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Synthesis of an O-glycosylated cell surface protein induced in yeast by α factor

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ABSTRACT **A number of cell surface glycoproteins can be specifically and completely released from intact cells of *Saccharomyces cerevisiae* with 0.5% mercaptoethanol. Among these proteins is one with a molecular mass of 22 kDa, which is synthesized only in haploid a cells treated with the peptide mating pheromone α factor. This protein could be radiolabeled *in vivo* with [2-^{3}H]mannose, [^{14}C]phenylalanine, and [^{35}S]sulfate. Its synthesis and export to the cell surface were not inhibited by tunicamycin. β-Elimination released almost all radioactivity from the [2-^{3}H]mannose-labeled protein, 36% of its radioactivity being recovered subsequently as mannose and 43% as a dimannoside. Evidence is presented that the 22-kDa O-glycosylated protein is a mating-type specific a cell agglutinin.**

Saccharomyces cerevisiae mating pheromone α factor causes a first-cycle growth arrest in G_1 when added to haploid a cells (1). In addition, the shape of the cells changes ("shmoo" formation), as does their cell surface, thereby increasing agglutinability with α cells, their mating partners (2–4).

So far, few biochemical changes correlating with α-factor treatment have been reported. Thus, the overall cell wall composition changes (5), and a significant increase in the amount of chitin synthesized (6, 7) and in chitin synthase activity (6) is observed.

Stetler and Thorner (8) have presented evidence that transcription of a number of genes is turned off, or in some cases on, after α factor administration. However, corresponding proteins have not been described up to now.

It will be shown here that a protein of 22 kDa is synthesized in *S. cerevisiae* a cells only when the cells are treated with α factor. This protein is solely O-glycosylated; it can be quantitatively removed from intact cells under mild conditions. Since the protein inhibits agglutination of a with α cells, it most likely is the agglutinin of a cells.

MATERIALS AND METHODS

Radiolabeling of Yeast Cells. Exponentially growing cultures with an initial OD_{578} of 1.2 were used in all experiments. *S. cerevisiae* line X2180-1A was grown in defined medium containing 0.6% sucrose (9); the bar1-1 mutant (carrying the *bar1* mutation)—unless indicated otherwise—were grown in a medium containing 1% yeast extract, 2% Bactopeptone, and 0.6% sucrose. Where appropriate, synthetic α factor (Bachem) was added to give a final concentration of 12 μM (cell line X2180-1A) or 6 nM (bar1-1 mutant); tunicamycin was added to X2180-1A to give a concentration of 8 μg/ml. Incubation was then continued for 10–15 min, whereupon [2-^{3}H]mannose (60 or 120 μCi/ml; specific activity, 11.4 Ci/mmol; 1 Ci = 37 GBq), [1-^{14}C]glucosamine (10 μCi/ml; 54 Ci/mol), [^{35}S]sulfate (20 μCi/ml; 2–4 Ci/mg), or L-[U-^{14}C]phenylalanine (2 μCi/ml; 20 mCi/mmol) was added. Radiolabeling was continued for 90–120 min, incorporation of the radioactivity being continuous under these conditions, and then cells were harvested by centrifugation.

Extraction of Cell Surface Glycoproteins. Cells from radiolabeled cultures were washed twice with 2 mM Tris·HCl (pH 8.0) and transferred to 1.5-ml capped Microfuge tubes. The cells were resuspended in 2 mM Tris·HCl (pH 8.0) containing 0.5% 2-mercaptoethanol, and the tubes were shaken vigorously and routinely for 2 hr at room temperature with an Eppendorf mixer. Then cells were sedimented by centrifugation, the supernatant was collected, and the cells were washed once with 0.2 ml of Tris·HCl (pH 8.0). The supernatants (mercaptoethanol extract) were pooled, $NaDodSO_4$/PAGE sample buffer was added, and the extract was concentrated in a Speed-Vac concentrator (Savant). After mercaptoethanol treatment, 0.25 ml of 20 mM Tris·HCl (pH 7.4) containing 10 μg of zymolyase 60,000 (Miles) per ml was added to the intact cells remaining after extraction with mercaptoethanol, and the suspension was shaken for 7 min at room temperature before centrifugation for 2 min. Few, if any, yeast cells are lysed by this treatment. $NaDodSO_4$/PAGE sample buffer was added to the supernatant, and this zymolyase-released material was concentrated. The amount of radioactivity in the fractions was determined, and samples of the fractions were submitted to $NaDodSO_4$/PAGE (10) on gels containing 10% or 12% acrylamide. Radiolabeled bands were detected by fluorography. To quantify the release of the 22-kDa protein by mercaptoethanol in the experiment of Table 1, fractions were separated by $NaDodSO_4$/PAGE, and the protein was located after fluorography. The areas of the dried gel corresponding to the 22-kDa protein were cut out, and their radioactivity was determined. The total radioactivity in the lane containing the 2-hr mercaptoethanol extract was also determined to correct for the recovery of radioactivity on the dried gel and so to allow estimation of the amount of the 22-kDa protein in the original extracts.

Agglutination Assay. Agglutination of pheromone-induced cells was measured by the procedure of Pierce and Ballou (11). X2180-1A (MAT a) or X2180-1B (MAT α) cells were incubated with α factor (6 μM) or a factor (20 units per ml), respectively, for 2 hr in a medium containing 1% yeast extract, 2% Bactopeptone, and 2% glucose. Incubation was stopped by adding cycloheximide at 15 μg/ml, and cell density was adjusted to 1.5×10^8 cells per ml. Then 50 μl of α cells, 125 μl of agglutination buffer, 0.1 M sodium phosphate (pH 6.0), and 25 μl of extract in increasing dilutions (2:1) were added into flat-bottom microtiter plates (15 × 17 mm wells). After 1 hr of rotary shaking (110 strokes of 1.5 cm per min), 50 μl of a cells were added. After 10 more min of shaking, agglutination can easily be seen in noninhibited

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Table 1. Time-dependence of the release of the α factor-induced 22-kDa glycoprotein by mercaptoethanol

Fraction	Radioactivity released in minutes of treatment, cpm × 10^{-4}			
	15	60	120	180
Mercaptoethanol				
Total fraction	77	240	380	380
22-kDa protein	1.9	2.3	3.9	3.7
Zymolyase				
Total fraction	390	630	830	730
22-kDa protein	0.5	0.2	<0.2	<0.2

A 10-ml culture of the bar1-1 mutant was preincubated with α factor for 10 min and then radiolabeled with [2-^{3}H]mannose for 2 hr. The cells from 2.5-ml portions were then extracted with mercaptoethanol for various times, after which they were treated with zymolyase as described. The fractions were then submitted to NaDodSO$_4$/PAGE. The area of the gel containing the 22-kDa glycoprotein was cut out, and its radioactivity was determined as described.

controls. One assay unit is defined as the minimal amount of extract that clearly inhibited agglutination under these conditions. In this assay, the crude mercaptoethanol extract of two induced **a** cells (*bar1*) inhibits agglutination of one pair of induced **a**/α cells.

β-Elimination. The mercaptoethanol extract of [2-^{3}H]mannose-labeled, pheromone-induced *bar1* cells was submitted to NaDodSO$_4$/PAGE on a gel containing 12% acrylamide. The area of the wet gel corresponding to the 22-kDa protein was cut out and eluted into 0.1% NaDodSO$_4$ solution. This solution was filtered, concentrated in a Speed-Vac concentrator, and washed twice with a 10-fold excess of ice-cold 1-butanol; 30 μl of 0.2 M NaOH was added to the dried pellet, and the mixture was incubated for 25 hr at room temperature. The suspension was then neutralized with 30 μl of 0.2 M HCl and submitted to paper chromatography on a Whatman 3 MM chromatography paper; the chromatogram was developed for 24 hr in ethyl acetate/1-butanol/acetic acid/water, 3:4:2.5:4 (vol/vol). The paper was cut into 1-cm strips, which were assayed for radioactivity in a liquid scintillation counter.

RESULTS

Release of Cell Surface Components from Intact Yeast Cells by Mercaptoethanol. When logarithmically growing **a** cells of *S. cerevisiae* X2180-1A were incubated with D-[2-^{3}H]mannose for 90 min, radiolabeled mannoproteins could be released subsequently from intact cells by treating them with mercaptoethanol, a method previously applied to solubilize external invertase (12). On NaDodSO$_4$/polyacrylamide gels, four proteins with molecular masses of 38, 49, 68, and 88 kDa could be resolved as relatively distinct bands (Fig. 1, lane 2). The 49-kDa and the 88-kDa proteins seemed to be N-glycosylated, since their synthesis was completely inhibited by tunicamycin (lane 3) and they both were radiolabeled in the presence of [^{14}C]glucosamine (lane 1). The 38-kDa and the 68-kDa proteins probably were exclusively O-glycosylated, although their synthesis was somewhat affected by tunicamycin. However, they were not radiolabeled with [^{14}C]-glucosamine. In addition, it is generally observed that tunicamycin inhibits O-mannosylation in yeast to some extent (13), although the mechanism of this inhibition is not yet understood. Besides these four proteins, 90% of the material radiolabeled with mannose and released with mercaptoethanol migrated on NaDodSO$_4$ gels with a molecular mass > 130 kDa (Fig. 1).

α-Factor Treatment Induces an Additional Protein That Is Released by Mercaptoethanol. After incubation with α factor (12 μM), a fifth, well-defined mannoprotein with an apparent

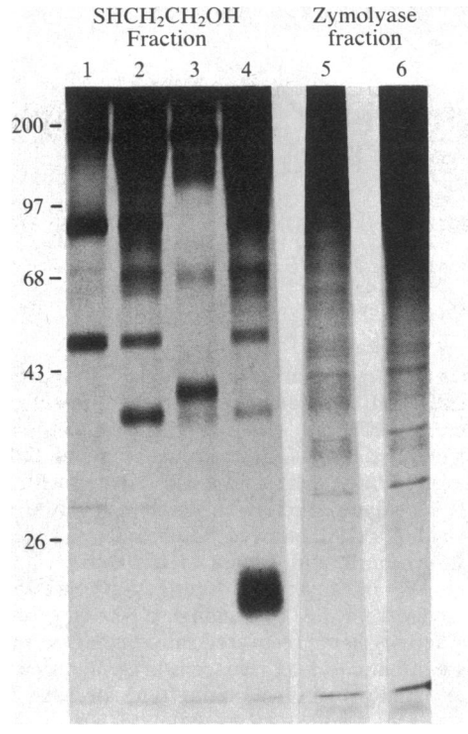

FIG. 1. Release of radiolabeled cell surface glycoproteins from *S. cerevisiae* X2180-1A by 2-mercaptoethanol and zymolyase. Cells were radiolabeled with [2-^{3}H]mannose or [1-^{14}C]glucosamine for 90 min, then extracted with 2-mercaptoethanol (for 2 hr), and subsequently treated with zymolyase as described. Extracts were submitted to NaDodSO$_4$/PAGE, and radiolabeled proteins were made visible by fluorography. Lanes: 1 and 2, material released by mercaptoethanol from control cultures labeled with [^{14}C]GlcN (lane 1) or [^{3}H]Man, respectively; 3 and 4, material released by mercaptoethanol from cultures radiolabeled with [2-^{3}H]Man in the presence of tunicamycin (TM) or α factor, respectively; 5 and 6, material released by zymolyase from mercaptoethanol-extracted cells radiolabeled with [2-^{3}H]Man in the absence and presence, respectively, of α factor. Sizes are shown in kDa.

mass of 22 kDa could be released from **a** cells by mercaptoethanol (Fig. 1, lane 4). It was completely absent from extracts of control cells not treated with α factor. The bar1-1 mutant, which does not degrade the α-factor peptide (14), synthesized the 22-kDa protein in the presence of 6 nM α factor per ml (see below and Fig. 2*A*). This protein was not synthesized when α cells were treated with α or **a** factor (data not shown).

All four or, from α-factor-treated **a** cells, five mannoproteins were quantitatively extracted with mercaptoethanol; even subsequent treatment with zymolyase (an endo-1,3-β-D-glucanase) did not release any more of these proteins. The material released by zymolyase was almost exclusively of high molecular weight, >100 kDa (Fig. 1, lanes 5 and 6). After α-factor treatment, these high molecular mass glycoproteins were somewhat smaller in size, as has been reported (15). The Coomassie-stained gel of Fig. 1 did not show any protein bands in lanes 1–4, whereas in lanes 5 and 6, a significant amount of protein released by the zymolyase treatment was visible (data not shown).

To optimize the release of material, cells of the bar1-1 mutant radiolabeled with [2-^{3}H]mannose in the presence of α factor were treated with mercaptoethanol for various times (Table 1). The 22-kDa protein was solubilized most easily, as indicated by the occurrence of the highest percentage of 22-kDa protein in the 15-min extract. Two hours in the presence of 0.5% mercaptoethanol was sufficient for complete release of the 22-kDa protein. Although subsequent

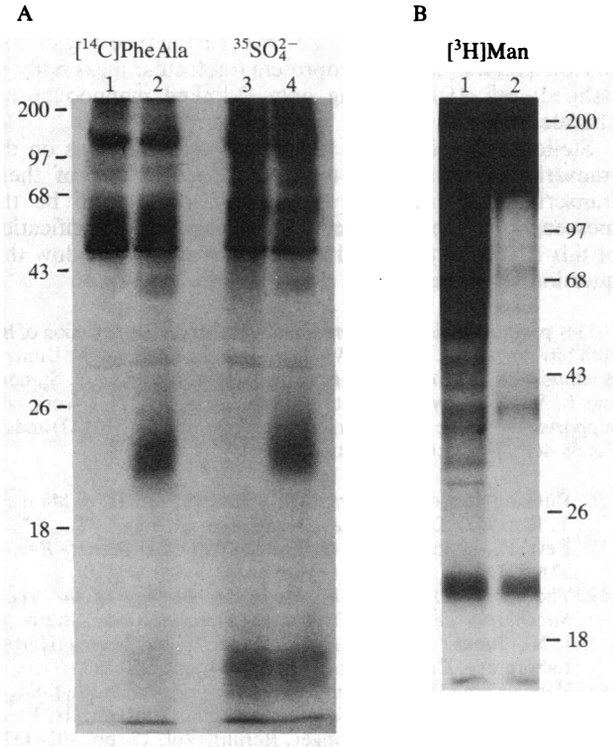

FIG. 2. Fluorogram of material extracted with mercaptoethanol and separated by NaDodSO$_4$/PAGE. (*A*) The 22-kDa protein is radiolabeled by [^{35}S]sulfate and by [^{14}C]phenylalanine. Cells (bar1-1 mutant) were preincubated with (lanes 2 and 4) or without (lanes 1 and 3) α factor for 15 min. The cultures were then radiolabeled with [^{35}S]sulfate (lanes 3 and 4) or with [^{14}C]phenylalanine (lanes 1 and 2) as described prior to extraction with mercaptoethanol. (*B*) Tunicamycin does not prevent synthesis or export of the α factor-induced cell surface glycoprotein. The bar1-1 mutant was preincubated with α factor for 15 min, whereupon tunicamycin (8 μg/ml) was added to half the culture (lane 2). This and the α-factor-treated control culture (lane 1) were then radiolabeled for 105 min with [2-^{3}H]mannose prior to extraction with mercaptoethanol.

treatment of the cells with zymolyase solubilized 2- to 4-fold more radioactivity, the 22-kDa protein was not completely extracted in this way, when it had not been fully removed by prior mercaptoethanol extraction (see 15-min values). The extent of release of cell surface material by zymolyase, on the other hand, depended on the length of time the cells were preincubated with mercaptoethanol.

It was necessary to prove that the radiolabeled material with an apparent mass of 22 kDa is protein in nature and to show that, in the presence of α factor, not only glycosylation reactions are activated, but also the whole molecule is synthesized. Therefore, cells were radiolabeled with [^{14}C]phenylalanine or with [^{35}S]sulfate under the same conditions as in Fig. 1 except that *bar1* cells and 10 ng of α factor were used. It could be demonstrated clearly (Fig. 2*A*) that with both precursors the same band on the gel was radiolabeled, that this material was completely released by 0.5% mercaptoethanol, and that this product was not formed in control cells.

The 22-kDa Protein Is Synthesized and Exported in the Presence of Tunicamycin. Since the 22-kDa protein was never observed when cells were labeled with [^{14}C]glucosamine, it seemed possible that this protein does not contain any asparagine-linked oligosaccharides. However, this lack of incorporation could have been due to the strong intracellular dilution of [^{14}C]glucosamine with a nonradioactive precursor that is caused by α factor (7). To clarify this point, cells were incubated with [2-^{3}H]mannose in the presence of α factor and tunicamycin. Clearly, the α factor-induced cell surface component was also synthesized under these conditions (Fig. 2*B*).

The 22-kDa Protein Is O-Glycosylated. Since 22-kDa material eluted from gels and treated with endoglycosidase H also did not change its molecular weight (data not shown), it appears that the protein is solely O-glycosylated. All saccharides should be released, therefore, by mild alkaline treatment (β-elimination). When this was carried out and the released radioactive material was separated by paper chromatography, the profile in Fig. 3 was obtained. The two main saccharide components released were a dimannoside (43% of the radioactivity) and a mannoside (36%); in addition, about 15% was present as tri- and tetrasaccharide. Only 5% of the radioactivity remained at the origin of the chromatogram, where asparagine-linked oligosaccharides still attached to protein under this condition would be located.

Evidence That the 22-kDa Protein Is an Agglutinin. Yanagishima and coworkers (4, 16) obtained a protein of 23 kDa by autoclaving whole cells for a short time. This protein prevented agglutination of **a** and α cells. The authors used haploid **a** cells that were constitutive for agglutination with α cells for these experiments, and they assumed that the protein released was responsible for the agglutination reaction associated with mating. To see whether the present O-mannosylated cell surface protein, synthesis of which is induced by α factor in **a** cells, is an agglutinin, we checked whether it prevents agglutination of **a** cells with α cells. The test was carried out as described. The fraction released by mercaptoethanol strongly inhibited agglutination of **a** cells with α cells, both having been induced previously with the reciprocal mating hormone. The corresponding extract obtained from **a** cells that had not been pretreated with α factor before extraction showed a lower inhibitory activity by a factor of 30. Since the only component observed under the various labeling conditions (Fig. 1 and 2*A*) that differed in the two extracts was the 22-kDa component, it seems likely that it is responsible for inhibiting agglutination by reacting with and partly blocking a counterpart in α cells. This conclusion was supported by the result that only material extracted from the corresponding region of the gel showed inhibitory activity (Table 2). This result also demonstrates that the glycoprotein retains its biological activity even after treatment with NaDodSO$_4$; >60% of the activity applied to the gel could be recovered again.

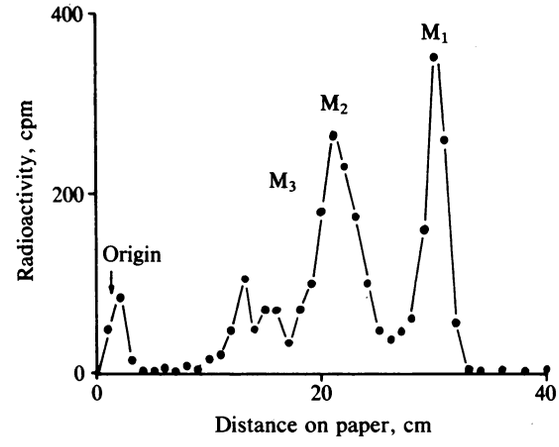

FIG. 3. β-Elimination of O-glycosidically linked sugar chains of the 22-kDa protein. [2-^{3}H]Mannose-labeled 22-kDa protein purified by NaDodSO$_4$/PAGE underwent β-elimination with 0.2 M NaOH for 25 hr. After neutralization, the material was applied onto paper for chromatography, and the radioactivity was determined as described. The peaks designated M_1, M_2, and M_3 coincide with the positions of added standards mannose, maltose, and raffinose.

Table 2. Biological activity extracted from pheromone-induced **a** cells after $NaDodSO_4$/PAGE

Excised region	Biological activity, assay units
Front–18 kDa	40
18–26 kDa	640
26–50 kDa	0
50–200 kDa	0

The excised regions of the gel were extracted overnight with 0.1% $NaDodSO_4$. The gel extracts were freeze-dried, extensively washed with ice-cold 1-butanol to remove $NaDodSO_4$, dialyzed, and finally concentrated for the biological assay.

DISCUSSION

Although there are a number of reports of general changes in yeast cell wall composition caused by the action of α factor (5, 6, 15, 17), no specific changes in cell wall glycoproteins have so far been identified. Since it is known that cell surface components can be released from intact yeast cells by mercaptoethanol (12) without affecting their viability at all (W.T., unpublished data), this method in combination with $NaDodSO_4$/PAGE was used to find glycoproteins whose synthesis is possibly specifically induced or repressed by mating pheromones. Whereas four components, well defined at least in terms of molecular weight, were solubilized in this way from control **a** cells, the existence of an additional protein (22 kDa) was observed after α-factor treatment. This latter protein and two other ones (38 and 68 kDa) seem to be exclusively O-glycosylated. β-Elimination of the [2-^{3}H]mannose-labeled 22-kDa protein released 95% of its radioactivity. Unusual was the ratio of ^{3}H-labeled dimannoside/[^{3}H]mannose of 1.2, since this ratio usually is >2.5 in O-linked chains from bulk yeast glycoprotein (13). The 22-kDa protein can be labeled with [^{35}S]sulfate and could possibly be linked, therefore, by a disulfide bridge to other cell wall components. However, the possibility that the various proteins released are only indirectly affected by mercaptoethanol because of a general cell wall loosening cannot be excluded (18).

Yanagishima and coworkers (4, 16) have isolated two proteins from **a** cells constitutive for sexual agglutinability that prevent agglutination when added to a mixture of agglutination-competent **a** and α cells. One of these proteins (23 kDa) is considered a cell wall constituent, and the other (130 kDa), a cytoplasmic one (19). Obviously, the 23-kDa protein could be identical to the α-factor-induced cell surface protein described here, although the observation that the 23-kDa protein is inactivated by mercaptoethanol and is thought to consist of two subunits (16, 19) is at variance with this conclusion.

It has been reported that tunicamycin added to **a** cells during α-factor treatment does not inhibit the induction of agglutination (20, 21). This observation supports the conclusions drawn here concerning the nature and function of the 22-kDa protein. The synthesis of this protein and its transport to the cell surface is not grossly affected by tunicamycin (Fig. 2*B*). Finally, it should be pointed out that the type 5 agglutinin of *Hansenula* is also a glycoprotein (molecular mass not well defined; ref. 4) containing only O-linked mannooligosaccharides (22).

Stetler and Thorner (8) found that α factor turns on the transcription of at least two genes; either one of these transcripts, 450 and 650 base pairs long, could be the messenger for the gene product described here. Purification of this 22-kDa protein and sequence studies will allow this question to be answered.

This paper is dedicated to Dr. Luis F. Leloir on the occasion of his 80th birthday, Sept. 6, 1986. We thank Drs. R. Betz and W. Duntze, Bochum, F.R.G., for supplying purified **a** factor and Drs. P. Sijmons and F. Klis for advice in setting up the bioassay. This work was supported by the Deutsche Forschungsgemeinschaft (SFB 43) and by Fonds der Chemischen Industrie.

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