Sexual agglutination substances require a 'carrier' glycoprotein for integration into the cell wall of *Saccharomyces cerevisiae*

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Sexual agglutination, caused by agglutination substance (AS) on a and α cell walls, is the first indispensable step of the mating reaction in ascosporogenous yeasts including *Saccharomyces cerevisiae*. The AS biosynthetic process in *S. cerevisiae* was investigated by pulse label-chase experiments with analysis by polyacrylamide gel electrophoresis (PAGE) for 16 h in the presence of urea. Because of its low mobility, AS can be separated from other proteins by prolonged PAGE. Nascent AS was integrated into cell walls after it linked covalently to a 'carrier' glycoprotein. The results suggest that the 'carrier' is synthesized stepwise through three distinct precursors (III $\rightarrow$ II $\rightarrow$ I). The 'carrier' glycoprotein (I) and its precursors (II, III) were synthesized in both a, α haploid and a/ α diploid cells. The N-glycosylation linkage inhibitor, tunicamycin, and protein synthesis inhibitor, puromycin, inhibited the III to I maturation. The results indicated that both the 'carrier' and the nascent active site of AS linked to the 'carrier' are integrated into the wall in a haploid cell while the 'carrier' alone is integrated in a diploid cell.

Keywords: *Saccharomyces cerevisiae*, sexual agglutination, agglutination substance, biosynthesis, carrier glycoprotein

INTRODUCTION

The first step in the mating reaction in ascosporogenous yeasts is sexual cell agglutination, which is caused by agglutination ligand substances (agglutination substances, ASs, or agglutinins) on the cell surface (Yanagishima, 1984; Yoshida *et al.*, 1989). In *Saccharomyces cerevisiae*, the co-dominant alleles *MATa* and *MAT α* confer a- and α -mating types, respectively. The *cis*-acting *MAT* locus controls expression of various mating-type-specific genes (MacKay & Manney, 1974; Strathern *et al.*, 1981), including production of the cell-surface AS (Tohoyama & Yanagishima, 1982). Sexual ASs from the cell wall (wAS) (Yoshida *et al.*, 1976; Yanagishima & Yoshida, 1981) and cytoplasm (cAS) (Yamaguchi *et al.*, 1982, 1984) have been isolated and characterized. The structural gene (*AG α 1*) of AS is located on chromosome

X (Suzuki & Yanagishima, 1986). Recently, the AS gene was cloned and sequenced (Lipke *et al.*, 1989).

In this paper, we describe the possible synthetic process of AS in *S. cerevisiae*.

METHODS

Strains. Prototrophic haploid strains of *S. cerevisiae*, T55 (a) and T56 (α), having constitutive sexual agglutinability, and diploid strain T556 (a/ α), were used (Tohoyama & Yanagishima, 1982).

Enzymes and chemicals. Tunicamycin was purchased from Sigma, cycloheximide, puromycin and bovine pancreas trypsin from Boehringer-Mannheim, Zymolyase-60000 (β -1,3-glucanase) from Seikagaku Kogyo, Tokyo, Japan, L-[³⁵S]methionine from New England Nuclear, and D-[2-³H]mannose from Amersham.

Labelling of cells with radioisotopes. In order to label the cells with [³H]mannose, 50 μ l of cell suspension, cultured on synthetic complete medium (SC medium) (Yamaguchi *et al.*, 1984) to the mid-exponential phase (about 5×10^6 cells ml⁻¹), was inoculated into 5 ml SC medium with [³H]mannose (0.444 MBq ml⁻¹) and cultured with stirring at 28 °C for 16 h. In the pulse-chase experiments with [³⁵S]methionine, cells that had been cultured in 5 ml SC medium to the mid-exponential phase (about 5×10^6 cells ml⁻¹), were pulse-labelled with

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Abbreviations: AS, agglutination substance; PAS, periodic acid/Schiff; PBS, 10⁻² M phosphate buffer solution, pH 5.5.

[^{35}S]methionine ($0.222 \text{ MBq ml}^{-1}$). The cells were then washed twice with 130 mM unlabelled methionine and once with SC medium. The washed cells were chased in SC medium without radioactive label. If inhibitors were added, cells were first converted to protoplasts as described below, and then were cultured in SC medium containing $5 \text{ ml } 0.8 \text{ M}$ sorbitol owing to the difficulty in inhibitor uptake.

Separation and determination of labelled agglutination substance. Labelled cells were washed twice with 130 mM unlabelled methionine and once with protoplast buffer (0.1 M citric acid/ 0.2 M Na_2HPO_4 buffer containing 0.8 M sorbitol; $\text{pH } 6.0$). To the cells, 2 ml of an isotonic protoplast buffer containing cycloheximide ($100 \mu\text{g ml}^{-1}$) and $200 \mu\text{l}$ Zymolyase solution (0.6 mg ml^{-1}) (containing 2 mM EDTA; $\text{pH } 7.2$) were added. Cell suspensions were incubated at 28°C for 1 h to allow cell wall digestion. The protoplasts were washed twice with protoplast buffer, and then 1 ml PBS containing 2 mM phenylmethylsulfonyl fluoride (PMSF) was added. After standing at 0°C for 30 min , the protoplast suspension was sonicated for cell disruption. Since AS is heat-stable, the sonicated suspension was heated at 100°C for 7 min in a water bath to inactivate degradative enzymes and was immediately centrifuged at 1500 g for 10 min (Yamaguchi *et al.*, 1982). The pH of the resultant supernatant fraction was adjusted to 2 with conc. HCl , and then the fraction was centrifuged at 56000 g for 30 min at 4°C . The supernatant fraction was neutralized with 10 M KOH and was adjusted to a final urea concentration of 8 M . The resultant sample was subjected to PAGE with a $0.5 \times 8 \text{ cm}$ disc in the presence of 8 M urea at 3 mA per gel for 16 h (Yamaguchi *et al.*, 1982, 1984). The prolonged electrophoretic run separated low-mobility AS from other contaminant proteins, which ran through. We adopted disc gels because of the convenience in sample extraction from gel slices and the higher loading capacity.

After the electrophoretic run, the gel was frozen using dry ice and cut into 1 mm slices with a razor blade. Half of the slices were used for radioactivity measurements. Each slice was solubilized in $0.5 \text{ ml } 30\%$ (v/v) hydrogen peroxide at 60°C . To the solution, 3 ml ACS-II (Amersham) was added, and then the radioactivity was measured in a liquid scintillation spectrometer (LKB 3800).

Preparation of crude α pheromone. Ten millilitres of T55 (a) cell culture, grown to early exponential phase in YHG medium (Tohoyama & Yanagishima, 1982), was inoculated into 10 litres of MMT medium, followed by aeration for 2 d (Yoshida *et al.*, 1976). Cells were removed by centrifugation and the culture supernatant was applied onto a CG 50 column to prepare partially purified α pheromone according to Tohoyama & Yanagishima (1982).

Binding activity of labelled agglutination substance to cells. PAGE gel slices corresponding to labelled AS were homogenized and centrifuged. The supernatant was added to sexually agglutinable α or α tester cells ($10^7 \text{ cells ml}^{-1}$) as described by Yamaguchi *et al.* (1982). The treated tester cells were collected onto Whatman 3MM filter paper, washed three times with 5% (w/v) TCA and once with ethanol, and then the radioactivity was measured using PPO-POPOP xylene-based scintillator.

Peptide mapping. The half of the labelled gel slices not used for radioactivity measurement were used for this procedure. Slices corresponding to AS were collected and submerged in a $150 \mu\text{l}$ solution of trypsin (1 mg ml^{-1}) in 50 mM ammonium hydrogen carbonate (NH_4HCO_3) ($\text{pH } 8.4$). The gel slice was macerated and incubated at 37°C for 24 h . After centrifugation, the

supernatant fraction was freeze-dried and subjected to PAGE for 2 h (until a bromophenol blue marker moved 4 cm using an 8 cm long gel at 3 mA per gel). The gel was cut into 1 mm slices, and the radioactivity of each slice was measured.

RESULTS

Presence of precursors of cytoplasmic α agglutination substance

For analysis of the biosynthesis of AS, its labelling in α cells was measured. *S. cerevisiae* cells in nutrient-poor media generally lose sexual agglutinability. However, they retain high agglutinability in SC medium or other standard rich media, such as YHG and YPD (Yanagishima & Yoshida, 1981; Yamaguchi *et al.*, 1984). Therefore, SC medium was chosen for labelling throughout this study.

Fig. 1. Pulse label-chase analysis of the cytoplasmic α AS. T56 (α) cells were subjected to 15 min pulse label/ 3 h chase with [^{35}S]methionine. Each $100 \mu\text{l}$ of cell lysate was subjected to prolonged PAGE. The gel was cut into slices and analysed. (a) 5 min pulse label; (b) 5 min pulse label/ 10 min chase; (c) 15 min pulse label/ 3 h chase. The values of radioactivity in double-thickness slices at the ends of the histogram are mean values of two activities.

Fig. 2. Coincidence of [^{35}S]methionine-labelled band with purified cytoplasmic αAS . Purified αAS (10 μg) and 100 μl [^{35}S]methionine-labelled cell lysate were subjected to prolonged PAGE and analysed. The gel stained with PAS reagent is shown at the top of the Figure. The gel was cut into 1 mm segments and the radioactivity of each segment was measured. Arrows I to III correspond to peaks I to III in Fig. 1.

Fig. 3. Effect of puromycin on αAS synthesis. Protoplasts were labelled with [^3H]mannose (0.444 MBq ml^{-1}) for 1 h. After washing twice with 0.8 M sorbitol containing 200 mM mannose, puromycin (4 mg ml^{-1}) was added and the mixture was incubated for 1 h. After washing twice with 0.8 M sorbitol containing 200 mM mannose, the protoplasts were labelled with [^{35}S]methionine for 1 h. The double-labelled sample was separated by prolonged PAGE. Arrows I to III correspond to peaks I to III in Fig. 1. $\circ$, [^3H]Mannose radioactivity; $\bullet$, [^{35}S]methionine radioactivity.

The cytoplasmic agglutination substance (cAS) was successfully separated as a single band by prolonged PAGE for 16 h since contaminant proteins ran through. This separation technique was based on the known low mobility of cAS in PAGE (Yamaguchi *et al.*, 1982, 1984).

In order to determine the presence of AS precursors and their maturation process, the cells were subjected to a

5 min pulse label with [^{35}S]methionine, a 5 min pulse label followed by a 10 min chase, and a 15 min pulse label followed by a 3 h chase, all at 28 $^{\circ}\text{C}$. The cytoplasmic extract of labelled cells was analysed by PAGE. After a 5 min pulse, three major peaks (I, II, III) were detected (Fig. 1a). Peak III diminished and peak II increased after a 10 min chase (Fig. 1b). After a 3 h chase, peak II decreased and was replaced by peak I, probably owing to an increased molecular mass (Fig. 1c). The results were interpreted as follows: the disappearance of peak I, which contains cAS, indicates the integration of AS into the wall, while the disappearance of peak III indicates its conversion into peak II, which is subsequently converted into peak I. The proposed conversion process is discussed in more detail later.

The cell extract from pulse-labelled/3 h chase cells was mixed with purified αAS (Yamaguchi *et al.*, 1982) and was then subjected to PAGE. After staining with PAS reagent to detect glycoproteins, the gel was cut into slices, and radioactivity measured. The peak of ^{35}S labelling (peak I of Fig. 1) always coincided with the band stained with PAS reagent (Fig. 2). When cell extract labelled with [^3H]mannose was analysed, the labelled peak again corresponded to peak I of Fig. 1 (data not shown). These data indicate that cAS contained a glycoprotein.

The labelled peak from 15 min pulse label/3 h chase cells corresponded to cAS, which must have matured and migrated to the cell surface in about 3 h after synthesis. The identification of AS was confirmed by the detection of labelled WAS separated by the autoclave method (Yoshida *et al.*, 1976) after a 3 h chase (data not shown).

Effect of inhibitors on the synthesis of α agglutination substance

Tunicamycin, a selective inhibitor of asparagine-linked glycosylation, was added to cells 30 min before they were pulse-labelled with [^{35}S]methionine. Tunicamycin treatment gave rise to broad peaks of labelling of higher electrophoretic mobility and thus of lower apparent molecular mass (data not shown). This suggests that the inhibition of *N*-glycosylation results in a smaller AS size.

Puromycin treatment gave rise to broad peaks of [^3H]mannose and [^{35}S]methionine labelling (Fig. 3). This was essentially the same electrophoretic profile as that seen after tunicamycin treatment. Because puromycin inhibits protein synthesis (Melcher, 1971), producing peptide fragments of various sizes, the broad peaks suggest that proteins with different sizes were linked to saccharides.

Effect of temperature and sex pheromone on the synthesis of α agglutination substance

T56 (α) cells lose agglutinability at 37 $^{\circ}\text{C}$ because the *AG α 1* gene is temperature-sensitive (Yanagishima & Yoshida, 1981). However, the agglutinability can be restored by the addition of pheromone, a process inhibited by cycloheximide (Tohoyama *et al.*, 1979). The effect of

Fig. 4. Effects of temperature shift and a pheromone on the synthesis of α AS. T56 (α) cells cultured at 37 °C were treated with a pheromone and analysed by prolonged PAGE. (a) α cells were cultured at 37 °C, and 30 μ l a pheromone was added to the cell suspension. After 30 min incubation, the cells were labelled with [35 S]methionine for 12 h. (b) After α cells had been cultured at 37 °C, the cells were labelled with [35 S]methionine for 12 h. (c) After α cells had been cultured at 28 °C, the cells were labelled with [35 S]methionine for 12 h. Arrows I to III correspond to peaks I to III in Fig. 1. The values of radioactivity in double-thickness slices at the ends of the histogram are mean values of two activities.

pheromone and temperature on AS synthesis was examined. As shown in Fig. 4, there were no distinct differences in the incorporation of radioactivity between the samples from cells cultured at 28 °C, at 37 °C, and at 37 °C in the presence of the α pheromone, although the incorporation of radioactivity was lower at 37 °C. In T556 (α/a) diploid cells, peak I (corresponding to cAS) had high radioactivity after culture at 28 °C (Fig. 5a) and at 37 °C (data not shown).

To examine the biological activity of each peak I, the gels shown in Figs 1(c), 4 and 5 were cut into slices and the eluates of slices corresponding to the peak of radioactivity were tested. The eluate from peak I slices in agglutinable haploid cells showed about three times greater adsorption to α cells than to α cells, although there was some background caused by non-specific adsorption (data not shown).

These results indicate that both haploid and diploid cells synthesize peak I substance to which cAS anchors in agglutinable haploid cells.

Fig. 5. The presence of peak I moiety in diploid cells. [35 S]Methionine-labelled cell lysate was prepared. Each 100 μ l of labelled lysate was subjected to prolonged PAGE and analysed. (a) Sample from T556 (diploid, α/a) cells; (b) sample from T56 (α) cells. Arrows I to III correspond to peaks I to III in Fig. 1. The values of radioactivity in double-thickness slices at the ends of the histogram are mean values of two activities.

Fig. 6. Peptide mapping of peak I substance from haploid cells and diploid cells. The radioactive peak I from (a) T556 (diploid, α/a) or (b) T56 (α) cells in Fig. 5 was analysed by PAGE after trypsin digestion.

Fig. 7. Possible scheme of AS biosynthesis. In α and α haploid cells, and α/α diploid cells, a common 'carrier' ($\square$) and its precursor ($\boxminus$) are synthesized in the cytoplasm. The nascent active site of a ($\blacktriangle$) or α ($\blacktriangle$) protein moiety becomes cAS to link peak I ($\boxplus$) and is subsequently integrated into the cell wall. By the autoclave method or enzymic method (Yoshida & Yanagishima, 1981), the AS substance on the cell wall is released with a part of the 'carrier'. Although the biosynthesis of nascent active sites themselves is influenced by inhibitors, we omitted it from this scheme. The T-type arrows ($\top$) indicate inhibition sites. The open arrows ($\uparrow$) indicate activation sites. I, II, III correspond to peaks I, II, III in Fig. 1. Abbreviations: CH, cycloheximide; TM, tunicamycin; PM, puromycin.

The α AS has an active site in its protein moiety (Yanagishima & Yoshida, 1981; Yamaguchi *et al.*, 1982). In order to examine the difference between peak I substances from agglutinable haploid cells and non-agglutinable diploid cells (Fig. 5), these were subjected to peptide mapping. Haploid cells and diploid cells were labelled with [35 S]methionine, and after separation by PAGE with SDS, peak I was completely digested with trypsin. The peak I digests of haploid and diploid cells were distinct (Fig. 6), indicating that the peak I substances of agglutinable haploid cells and nonagglutinable diploid are different. The difference is due to the presence or absence of cAS in peak I.

While investigating the presence of precursors of AS, we found peak I substances in the cytoplasm of *S. cerevisiae* cells (Fig. 1). Peak I substances were radiolabelled in non-agglutinable haploid cells (Fig. 4) as well as in diploid cells (Fig. 5a), but AS activity in peak I was present in only agglutinable haploid cells. Since our prolonged PAGE cannot resolve subtle differences in the size of large glycoproteins with high carbohydrate content such as cAS (Yamaguchi *et al.*, 1982, 1984) because of their very low mobility, we cannot separate AS active peak I from non-AS active peak I. However, peptide mapping clearly

AS has a very high saccharide content and the active site is in the protein moiety (Yoshida *et al.*, 1976; Yanagishima & Yoshida, 1981; Yamaguchi *et al.*, 1982, 1984). Lipke *et al.* (1989) showed that the *AGα1* gene encodes a 650-amino-acid protein with a putative signal sequence, 12 possible *N*-glycosylation sites, and a high content of serine and threonine residues. We previously isolated active αAS and estimated it to be about 200 kDa, with a carbohydrate content of more than 70% (Yamaguchi *et al.*, 1982). The discrepancy in molecular size is due to the polysaccharide moiety in αAS. Recent data by Cappellaro *et al.* (1991) are consistent with our high molecular mass of AS. They also indicate that the histidine-containing active protein region of αAS is essential for interaction with aAS but the saccharides are not essential.

shown in Fig. 1, where the experiments were done at 28 °C, except that no radioactivity was apparent in peak I in Fig. 1(a) (data not shown). This indicates that the relatively high pulse radioactivity of peak I in Fig. 1(a) is due to the radioactivity of AS but not to the 'carrier'. The disappearance of peak I in Fig. 1(b) is explained by the integration of AS into the wall. The 'carrier' may mature through III to I. Both the 'carrier' and cAS ('carrier'-bound AS active site) fall in peak I by prolonged PAGE. In other words, the binding of a relatively small AS active site may not increase the electrophoretic mobility because the size of the 'carrier' is estimated to be more than 140 kDa with a carbohydrate content of more than 70% (Yamaguchi *et al.*, 1984). After the active site of AS is covalently linked to the 'carrier', the matured AS now may be anchored into a cell wall. This is shown schematically in Fig. 7.

The puromycin experiment (Fig. 3) indicates that the 'carrier' for AS matured from its precursors, and its maturation required the covalent linking of newly synthesized protein. If no such common 'carrier core' existed in AS, newly synthesized proteins (³⁵S peak), present after the removal of puromycin, could not be linked to the glycoproteins (³H peak) synthesized before the addition of puromycin, so the peaks of ³⁵S and ³H would not be coincident with each other. However, ³⁵S and ³H were in fact reasonably coincident with each other over a relatively broad range (Fig. 3). This supports the idea of a 'common carrier' in Fig. 7.

In a study using secretion-deficient *sec* mutants (Tohoyama & Yanagishima, 1987), a pheromone induced the agglutinability of wild-type cells at 37 °C but not that of mutant cells. The study also indicated that both aAS and α AS may be secreted through a common path shared by invertase and acid phosphatase. This suggests that ASs as well as these secretory enzymes are concentrated in the top portion of the shmoo during conjugation. In fact, Watzele *et al.* (1988) have shown that the binding fragment of aAS localizes at the growing shmoo tip of a cells after exposure to α factor. Furthermore, the *FUS* product, which functions in the fusion of a and α cells to produce a zygote, shows a similar localization after exposure to pheromone (McCaffrey *et al.*, 1987; Truehart *et al.*, 1987). The shmoo tips are likely to correspond to the regions of the a cell and α cell that fuse to form a zygote; a high local concentration of proteins involved in fusion may therefore be required in this region to promote efficient fusion. Similar localization of α AS in the shmoo tip by exposure to a pheromone could also account for the discrepancy between the observed 20-fold induction of *AG α 1* RNA and only a 1.5-fold increase in cellular agglutinability (Lipke *et al.*, 1989; Wojciechowicz & Lipke, 1989).

If our present data are considered together with the AS-localization in shmoo tips (see above) and the rigidity of the cell wall, it is plausible that proteins, including AS, are not directly inserted into the cell wall but are inserted by a common glycoprotein 'carrier' (Fig. 7). The results presented here suggest that the biosynthesis of AS is different from those of glycoprotein enzymes. The matu-

ration time of secretory invertase and carboxypeptidase Y in yeasts is in the range of 50–60 min (Novick *et al.*, 1981; Stevens *et al.*, 1982), while that of α AS was about 3 h. Although no such precursors have been observed for the other secretory proteins discussed, the 'carrier' for AS matured stepwise through three precursors (peaks I, II, III) (Fig. 1).

Therefore, it is most likely that the nascent active site of AS is linked to a 'carrier' glycoprotein and subsequently the 'carrier' is introduced into specific portions such as the shmoo in cell walls.

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