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α-AGGLUTININ EXPRESSION IN *Saccharomyces cerevisiae*

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A polyclonal antiserum raised against purified α-agglutinin was made specific for α-agglutinin after adsorption with **a** cells. The adsorbed antiserum identified α-agglutinin peptides on Western blots and bound to cell surface α-agglutinin, inhibiting the binding of α cells to **a** cells. Using the antibody, we have determined that 1) the surface distribution of α-agglutinin on α cells is polar, 2) about 5 x 10^4 molecules/cell are constitutively expressed on strain X2180-1B (α) cells, and 3) treatment of α cells with the sex pheromone a-factor causes an increase in cell surface α-agglutinin, consistent with the a-factor induced increase in cell agglutinability.

Cells of the two mating types of *S. cerevisiae*, **a** and α, express cell surface glycoprotein agglutinins that mediate the binding of one mating type to the other. Adhesion of the two cell types is an early step in a cascade of events which culminates in the fusion of the two haploid cells to form a diploid zygote (1).

α-agglutinin is constitutively expressed on the surface of α cells. The agglutinability of α cells increases slightly (1.1 to 1.3 fold) following treatment with the **a** cell derived sex pheromone a-factor (2,3,4). α-agglutinin has an apparent molecular weight of 155 kD (as determined by density gradient centrifugation) and contains about 50% N-linked carbohydrate by weight (5). Purified α-agglutinin can specifically bind to **a** cells (6). When α-agglutinin is deglycosylated with endo-N-acetyl glucosaminidase H, polypeptides of 72, 105, 145 and 160 kD are generated, all of which can bind to **a** cells (6).

In an effort to further characterize α-agglutinin expression, antibodies were raised against endo-N-acetyl glucosaminidase H treated preparations of the glycoprotein. After adsorption with **a** cells, the antiserum was used to determine the spatial distribution of α-agglutinin on α cells, and to quantitate α-agglutinin on the cell surface.

Materials and Methods

Strains and growth: X2180-1A (**a**) and X2180-1B (α) were grown in minimal medium (Yeast Nitrogen Base with glucose) to log phase and induced with **a** or α-factor as

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described previously (4). Strains W303-1A (**a**) and W303-1B (α) were grown in enriched yeast extract-peptone-glucose medium. W303-1B was induced with equal amounts of medium and spent **a** cell enriched medium which was buffered to pH 4.25 with sodium citrate. Cells were harvested by centrifugation at 4,000 x g for 5 minutes and stored at 4°C in 100 mM sodium acetate pH 5.5 with 10 ug/ml cycloheximide (buffer A) at a cell density of 1 x 10^8 cells per ml.

General Methods: α-agglutinin was isolated as described (5). Treatment of purified α-agglutinin with endo-N-acetyl glucosaminidase H employed 0.001 U of enzyme for 16 hours at 25°C in 10 mM sodium acetate pH 5.5, 1 mM phenyl methyl sulfonyl fluoride and a weight of SDS equal to the amount of protein being treated. Quantitative agglutination assays were performed as previously described (4). SDS-polyacrylamide gel electrophoresis was performed as described (7) using 7.5% polyacrylamide gels.

Production and Adsorption of Antiserum: Antibodies to α-agglutinin were produced by a primary subcutaneous injection of 100 ug of endo-N-acetyl glucosaminidase H treated α-agglutinin in Freund's complete adjuvant followed by booster intravenous injections of 100 ug of treated α-agglutinin in phosphate buffered saline. Antiserum was adsorbed against an equal volume of **a** or α cells which were grown in minimal medium, washed and heat killed at 60°C for 1 hour. The antiserum was adsorbed twice for a total of 12 hours, retrieved by centrifugation, and stored at -20°C.

Immunodetection of α-agglutinin by Western Blotting: Transfer of protein from polyacrylamide gels to nitrocellulose membranes was carried out as described (8). Antibody dilutions and washings employed 140 mM NaCl, 10 mM Tris, pH 7.4 (buffer B). Blots were first incubated in buffer B with 3% (w/v) bovine serum albumin for 30 minutes followed by incubation in anti-α-agglutinin (diluted 1:100) for 2 hours. The blots were washed and incubated in goat anti-rabbit IgG (diluted 1:100) for 30 minutes followed by washing and incubation in rabbit peroxidase-anti-peroxidase (diluted 1:1000) for 45 minutes. Blots were washed and placed in 4-chloro-1-naphthol to visualize bound antibody.

Antibody Inhibition Assay: Cells were equilibrated in 140 mM NaCl, 10 mM Tris, pH 7.4 with 10 ug/ml cycloheximide (buffer C). 100 ul of α cell suspension was centrifuged (1000 x g for 5 minutes) in 13 x 100 mm glass tubes and the cells were resuspended in 200 ul of antiserum diluted in buffer C. This mixture was incubated for 90 minutes at room temperature on a platform rotator. The cells were centrifuged and the spent antiserum removed. The cells were washed once in 500 ul of buffer C and resuspended in 2.9 ml of buffer A. **a** cell suspension (100 ul) was then added. The cells were mixed and centrifuged. Cells were resuspended using a constant speed mixing device and left undisturbed for 20 minutes. The turbidity of the mixture was determined using a Spectronic 21 spectrophotometer at 660 nm (4). To demonstrate competition for antibody inhibition by purified α-agglutinin, increasing amounts of α-agglutinin (equilibrated in buffer C) were added simultaneously to α cells and antibody. The remainder of the inhibition assay was carried out as described above.

Indirect Fluorescent Antibody Assay: Cells from 25 ul of suspension were washed in buffer C and incubated in anti-α-agglutinin (diluted 1:20) in 100 ul of 140 mM NaCl, 30 mM Tris, pH 7.4, 10 ug/ml cycloheximide with 1% gelatin (w/v) (buffer D). After a 90 minute incubation on a platform rotator the cells were washed 3 times in buffer D and incubated for 45 minutes in 50 ul of anti-rabbit IgG conjugated with fluorescein (diluted 1:10). The cells were washed 3 times and resuspended in deionized water. The cells were then added to microscope slides coated with polylysine (70 kD) and allowed to adhere for 10 minutes. Slides were then washed with deionized water to remove unbound cells. Cells were observed and photographed using an UV microscope (Zeiss). Micrographs were taken using Tri X-pan 400 film (Kodak).

Enzyme Immunoassay: Antibody incubations in 13 x 100 mm tubes were identical to those described in the fluorescent antibody assay except that the labelled second antibody was a 1:250 dilution of anti-rabbit IgG conjugated to alkaline phosphatase. Upon completion of the second antibody incubation, the cells were washed and placed in 800 ul of substrate solution (0.1 M glycine pH 10.4, 0.01 mM $CaCl_2$, 0.01 mM $MgSO_4$ and 1 mg/ml of p-nitrophenyl phosphate). The mixture was then placed at 37°C for 1 hour. NaOH (1 M, 200 ul) was added followed by 2 ml of deionized water. The cells were

centrifuged (1000 x g for 5 minutes) and the absorbance of the supernatant was determined at 405 nm.

Results and Discussion

1. Antibody specificity and reactivity

Antiserum raised against purified α-agglutinin labelled cell surface components of **a** and α cells in indirect fluorescent antibody assays, whereas preimmune serum did not react. To eliminate cross-reacting antibodies the antiserum was adsorbed twice with intact, heat-killed **a** cells. After two adsorptions the antiserum no longer reacted with **a** cells but still reacted with α cells (Fig. 1).

The adsorbed antiserum recognized native and endo-N-acetyl glucosaminidase H treated α-agglutinin on Western blots (Fig. 2) while preimmune serum failed to react (data not shown). The specificity of α cell binding by antibody was tested by incubating the antiserum with purified α-agglutinin prior to assay. The preincubated antiserum did not bind to α cells in indirect fluorescent antibody assays (data not shown). This result indicated that the antibody which bound to α cells was directed against α-agglutinin-specific antigenic determinants.

The adsorbed antibody was also tested for its ability to inhibit the binding of α cells to **a** cells. Antiserum inhibited the agglutinin-mediated binding of the two mating types when it was preincubated with α cells (Fig. 3), but not if the preincubation was with **a** cells (data not shown). Neither preimmune serum (data not shown), nor antiserum

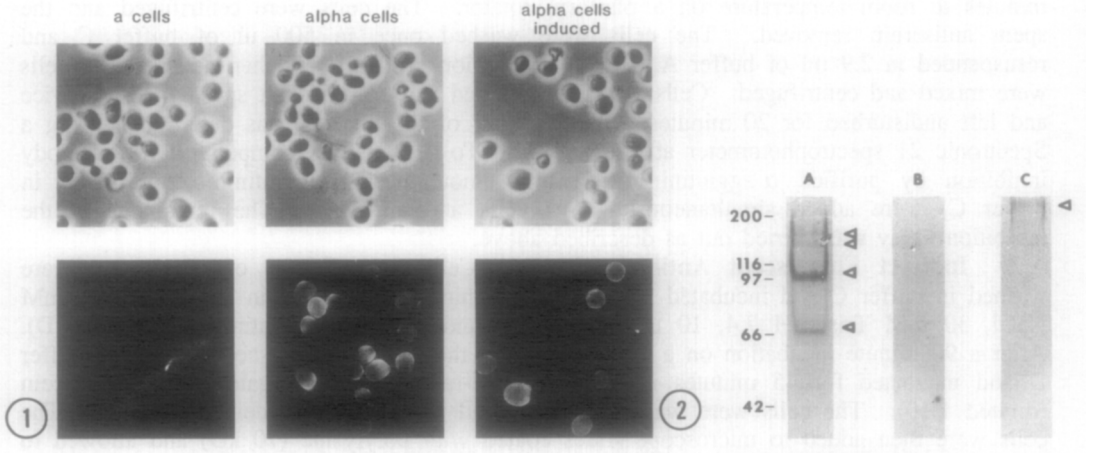
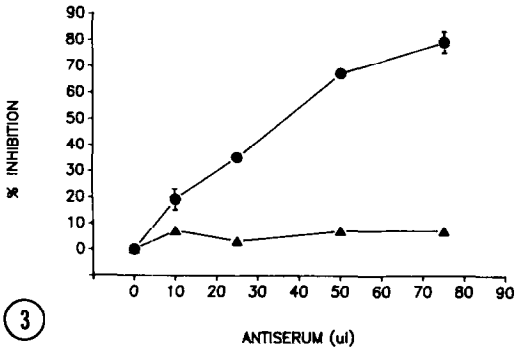


Fig. 1 Specificity of antibody and cell surface distribution of α-agglutinin. Phase micrographs (Top) and matching fluorescent micrographs (Bottom). **a** Cells, α cells and α cells induced with a-factor were incubated with **a** cell adsorbed anti-α-agglutinin followed by incubation in anti-rabbit IgG conjugated with fluorescein.

Fig. 2 **a** Cell adsorbed antiserum recognizes purified α-agglutinin by Western blotting. 250 ng of α-agglutinin (Lane C) or endo-N-acetyl glucosaminidase H treated α-agglutinin (Lane A) were electrophoresed in a 7.5% SDS-polyacrylamide gel and transferred to nitrocellulose. The blot was incubated with anti-α-agglutinin followed by incubation with secondary antibodies. Arrowheads identify α-agglutinin peptides (5). Lane B, endo-N-acetyl glucosaminidase H alone. Numbers indicate kD.



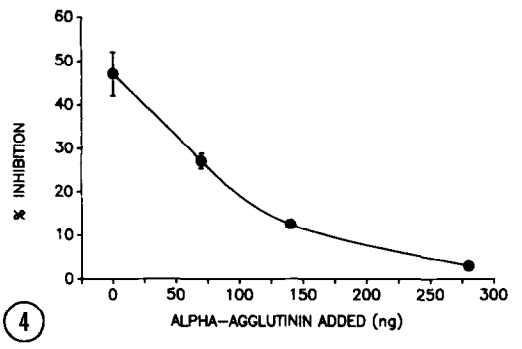


Fig. 3 Inhibition of agglutination by anti-α-agglutinin. Increasing amounts of a-cell-adsorbed antiserum (●) or α-cell-adsorbed antiserum (▲) were incubated with 1 x 10^7 α cells. The cells were then washed once with buffer and an equal number of **a** cells added. The agglutinability of the cell suspension was then assayed. Error bars (range for n=2) not visible are smaller than symbols.

Fig. 4 Competition of antibody inhibition of agglutination by purified α-agglutinin. Increasing amounts of purified α-agglutinin were incubated with 1 x 10^7 α cells and 15 ul of antiserum. The cells were then washed once with buffer and an equal number of **a** cells added. The agglutinability of the mixture was then assayed. Error bars (range for n=2) not visible are smaller than symbols.

adsorbed with α cells inhibited agglutination (Fig. 3). Inhibition effected by antibody was neutralized by adding purified α-aggluninin to compete with cell-bound α-agglutinin for antibody. Neutralization of antibody inhibition was found to be dependent upon the amount of purified α-agglutinin added to the mixture (Fig. 4). These results demonstrate that the **a**-agglutinin binding site on α-agglutinin is at least partially blocked by these α-agglutinin-specific antibodies.

2. Qualitative expression of α-agglutinin

The cell surface distribution of α-agglutinin was different for α cells pretreated for 90 minutes with the sex pheromone a-factor ("induced cells") and untreated α cells ("uninduced cells"). Most uninduced cells exhibited a polar pattern of fluorescence while induced cells fluoresced more evenly (Fig. 1).

Buds of uninduced cells seldom fluoresced (5 fluorescent buds in a sample of 50) (Fig. 5). The fraction of fluorescent buds did not appreciably change upon exposure to a-factor (4 out of 35). Smaller cells within the population also failed to bind antibody (Fig. 5). These results suggest that α-agglutinin is not expressed early in the process of cell formation. In contrast, a-agglutinin is expressed first on buds of **a** cells exposed to α-factor (9).

3. Quantitative expression of α-agglutinin

The results in Figure 4 were used to calculate the number of α-agglutinin molecules constitutively expressed. Because antibody is titrated by soluble α-agglutinin, the assay is analogous to a competitive binding radioimmunoassay (10) where soluble unlabelled antigen competes with labelled antigen for antibody. If one assumes that 1) antibody binding is

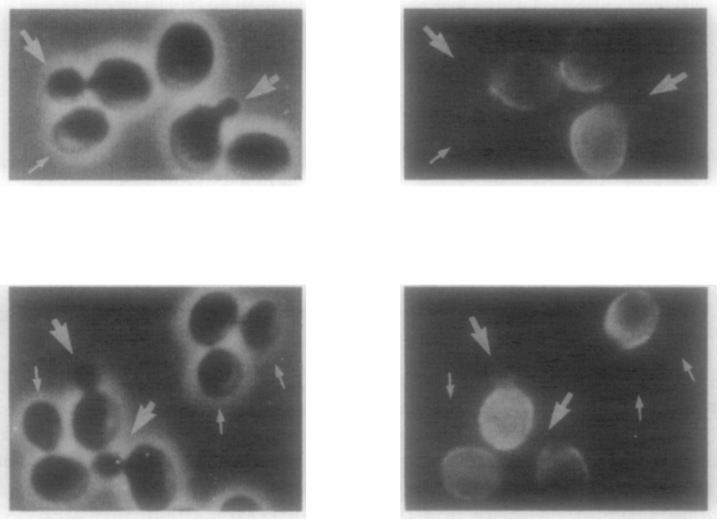


Fig. 5 α-agglutinin expression in uninduced α cells. Phase micrographs (left) and matching fluorescent micrographs (right) of log phase α cells. Buds (large arrows) and smaller cells (small arrows) which do not fluoresce are identified.

proportional to decrease in agglutinability and 2) that antibody can bind equally to both cell-bound and soluble α-agglutinin, then one can calculate the number of α-agglutinin molecules constitutively expressed on α cells. Given the peptide molecular weight of α-agglutinin to be approximately 70,000 (5), the number of α-agglutinin molecules expressed per α cell is 5 x 10^4. This value is the same as the value calculated by Watzele et al. using labelled a-agglutinin to titrate site number.

Changes in α-agglutinin expression upon exposure to a-factor were monitored by enzyme immunoasssay. Because induction of X2180-1B increases cellular agglutinability only 1.1 to 1.3 fold (2,3,4), W303-1B was used to study changes in α-agglutinin expression upon exposure to pheromone. After a 90 minute incubation in medium containing a-factor, cellular agglutination increased 2.4 fold while α-agglutinin expression increased 6.7 fold (Table 1) to 4 x 10^4 molecules per α cell. Induction of antibody binding sites in X2180-1B was 1.3-fold, consistent with the observed increase in cellular agglutinability (2,3,4).

Table 1

Comparison of Cell Agglutinability and Cell Surface Expression of α-agglutinin

Cells	Enzyme Immunoassay Substrate hydrolyzed (10^{-5} uMol/minute)*	Agglutination Index
uninduced	0.37	0.33
induced	2.45	0.79

*Values are means for triplicate determinations after subtraction of a cell controls.

The antibody has been shown to be specific for α-agglutinin and has been used to localize and quantitate this cell surface glycoprotein. Because the antibody binds to epitope(s) involved in binding to a-agglutinin, it will be used in an attempt to identify the binding domain of α-agglutinin.

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