters have been proposed (17–21). Current research in cellular recognition is centered on the identification and characterization of cell-surface aggregation factors isolated from diverse systems (22–33). In general, the information for the specificity of aggregation factors is considered to reside in the primary sequence of the protein moiety. However, the role played by the carbohydrate moieties may be nontrivial since most of the aggregation factors studied thus far either interact with carbohydrates or glycoproteins on the cell surface or are glycoproteins themselves. The latter is true for the mating factors isolated from sexually agglutinative mating types of the yeast *Hansenula wingei* (34). This mating system has been reviewed (34), and criteria for determining specificity in other cell-aggregating systems were proposed (35). The hypothesis that the haploid mating factors are mutually repressed in the nonagglutinative diploid (36) has been supported by recent work in which conditions for the differential induction of each glycoprotein mating factor in the diploid were discovered (37).

The mating factor from strain *5*, called 5-factor (5f) (38) or 5-agglutinin (39) is a mannan-protein, heterogeneous with respect to molecular weight (15,000 to 10^8 daltons) (34, 38) and with respect to carbohydrate content [50% (38) to 96% (40)]. The biological assay for 5f used by all these workers is agglutination of strain *21* cells. Because 5f is an agglutinin, it is considered multivalent; i.e., it must have more than one combining site to crosslink cells. In fact, Taylor and Orton (40, 41) have shown that 5f, isolated by subtilisin digestion of strain-*5* cells, has six binding sites per molecule of 16.7 S_{25}. Reduction of this molecule with 2-mercaptoethanol destroys agglutination activity and liberates six fragments of 1.75 S each.

The mating factor from strain *21*, called 21-factor (21f), is also a mannan-protein (34, 35) but is homogeneous (2.9 $S_{20,w}$). Since the 21f does not agglutinate strain-*5* cells but inhibits 5f activity, it is considered univalent (34, 35). If there is only one binding site on the 21f, then the 5f·21f complex should be soluble since networks of crosslinks cannot be formed. Since the free energy of binding of 5f to strain-*21* cells is high (−14.5 kcal/mol) (42), the 5f·21f complex should be quite stable during purification. To identify the 5f·21f complex in column fractions, we used a biological assay that is based on the fact that 21f is rapidly inactivated by alkali whereas 5f is unaffected. Thus, alkali treatment of complex results in recovery of 5f agglutination activity. This assay was first exploited to determine whether the 5f·21f complex was present in the *5* × *21* diploid hybrid (36). Since no 5f could be recovered from concentrated diploid extracts, it was proposed that both 5f and 21f synthesis is repressed in the diploid (36). However, until now it has not been proven that a physical complex *is* formed when 5f activity is neutralized by 21f preparations. The results presented here demonstrate that a soluble 5f·21f complex *does* exist, thereby substantiating the idea that these two molecules are complementary.

MATERIALS AND METHODS

Buffers. All cell-free extracts were prepared in 0.01 M KH_2PO_4 (pH 5.3), called standard buffer (SB). For gel filtration, 0.02% sodium azide (NaN_3) was added to SB to prevent microbial contamination.

Chemical Assays. Protein was estimated by the Lowry method (43) but in $^1/_5$th the recommended volume, with

Abbreviations: 5f and 21f, mating factor from strain *5* and strain *21*, respectively; SB, standard buffer (0.01 M KH_2PO_4, pH 5.3).

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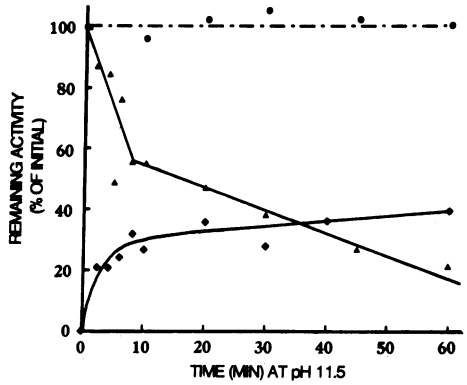

FIG. 1. Kinetics of inactivation of 21f and recovery of 5f activity from the complex. Preparations of 5f(*circles*) and 21f (*triangles*) were adjusted to pH 11.5 with 1 N NaOH, incubated at 30°, and then reneutralized to pH 5-6 with 1 N HCl at various times. The zero-time control was diluted with a volume of SB equal to the volume of NaOH + HCl added to the other samples. Complex was formed by titrating a 5f preparation with 21f until 5f agglutination activity was just neutralized. Recovery of 5f activity from the alkali-treated complex (*squares*) was calculated in terms of percent of 5f activity present in a 5f solution diluted with SB to the same extent as in the complex. Units of 5f and 21f activity, linear with dilution, were determined from standard curves of units against % agglutination on arithmetic probability graph paper (Codex Book Co., Inc., Norwood, Mass.) [Crandall (1968) Ph.D. Dissertation, Indiana Univ.].

bovine-serum albumin as standard. Carbohydrate was determined by the phenol–sulfuric acid method (44), with Dextran as standard.

Agglutination Assays. 5f and 21f were assayed as described (34) except that 880 mg/100 ml of NaCl instead of $MgSO_4$ was added to SB to maximize agglutination.

Chemicals. Trypsin (molecular weight 24,000) and lima bean trypsin inhibitor (molecular weight 10,000) were purchased from Worthington Biochemical Corp., Freehold, N.J.; ferritin from Calbiochem, Los Angeles, Calif.; myoglobin from Miles-Seravac (PTY) Ltd., Maidenhead, Berks, England; bovine-serum albumin, catalase, Dextran, thyroglobin, and ovalbumin from Sigma Chemical Co., St. Louis, Mo.; and Sephadex G-200 and Sepharose-6B from Pharmacia Fine Chemicals, Piscataway, N.J.

Yeast Strains. The origin of the haploid mutant strains used is explained in ref. 45.

Preparation of 5f. Cells of strain *5 cyh lys*, grown to stationary phase with vigorous aeration in 10 liters of YG medium (34) were homogenized in SB with 0.5-mm glass beads. RNA was precipitated with $MnCl_2$ and the supernatant was dialyzed against SB, concentrated, clarified by centrifugation, and applied to a column of Sephadex G-200. The excluded fractions of 5f activity (>200,000 daltons) were concentrated and stored frozen. Traces of smaller molecular weight 5f were discarded.

Preparation of 21f. Cells of strain *21 ade his*, grown to stationary phase in 10 liters of YG medium, were washed twice, resuspended in 5 liters of water, steamed in 200-ml aliquots for 30 min, cooled rapidly, harvested, washed twice more, and resuspended in 500 ml of 0.046 M tris(hydroxymethylaminomethane), pH 8.5, containing 11.5 mM $CaCl_2$. Trypsin was

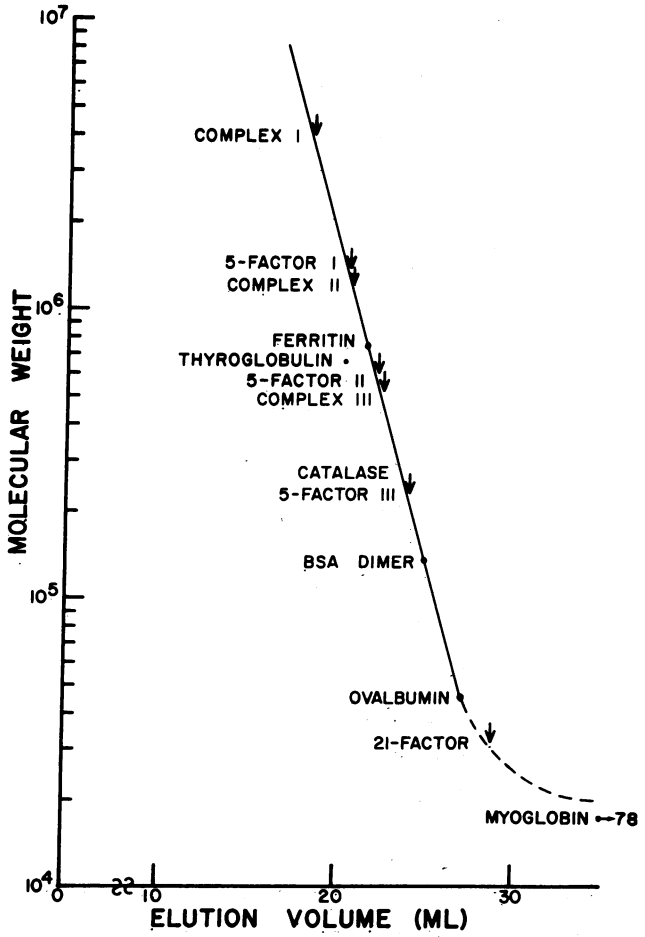

FIG. 2. Calibration of the Sepharose 6B column with standard proteins. Protein solutions at 10 mg/0.5 ml of SB were clarified and applied to the column (1.0 × 58 cm). The elution volume was measured from time of application to elution of peak protein concentration (*A* at 280 nm). Bovine-serum albumin (*BSA*) apparently dimerized in SB.

added to a final concentration of 100 μg/ml, the mixture was stirred at room temperature for 1 hr, and the cells were removed. The supernatant was neutralized to pH 5.5 in an ice bath; lima bean trypsin inhibitor was added to a final concentration of 30 μg/ml; then RNA was removed with $MnCl_2$. The supernatant 21f preparation was dialyzed against SB, concentrated, dialyzed again, clarified, and purified on a Sephadex G-200 column. The single peak of 21f activity was the largest molecular weight protein in the trypsin digest and came off just before the major protein peak (trypsin·lima bean trypsin inhibitor complex). Thus, the molecular weight of 21f is > 34,000. Pooled fractions of 21f were concentrated and stored frozen.

RESULTS

Kinetics of Inactivation of 21f and Recovery of 5f Activity from the Complex. 21f is rapidly inactivated by alkali whereas 5f is unaffected (Fig. 1). Recovery of 5f activity from the complex occurs with the same kinetics as loss of 21f activity.

Sepharose 6B Gel Filtration of 5f, 21f, and the 5f·21f Complex. Since the majority of 5f activity isolated from the cytoplasm was >200,000 in molecular weight, complex was purified

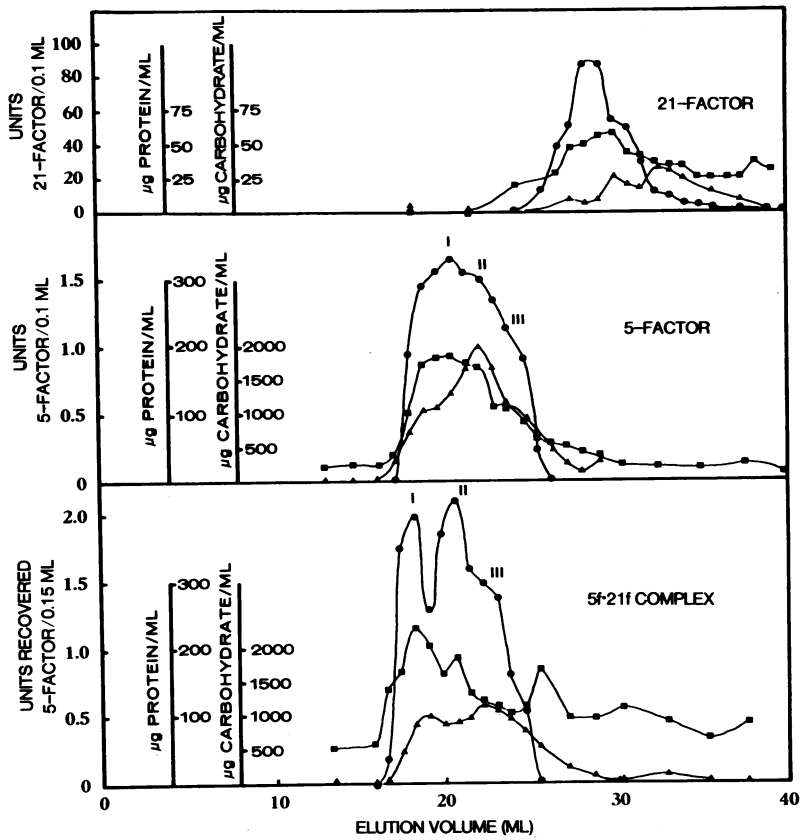

FIG. 3. Sepharose 6B gel filtration of 5f, 21f, and the 5f·21f complex. Protein (*squares*), carbohydrate (*triangles*), and biological activity (*circles*), were measured in each fraction. For the 5f·21f complex, aliquots of each fraction were first assayed for 5f activity; none was found. Then duplicate aliquots were adjusted to pH 11.5, incubated at 30° for 30 min, neutralized, and again assayed for 5f activity.

on Sepharose 6B (molecular weight exclusion limits for globular proteins between 100,000 and 4,000,000). 21f was previously reported to be 2.9 $S_{20,w}$ (34). This corresponds to a molecular weight of roughly 45,000. 21f was estimated to be >34,000 daltons from its purification on Sephadex G-200. On Sepharose 6B, 21f was below the lower exclusion limit (Fig. 2). Therefore, for later calculations the molecular weight of 21f will be assumed to be about 40,000.

5f was found to be heterogeneous (Fig. 3), as was reported previously (34, 38). The broad peak of 5f activity gave evidence of three components, with the corresponding molecular weights being: I = 1,300,000; II = 560,000; and III = 220,000. Most of the protein in this 5f preparation is >200,000, in agreement with its preliminary purification on Sephadex G-200.

The complex, identified by recovery of 5f activity after alkali treatment of each fraction, reflected the molecular weight heterogeneity of the 5f (Fig. 3). There were two major peaks of complex (I and II) of molecular weights 3,800,000 and 1,200,000, respectively, and a minor peak (III) of molecular weight 460,000. Clearly, the difference in molecular weight of 5f peaks I and II became more exaggerated as a result of complex formation with 21f.

Calculation of the Number of Combining Sites. The number of molecules of 21f bound to 5f in each peak of complex can be calculated by subtracting the molecular weight of 5f from the molecular weight of the corresponding complex and dividing the difference by the molecular weight of the 21f. Thus, for peaks I, II, and III, the calculated number of combining sites is 63, 16, and 6, respectively.

DISCUSSION

The existence of a physical complex between solubilized 5f and 21f was predicted by all the earlier studies (34–42) and is demonstrated here for the first time. By gel filtration and biological assays for 5f, 21f, and complex, it was possible to estimate the molecular weight of each peak of complex and 5f and calculate the number of combining sites. While it is clear that the three peaks of complex reflect the heterogeneity of 5f and that the number of combining sites increases roughly in proportion to molecular weight, it is also clear that the exact molecular weights cannot be determined accurately by the method used. First of all, the mating factors are glycoproteins (34, 38, 40) and globular proteins were used as molecular weight standards for calibration of the Sepharose 6B column. Perhaps Dextran fractions of various molecular weights would be better standards for future studies, especially since 5f is mostly carbohydrate (mannose) (38, 40). Secondly, peak I of the complex may be outside the upper exclusion limits of Sepharose 6B.

It would be interesting to know whether the several peaks of cytoplasmic 5f observed repeatedly [ref. 38; Crandall (1968) Ph.D. Dissertation, Indiana Univ.; Fig. 3] represent a loose association or a covalent aggregate of a smaller 5f component. It is certainly clear that this heterogeneous cytoplasmic factor is different from the homogeneous cell-wall 5f iso-

lated by Taylor and Orton (40), which is 16.7 S_{25} (about 10^6 molecular weight) yet contains only six combining sites as compared to peak I (Fig. 3), which is also about 10^6 molecular weight but has about 10 times the number of combining sites. It may be that subtilisin releases a repeating cell-wall unit that contains one molecule of 5f with six combining sites (perhaps peak III) together with extraneous cell-wall material.

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