Apparent inhibition of glycoprotein synthesis by *S.cerevisiae* mating pheromones

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Saccharomyces cerevisiae mating pheromones *a* and α factor strongly inhibit the incorporation of radiolabelled glucosamine into *N*-glycosylated proteins of corresponding haploid cells. This observation was erroneously interpreted as an inhibition of glycoprotein synthesis. It has turned out that α factor causes a 4–5-fold dilution of incorporated [^{14}C]glucosamine with non-radioactive endogenous precursor. In the case of the [^{14}C]chitin synthesized, which does not show inhibition by α factor, the lowering of the specific activity of the precursor is exactly compensated for by an increased rate of chitin synthesis caused by α factor.

α Factor *Chitin* *Glycoprotein* *Yeast*

1. INTRODUCTION

Saccharomyces cerevisiae mating pheromones (*a* and *α* factor), when added to corresponding haploid cells, cause a first cycle growth arrest in G1, in addition to changing the cell's shape and surface [1]. Recently we reported that the incorporation of [^{14}C]glucosamine into water-soluble and SDS-extractable glycoproteins of haploid *a* cells is strongly inhibited by *α* factor, whereas the incorporation of radiolabelled glucosamine into cell walls (mainly chitin) was not affected [2,3]. These results were interpreted as a specific inhibition of the synthesis of *N*-glycosylated proteins by *α* factor.

We report here that this interpretation is wrong. Further experiments have shown that *α* factor leads to a dilution of exogenous radiolabelled glucosamine by a factor of 4–5, thereby stimulating inhibition of glycoprotein synthesis. The rate of chitin synthesis, which was considered to constitute an endogenous control, did not show this decrease, since an increased rate of chitin synthesis caused by *α* factor exactly compensated for the lowered specific activity of the radiolabelled precursor.

2. MATERIALS AND METHODS

S. cerevisiae X-2180-1a cells were grown in a defined medium as described [2]. Radiolabelling with [^{14}C]phenylalanine (spec. act. 20 μCi/μmol) and with [^{14}C]glucosamine (spec. act. 54 μCi/μmol), cell breakage with glass beads, and fractionation into water-soluble, SDS-extractable and insoluble fractions, was carried out as in [2].

2.1. *Immune precipitation of carboxypeptidase Y*

After breaking the cells with glass beads, Triton X-100 and KCl were added to the crude extract to give final concentrations of 2% and 0.3 M, respectively. After incubation for 20 min at 30°C and subsequent repeated freeze-thawing, the extract was centrifuged at 48000 × *g* for 1 h. To 0.4 ml supernatant, 3 μg purified carboxypeptidase Y (CY) [4], 90 μl CY antibody [4], and 180 μl of buffer (50 mM Tris-HCl, pH 7.4, containing 2 M KCl and 2% Triton X-100) were added. After 15 h at 4°C, the precipitate was centrifuged and washed twice with 1 ml of 50 mM Tris-HCl, pH 7.4, containing 1.2 M KCl and 1.2% Triton X-100, and once with this buffer without detergent. The precipitate was separated by SDS-PAGE (10%) ac-

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cording to Laemmli and King [5]. Fluorograms of the gels were produced with Amplify (Amersham).

2.2. *Determination of the specific activity of [^{14}C]glucosamine in cell fractions*

S. cerevisiae X-2180-1*a* cells (40-ml cultures) were radiolabelled for 3.5 h with 0.5 μCi [1-^{14}C]glucosamine/ml in the presence or absence of 10 μg α factor/ml. Following cell breakage [2] the water-soluble fraction was dialysed 3 successive times for 24 h against cold water, and the dialysable and non-dialysable fractions were freeze-dried separately. Non-dialysable material was suspended in 1.5 ml ice-cold butan-1-ol, then centrifuged at 20000 × *g* for 30 min at 4°C. The resulting pellet (containing essentially all the radioactivity in this fraction) was washed twice with 1.5-ml portions of butan-1-ol. Material solubilized from total membranes in 1.5% (w/v) SDS was precipitated by the addition of 10 vols ice-cold butan-1-ol, and sedimented at 20000 × *g* as above. The precipitate was then washed 3 times with 10 ml cold butan-1-ol. The resulting pellet contained at least 80% of the radioactivity in the SDS-soluble fraction. The SDS-insoluble pellet of cell wall material was washed 4 times with 2.5-ml volumes of cold butan-1-ol. In one experiment, this material was then extracted twice by heating it with 0.5-ml volumes of 1.0 M NaOH in a boiling water bath, the insoluble material (mainly chitin) being retained.

Dialysable and butan-1-ol-precipitated non-dialysable material from the water-soluble fractions, and the butan-1-ol-precipitated, SDS-soluble material, was hydrolysed in 6 M trifluoroacetic acid (TFA) in vacuo at 80°C for 20 h. The acid was then removed by repeated evaporation from water under reduced pressure in a Speed-Vac concentrator. The recovery of radioactivity in TFA hydrolysates of all 3 fractions was 88–96%. Samples of the hydrolysates were submitted to descending paper chromatography for 16 h on Whatman no.1 paper in a solvent containing ethyl acetate/butan-1-ol/acetic acid/water (3:4:2.5:4, by vol.). Radiolabelled glucosamine, *N*-acetylglucosamine, glucose and mannose were run in parallel as standards. Radioactivity was located using a Berthold (Wildbad, FRG) LB 280 paper chromatogram scanner.

Acid hydrolysis of the SDS-insoluble fraction was performed in 0.25 M H_2SO in 95% (v/v) acetic acid as described by Stellner et al. [6]. To remove the acid, the hydrolysates were diluted to 2 ml, then applied to a column containing 2 ml Dowex 50 W (H^+ form), which was then washed with water. Glucosamine was then eluted with 8 M NH_4OH, and ammonia removed by repeated evaporation from water. The recovery of radioactivity in this eluate was 81%. Samples of the hydrolysate were submitted to thin-layer chromatography on Kieselgel 60 sheets (Merck), which were developed in pyridine/ethyl acetate/acetic acid/water (36:36:7:21, by vol.). Radioactivity was then located using a Berthold LB 282 TLC linear analyser. Alkali-extracted cell wall material was hydrolysed in 6 M TFA for 18 h at 60°C, at least 81% of the radioactivity being recovered.

The glucosamine content of acid hydrolysates was determined in a Biotronik LC 5000 amino acid analyser using the manufacturer's amino-sugar programme.

3. RESULTS

Though α factor always inhibited the incorporation of radiolabelled glucosamine into glycoproteins by at least 80% within 3 h, the inhibition of incorporation of [^{14}C]phenylalanine into these glycoproteins in general was less pronounced [3]. In addition, the inhibitory effects were also not as well reproducible as those with glucosamine. For direct comparison, therefore, parallel incubations were carried out with both radiolabelled precursors, and the incorporation of radioactivity into CY which was subsequently immunoprecipitated was measured. Fig.1 shows the result of such an experiment. A strong inhibition of [^{14}C]glucosamine incorporation in the presence of α factor is obvious, but it is not at all matched by a corresponding inhibition of [^{14}C]phenylalanine incorporation. The lack of glucosamine incorporation when α factor is present is not explainable, however, by specific inhibition of glycosylation, since in this case the size of CY in lane 2 should have changed to that of the non-glycosylated form [4]. This result clearly suggested, therefore, that the same amount of fully glycosylated CY is synthesized in the presence or absence of α factor, and that the decreased glucosamine radioactivity seen

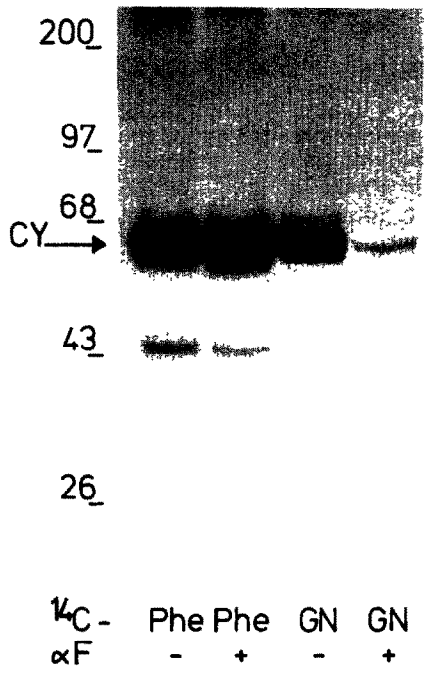

Fig.1. Fluorogram of SDS-PAGE separation of immune-precipitated radiolabelled carboxypeptidase Y (CY). A culture of *S. cerevisiae* X-2180-1*a* (A_{578} = 1.2) was divided into 4 15-ml portions. Two of them were pre-incubated with 13 μg α factor/ml for 30 min, whereupon they were radiolabelled with [^{14}C]phenylalanine (1 μCi/ml) or [1-^{14}C]glucosamine (2 μCi/ml) for 2 h. Control cultures, without α factor, were radiolabelled in parallel. Cells were then harvested, CY immune-precipitated as described in section 2, and radiolabelled bands made visible by fluorography. Lanes 1 and 2 show [^{14}C]phenylalanine (Phe) labelled CY and lanes 3 and 4 [1-^{14}C]glucosamine (GN) labelled CY from control and α factor treated cells, respectively.

in the CY band (lane 4), has to be due to a considerably decreased specific activity of the radiolabelled precursor. The latter must have been diluted by a large supply of non-radioactive glucosamine, a process caused in some way by α factor.

To prove this point, the water-soluble fraction, the SDS extracts of the total membranes, and the insoluble cell wall fractions were hydrolyzed after radiolabelling haploid *a* cells with glucosamine in the presence and absence of α factor. The radioactivity, as well as the total amount of glucosamine present in these fractions, and thus its specific radioactivity, was determined. In table 1 the data

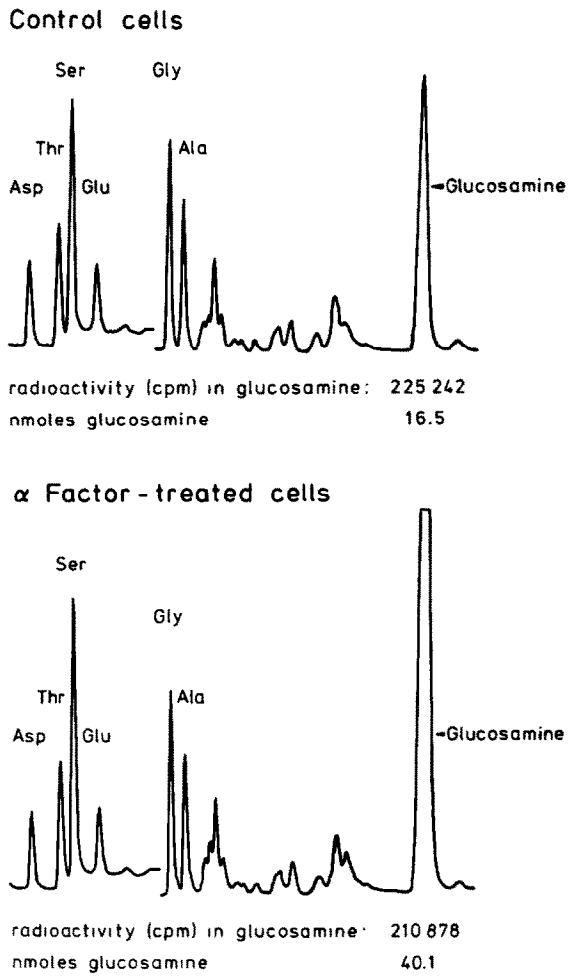

Fig.2. Increase in incorporation of glucosamine and lowering of the specific activity of [1-^{14}C]glucosamine in the acid hydrolysate of SDS-insoluble cell wall material from cells of *S. cerevisiae* X-2180-1*a* treated with α factor. SDS-insoluble fractions from cells radiolabelled with [1-^{14}C]glucosamine in the presence and absence of α factor were submitted to acid hydrolysis, and the glucosamine contents of hydrolysates determined as detailed in section 2. After thin-layer chromatography and scanning for radioactivity as described in section 2, the proportions of the radioactivity with the chromatographic mobility of glucosamine were determined to be 96 and 97% for control- and α factor-treated cells, respectively. The results illustrated here are for samples representing 7.4% of the total SDS-insoluble material. The positions of selected amino acids and of glucosamine are indicated on the amino acid analyser traces above. The break in the traces reflects a change in the absorbancy range, introduced by the programme for the analysis.

Table 1

α Factor lowers the specific activity of [1-^{14}C]glucosamine in water and SDS-soluble cell fractions of *S. cerevisiae* X-2180-1*a*

Cell fraction	nmol glucosamine in hydrolysate		Radioactivity (cpm) in glucosamine in hydrolysates		Specific activity of glucosamine (cpm/nmol)	
	Control	+α factor	Control	+α factor	Control	+α factor
Water-soluble						
Dialysable	36.3	38.8	495090	144565	13639	3726
Non-dialysable	44.3	43.3	591584	88139	13354	2036
SDS-soluble	19.4	20.6	314491	69442	16211	3371

Cells were radiolabelled with [1-^{14}C]glucosamine and fractionated, fractions submitted to acid hydrolysis, and glucosamine contents of hydrolysates determined, as detailed in section 2. The proportion of material in hydrolysates of each fraction that had the chromatographic mobility of glucosamine is as follows, in each case for control- and α factor-treated cells, respectively: dialysable, water-soluble material, 90 and 91%; non-dialysable, water-soluble material, 90 and 86%; SDS-soluble material, 81 and 82%. These values were used to calculate the amount of radiolabelled glucosamine in hydrolysates. Figures presented are for the glucosamine content of the entire fractions obtained from the radiolabelled cultures

are given for the membrane and the water-soluble (= non-dialyzable) glycoproteins. Clearly, the specific radioactivity of glucosamine is much lower in the glycoproteins extracted from α factor-treated cells: 2000–3300 vs 13300–16200 cpm/nmol in the controls. The same phenomenon is also observed in the water-soluble, dialyzable fraction, which seems to consist mainly of phosphorylated derivatives of glucosamine.

Fig.2 shows the separation of cell wall hydrolysates on an amino acid analyzer. The [^{14}C]glucosamine in this fraction arises mainly from chitin [2]. The amount of radioactivity incorporated into chitin is not affected by α factor. Again, however, the specific activity is lowered, although only by a factor 2.5; this factor increases to about 4, however, if half the total chitin in the control cells (8 nmol glucosamine) is subtracted from the control and α factor sample, since approximately this amount is present before α factor is added. This then means that in the case of chitin, the lowered specific activity of the precursor glucosamine is completely compensated for by an increased rate of chitin synthesis. That α factor increases the rate of chitin synthesis about 3-fold in *a* cells has indeed been reported before by Schekman and Brawley [7].

4. DISCUSSION

Our data confirm the report by Schekman and Brawley [7] that α factor increases the rate of chitin synthesis. It was shown [7] that this chitin is no longer localized in the division septum. Why the amount of chitin increases when budding is prevented is unclear. However, this increased rate of chitin synthesis might actually be the reason for the extensive dilution of [^{14}C]glucosamine reported here: α factor may not only increase the activity of chitin synthase [7], but also increase the rate at which *N*-acetylglucosamine is supplied. The chitin precursor may well be generated in a rather specific conversion of reserve carbohydrate to GlcNAc after α factor treatment. Radiolabelled mannose, for example, does not seem to be diluted significantly after treatment of *a* cells with α factor (Arnold, unpublished). The inhibition of [2-^{3}H]mannose incorporation into mannoproteins by α factor reported previously [2] might therefore indeed reflect an inhibition of protein glycosylation.

If the specific activity of the [^{14}C]glucosamine supplied (100000 cpm/nmol) is compared with those in the water-soluble and SDS-extractable fraction after correction for the non-radioactive

material already present at the outset of the labelling experiment (≈50%), an average value of 28000 in the control and 6000 in α factor-treated cells is obtained. The corresponding values for chitin are 26500 and 6500. Thus, exogenous glucosamine is not preferentially incorporated into either glycoproteins or into chitin; it is also neither dissimilated nor converted to other sugars to any significant extent. Under the experimental conditions used here, this exogenous radiolabelled glucosamine is mixed with about 3 parts (control) and 14 parts (α factor-treated cells) of non-radioactive endogenous glucosamine.

It has been shown previously that tunicamycin arrests yeast cells at the same time point in their cell cycle as α factor does [8]. This effect was also observed with a temperature-sensitive *N*-glycosylation mutant (alg 1-1) at its non-permissive temperature [9]. It was postulated, therefore, that *N*-glycosylated proteins are required for G1/S phase transition in yeast [8–10]. Similar observations have been made for mammalian cells [11,12] and plant cells ([13]; Ettlinger and Lehle, unpublished). Although the yeast mating pheromones do not interfere with the synthesis of *N*-glycosylated proteins in a general way, as was suggested by our previous data [2,3], these peptides might still prevent the synthesis of specific glycoproteins. It has recently been reported that α factor turns off transcription of two genes [14].

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