The EMBO Journal vol.10 no.13 pp.4081-4088, 1991

Saccharomyces cerevisiae a- and α-agglutinin: characterization of their molecular interaction

Corinna Cappellaro, Karin Hauser, Vladimir Mrša, Manfred Watzele, Gabriele Watzele, Claudia Gruber and Widmar Tanner

Lehrstuhl für Zellbiologie und Pflanzenphysiologie, Universität Regensburg, Universitätsstrasse 31, 8400 Regensburg, FRG

Communicated by W.Tanner

An O-glycosylated protein of ~18 kDa responsible for mating type specific agglutination has been isolated from *Saccharomyces cerevisiae a* cells, purified to homogeneity and via peptide sequences the gene was cloned by PCR. An open reading frame codes for a protein of 69 amino acids. A minimum of five serine and five threonine residues of the mature protein are glycosylated. α-Agglutinin is a highly N-glycosylated protein of ~250 kDa. Both purified agglutinins form a specific 1:1 complex *in vitro*. Pretreatment of α-agglutinin, but not of *a*-agglutinin, with diethylpyrocarbonate (DEPC) prevents formation of the complex; treatment of α-agglutinin in the presence of *a*-agglutinin protects the former from DEPC inactivation. By carboxy terminal shortening of the α-agglutinin gene and by replacing three of its eight histidyl residues by arginine, the active region of α-agglutinin for interaction with *a*-agglutinin has been defined. Neither the N- nor the O-linked saccharides of the two agglutinins seem to be essential for their interaction.

Key words: DNA sequence of *a*-agglutinin/protein-protein interaction/diethylpyrocarbonate inhibition

Introduction

Haploid *Saccharomyces cerevisiae* cells synthesize inducible mating type specific agglutinins (Betz *et al.*, 1978; Fehrenbacher *et al.*, 1978; Doi *et al.*, 1979). Mating type *a* cells for example, when treated with α factor, increase the amount of an O-glycosylated cell surface molecule of 18 kDa apparent molecular weight by 30-fold (Orlean *et al.*, 1986). This protein can be released from intact *a* cells by SH compounds. The solubilized protein has been purified to homogeneity; it inhibits agglutination of *a* with α cells at a concentration of 4×10^{-9} M (Watzele *et al.*, 1988).

Genetic analysis of *a* cells showed that agglutination-negative mutants consisted of two complementation groups (Roy *et al.*, 1991). The *AGA1* gene encodes an unusual 73 kDa protein consisting to >50% of serine and threonine residues; it constitutes the cell surface attachment subunit for the *a*-agglutinin binding fragment (Roy *et al.*, 1991). The gene of the latter (*AGA2*) has been cloned and sequenced; the results are reported herein.

The α-agglutinin has been purified to homogeneity (Hauser and Tanner, 1989) and the corresponding gene has been cloned and sequenced (Lipke *et al.*, 1989; Hauser and Tanner, 1989). It increases the efficiency of conjugation in liquid culture by a factor of 10^5, although the protein is not essential for conjugation as such (Lipke *et al.*, 1989).

Thus the components of *S.cerevisiae* responsible for mating type recognition and agglutination are fairly well defined both as genes and as highly glycosylated cell surface proteins. It is possible, therefore, to study this cell-cell interaction at the molecular level. Besides the DNA sequence of the *a*-agglutinin binding fragment, we report the *in vitro* formation of a specific 1:1 complex of the two deglycosylated agglutinin proteins and present evidence that His273 of α-agglutinin is essential for the protein-protein interaction.

Results

Purification of a-agglutinin and preparation of tryptic peptides

S.cerevisiae strain 4277-7 (MAT*a*, *bar 1-1*) was grown in YPD medium to an OD_{578} value of 5; then 40 μg/l of α factor was added and the incubation continued for 2 h. *a*-Agglutinin was released by treating intact cells of a 10 l culture with DTT as described previously (Watzele *et al.*, 1988). The purification was also carried out as before except that the HPLC sizing column was replaced by an ion exchange column (Mono Q, see Materials and methods). The protein obtained in this way was shown to be pure by silver staining on SDS-PAGE (Figure 1a).

The N-terminal sequence of two different preparations is given in Table I. In addition a number of tryptic peptides separated on RP-304 and RP-18 columns were sequenced; four peptides yielded information (Table I). To remove O-linked mannosyl residues (Orlean *et al.*, 1986; Watzele *et al.*, 1988) peptides 2 and 4 were treated with α-mannosidase and partially demanosylated as can be seen by comparing positions 3 and 6 of peptides 1 and 2.

Cloning of the a-agglutinin gene (AGA2) and its sequence

According to peptide 3 the following 27 bp antisense oligonucleotide was synthesized: 5'-TTT GTA GTA TTC GAA NAC NCC TTG CAT. This nucleotide hybridized to an α factor inducible 0.5 kb RNA (data not shown). A positive 100 bp signal was obtained by PCR using the above oligonucleotide and the following oligonucleotide conceived from the N-terminus 5'-GAG TTG GAA TCG ATT TCT GAA/G CAA/G ATT/C/A CC. This PCR signal was obtained only with mRNA from α factor treated *a* cells. In addition a PCR band of 350 bp was obtained, when the oligonucleotide corresponding to the N-terminus was used together with oligo(dT). When the 350 bp piece was subcloned in PIC20H and sequenced, the sequence contained all the peptide sequence information of Table I except two

errors at the C-terminal end (amino acids 66 and 67; see Figure 2). Also the Northern analysis yielded a mating type specific and strongly inducible signal corresponding to a 500 bp mRNA (Figure 1b).

The full sequence including the signal peptide was obtained by carrying out PCR with 3′-poly(A) extended DNA fragments obtained by cutting total yeast DNA by *Alu*I and attaching oligo(A) at the 3′-ends of it (see Materials and methods). Figure 2 shows the full sequence of *a*-agglutinin. The sequences corresponding to those gained from peptide sequencing are underlined. The 18 amino acid signal peptide contains the typical positive charge (Arg in position −14), followed by 13 uncharged amino acids (nine of which are lipophilic) and a typical splitting configuration with V at −3 and A at −1 (Heijne, 1985). Unfortunately the sequence available does not contain a stop codon in front of the ATG, leaving open the unlikely possibility that the signal peptide may be considerably longer.

Southern analysis demonstrated that only one copy of the *a*-agglutinin gene (*AGA2*) is present in the haploid genome (data not shown). When the gene was disrupted with a *URA3* construct (see Materials and methods), these cells no longer produced the cell surface antigen as shown by immunoblots of cell surface extracts and the cells no longer agglutinated with α-cells (data not shown).

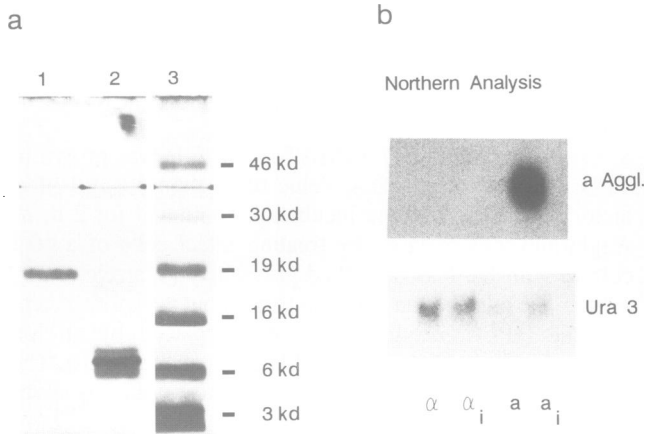

Fig. 1. (**a**) Silver-stained SDS−PAGE of purified *a*-agglutinin binding fragment. Lane 1, intact *a*-agglutinin; lane 2, *a*-agglutinin deglycosylated by HF (the faster running material may be a protein breakdown product); lane 3, low molecular weight prestained standards insulin A and B chains (2 and 3 kDa), bovine trypsin inhibitor (6 kDa), lysozyme (15.5 kDa), β-lactoglobulin (19 kDa), carbonic anhydrase (30 kDa), ovalbumin (46 kDa). (**b**) Northern analysis using the 350 bp PCR band subcloned in PIC20H. a and α, total RNA (50 μg) from *a* and α cells; a_i and α_i, total RNA (50 μg) from *a* and α cells treated for 20 min with pheromone.

The a-agglutinin: size and glycosylation

Using low molecular weight standards yielded values of ~18 and 7 kDa for the glycosylated and the deglycosylated form, respectively (Figure 1a). The values published previously (Watzele *et al.*, 1988) were too large. The sequence codes for a 7.48 kDa protein, 69 amino acids long without the signal peptide.

Twenty-one of the 69 amino acids are serine/threonine residues, at least 10 of which are glycosylated, based on the appearance of *X* in the peptide sequence where the DNA sequence predicts a Ser or Thr residue. Non-glycosylated hydroxy amino acids like S52, S55, T56, S58, and T65 were detected in partially demannosylated peptides; thus they may very well be glycosylated in the intact protein.

The amino acids S17, T18 and S23 are the only hydroxy amino acids recovered unglycosylated during N-terminal sequencing. They may not, however, be unmodified within each individual molecule, since a certain degree of molecular heterogeneity has to be deduced from the diffuse running behaviour on SDS gels. Only S17 most likely is not glycosylated at all, since it has been shown *in vitro* that mannosyl transfer to hydroxy amino acids next to acidic amino acids is not catalysed by the mannosyl transferase (Strahl-Bolsinger and Tanner, 1991). The protein does not contain any potential N-glycosylation sites in accordance with previous results with tunicamycin (Orlean *et al.*, 1986).

The pheromone induced agglutinins react with each other

In vivo observation. That the two mating type specific agglutinins interact with each other and not with a wall component constitutively expressed at the surface of the partner cell, has generally been assumed (Hagiya *et al.*, 1977; Betz *et al.*, 1978; Terrance and Lipke, 1981). It has to be inferred from the lack of agglutination of pheromone treated cells with control cells (Figure 3). When both mating types are pretreated with pheromone, the formation of agglutinates can be seen within 2−5 min and this is independent of whether cycloheximide is included in the incubation or not. Cells not pretreated with pheromone show strong agglutination after 30−40 min, but do not do so in the presence of cycloheximide; the agglutinins have first to be synthesized and exposed at the cell surface (Betz *et al.*,

Table I. N-terminal and peptide sequences obtained from purified a-agglutinin

	N-terminus
Sample 1	Q/AE L X X I X E Q I P X P (D) L E[a]
Sample 2	X E L E S I (S) E Q I P X G/PX L E S T P Y X L S
	Tryptic peptides
Peptide 1	X V X F V X N N X S H
Peptide 2	X V T F V S N Y G S H P S T X S K G S[b]
Peptide 3	A M Q G V F E Y Y K
Peptide 4	X X P I (N) T G T V[b]

[a]The N-terminus of sample 1 was sequenced by Dr J.Hoppe, Würzburg; all other sequences were obtained by Dr R.Deutzmann, Regensburg.
[b]These peptides were partially demannosylated with α-mannosidase.

1978). When only one mating type is pretreated with pheromone (wells 2 and 3) the mixed population behaves as the untreated control (well 4); the slight difference in time is most likely due to the increased pheromone production of the pretreated cell type (Strazdis and MacKay, 1983; Achstetter, 1989). These results indicate that the two inducible agglutinins interact with each other.

1:1 complex formation in vitro. To investigate this question further it was tried whether the two purified proteins react with each other *in vitro*. The α-agglutinin has been shown to consist of a protein moiety 631 amino acids long with a molecular weight of 68.2 kDa. Maximally 12 N-linked saccharide chains increase the apparent M_r by 200 kDa as estimated from SDS–PAGE (Hauser and Tanner, 1989). The purified glycoprotein yields up to six peptides after deglycosylation, which possess identical N-termini and are all biologically active (Hauser and Tanner, 1989). The biologically active part of *a*-agglutinin consists of the 69 amino acid protein, which in its glycosylated form has a molecular weight of ~18 kDa (Figure 1a). That these two proteins are the two mating type specific agglutinins is based on the observations (i) that both these proteins inhibit the agglutination of *a* with α cells (Orlean *et al.*, 1986; Hauser and Tanner, 1989); (ii) that the disruption of the corresponding genes yields non-agglutinating mutants (Lipke *et al.*, 1989; and see above); (iii) that polyclonal antibodies obtained against both proteins inhibit agglutination (M.Watzele and K.Hauser, unpublished); and (iv) that the antibodies react with *S.cerevisiae* zygotes in the predicted mating type specific manner, whereas the new diploid zygote bud no longer reacts with either antibody (Figure 4).

When purified deglycosylated α-agglutinin is incubated with *a*-agglutinin, crosslinked with glutaraldehyde, then

```
                .         .         .         .         .         .
    CTCAACAGTCACTTGTGTGCCAAAATATTATACCTACTTCATACATTTTCAATTAAGATG
                                                              M     1
                .         .         .         .         .         .
    CAGTTACTTCGCTGTTTTTCAATATTTTCTGTTATTGCTTCAGTTTTAGCACAGGAACTG
  2 Q  L  L  R  C  F  S  I  F  S  V  I  A  S  V  L  A  Q  E  L    21
                .         .         .         .         .         .
    ACAACTATATGCGAGCAAATCCCCTCACCAACTTTAGAATCGACGCCGTACTCTTTGTCA
 22 T  T  I  C  E  Q  I  P  S  P  T  L  E  S  T  P  Y  S  L  S    41
                .         .         .         .         .         .
    ACGACTACTATTTTGGCCAACGGGAAGGCAATGCAAGGAGTTTTTGAATATTACAAATCA
 42 T  T  T  I  L  A  N  G  K  A  M  Q  G  V  F  E  Y  Y  K  S    61
                .         .         .         .         .         .
    GTAACGTTTGTCAGTAATTGCGGTTCTCACCCCTCAACAACTAGCAAAGGCAGCCCCATA
 62 V  T  F  V  S  N  C  G  S  H  P  S  T  T  S  K  G  S  P  I    81
                .         .         .         .         .         .
    AACACACAGTATGTTTTTTGAGCCTTCCCCCCCAAGTTTCCATTTCATCGTTCGTGACAC
 82 N  T  Q  Y  V  F                                              101
                .         .         .         .         .         .
    TTATAAACTAGTGTAAGATTATTTACCTATATATAATTTGATAATAATAGCAAAAAAAAA
                .         .
    AAAAAAAAAAAAAAAAAAAAA
```

Fig. 2. Nucleotide sequence and deduced amino acid sequence of the *AGA2* gene. The sequenced peptides (Table I) are underlined.

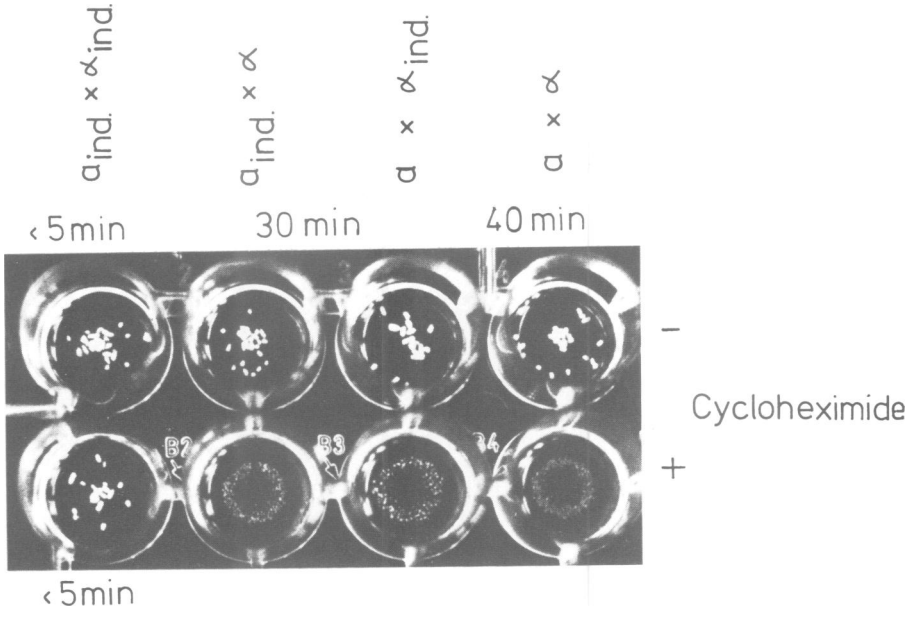

Fig. 3. Agglutination test of *a* and α cells (X2180) pretreated or not pretreated with α and *a* factor respectively: a_{ind} α_{ind}, cells pretreated with the corresponding mating pheromone (10 μg/ml of α factor and 10 units/ml of a factor) for 90 min; a and α, cells not pretreated. The times indicated correspond to the time required until the first large agglutinates could be seen. Where indicated cycloheximide are present (10 μg/ml); other conditions see Materials and methods.

separated on SDS−PAGE and an immuno−blot prepared with anti-*a*-agglutinin antibody, the picture shown in Figure 5a is obtained. Whereas in all controls immuno-positive material is visible only slightly above 18 kDa, two additional bands of ~77 and 92 kDa are visible in the experimental sample (Figure 5a, right). The two major peptides of deglycosylated α-agglutinin run at 72 and 57 kDa (see silver-stained SDS−PAGE; Figure 5b, left) and two new bands are formed in the presence of *a*-agglutinin (Figure 5b, right, arrow heads); the latter two bands react with the anti-*a*-agglutinin antibody. The ~20 kDa larger new bands are the 1:1 complex of both agglutinins. The same result is obtained with crude extracts from *a* and α cells (data not shown); this shows the specificity of the interaction and also that glutaraldehyde crosslinking does not give rise to additional non-specific protein−protein complexes.

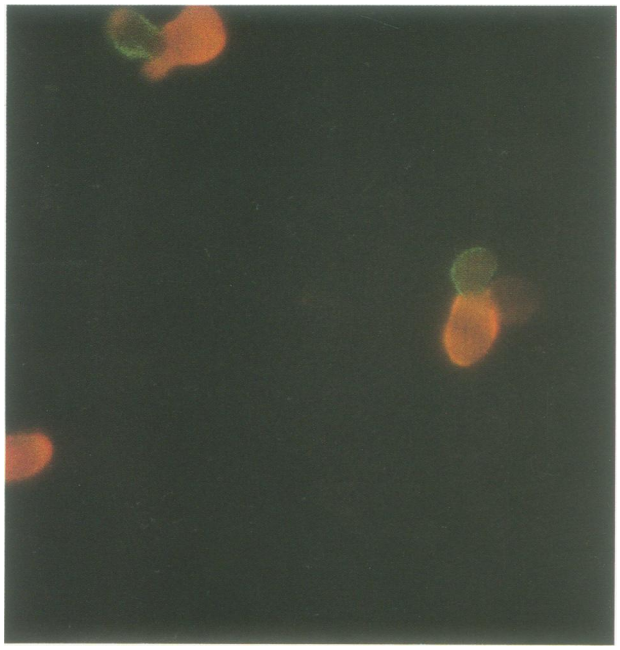

Fig. 4. Yeast zygotes stained with FITC labelled anti-*a*-agglutinin antibody and rhodamine labelled secondary antibody directed against anti-α-agglutinin antibody (see Material and methods). The bud representing the first diploid offspring contains neither *a*- nor α-agglutinin.

The formation of the a−α-agglutinin complex is inhibited by diethylpyrocarbonate

When the α-agglutinin was pretreated for 10 min with the histidyl modifying agent diethylpyrocarbonate (DEPC) (Miles, 1977) before addition of *a*-agglutinin and cross-linking, the *in vitro* complex was no longer formed (Figure 6). Pretreatment of *a*-agglutinin in the same way did not prevent complex formation. When the α-agglutinin was treated with DEPC in the presence of *a*-agglutinin, the complex was formed (Figure 6), indicating that *a*-agglutinin as 'substrate' of the α-agglutinin protected the active site of the latter from the action of DEPC. When analogous experiments were carried out with *N*-ethylmaleimide (NEM) no inhibitory action was observed.

Identification of the critical histidyl residues within the α-agglutinin

The protein sequence deduced from the α-agglutinin gene contains eight histidyl residues (Hauser and Tanner, 1989; Lipke *et al.*, 1989). Carboxy terminal deletions up to one third of the gene still resulted in biologically active protein (Figure 7). When only the N-terminal one third was expressed no biologically active extract was obtained anymore. Lipke *et al.* (1989) concluded from an inhibitory antibody raised against a fusion protein that the region between amino acids 128 and 356 is important for agglutination. From both these observations it was expected that either the histidyl residue 164, 178 or 273 participates in the interaction with *a*-agglutinin. By site directed mutagenesis three mutants were constructed, each with one of the three histidines replaced by arginine. Only the replacement of

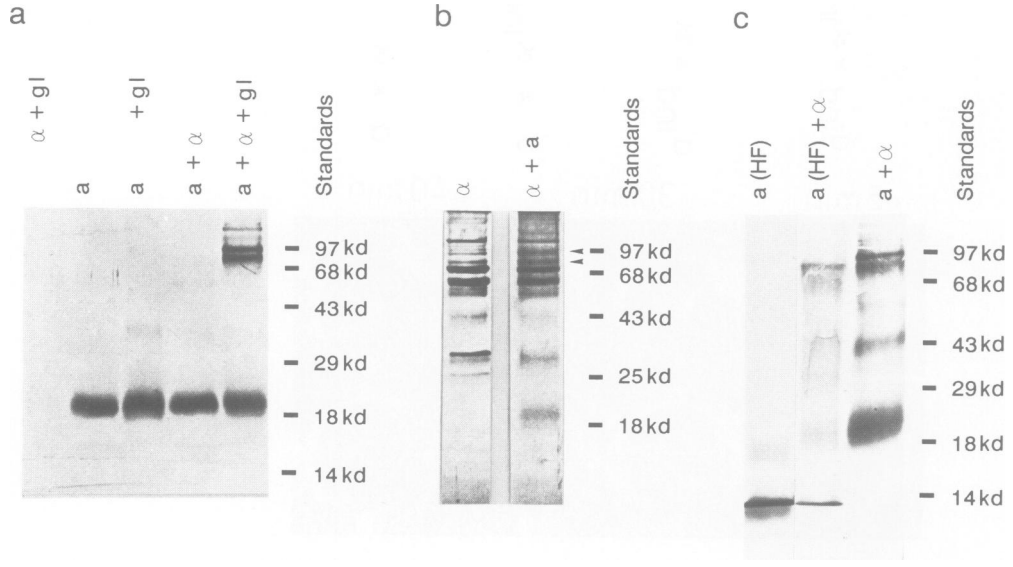

Fig. 5. Complex formation of *a*- and α-agglutinin *in vitro*. (**a**) Anti-*a*-agglutinin immunoblot of *a*-agglutinin crosslinked to deglycosylated α-agglutinin with glutaraldehyde (a + α + gl); the other four lanes are controls. (**b**) Silver-stained SDS−PAGE of deglycosylated α-agglutinin (α) and the same material after crosslinking to a-agglutinin (a + α). The two new bands (arrow heads) are identical with the immuno-positive double band in panel a. (**c**) Anti-*a*-agglutinin immunoblot: a + α, *a*-agglutinin crosslinked to deglycosylated α-agglutinin; a(HF), deglycosylated *a*-agglutinin a(HF) + α, both deglycosylated agglutinins crosslinked.

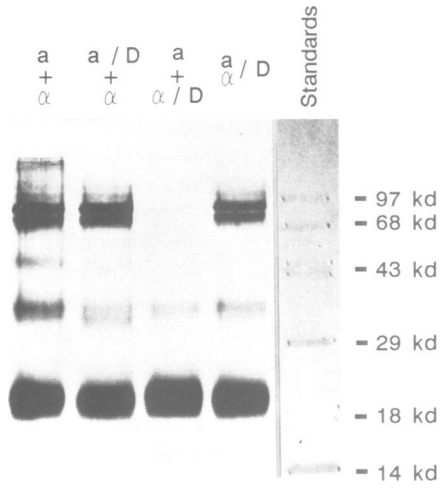

Fig. 6. Inhibition of *a*- and α-agglutinin interaction by DEPC; immunoblot with anti-*a*-agglutinin antibody. Lanes from left to right: a + α, control; a/D + α, *a*-agglutinin pretreated with DEPC; a + α/D, α-agglutinin pretreated with DEPC; $\frac{a}{\alpha}$/D, α- and *a*-agglutinin together treated with DEPC. Other conditions as in Figure 5 and Materials and methods.

His273 gave rise to an almost inactive protein, whereas the other two mutants behaved like wild-type (Figure 7). Therefore, the region NVNTIDHALEFQY around H273 is a likely site for directly binding to *a*-agglutinin.

The role of carbohydrates in the agglutinin interaction

As pointed out previously already (Terrance *et al.*, 1987; Hauser and Tanner, 1989) the N-linked carbohydrate moieties of α-agglutinin, which amount to more than two thirds of the molecular mass of the intact glycoprotein, are not necessary for biological activity. In addition the α-agglutinin does not seem to possess O-linked saccharides (K.Hauser, unpublished). The *a*-agglutinin on the other hand contains 29% by weight of O-linked short oligosaccharides consisting of mannosyl residues only (Watzele *et al.*, 1988). Are they required for the specific interaction of the two agglutinins? Since mild periodate treatment of *a*-agglutinin partially destroyed its ability to inhibit *a* and α cell agglutination, a possible role of the sugars had been postulated (Watzele *et al.*, 1988). When HF treated α-agglutinin, however, was tested by the *in vitro* complex formation described above, it still reacted with α-agglutinin (Figure 5c). Similar results were obtained when the sugars

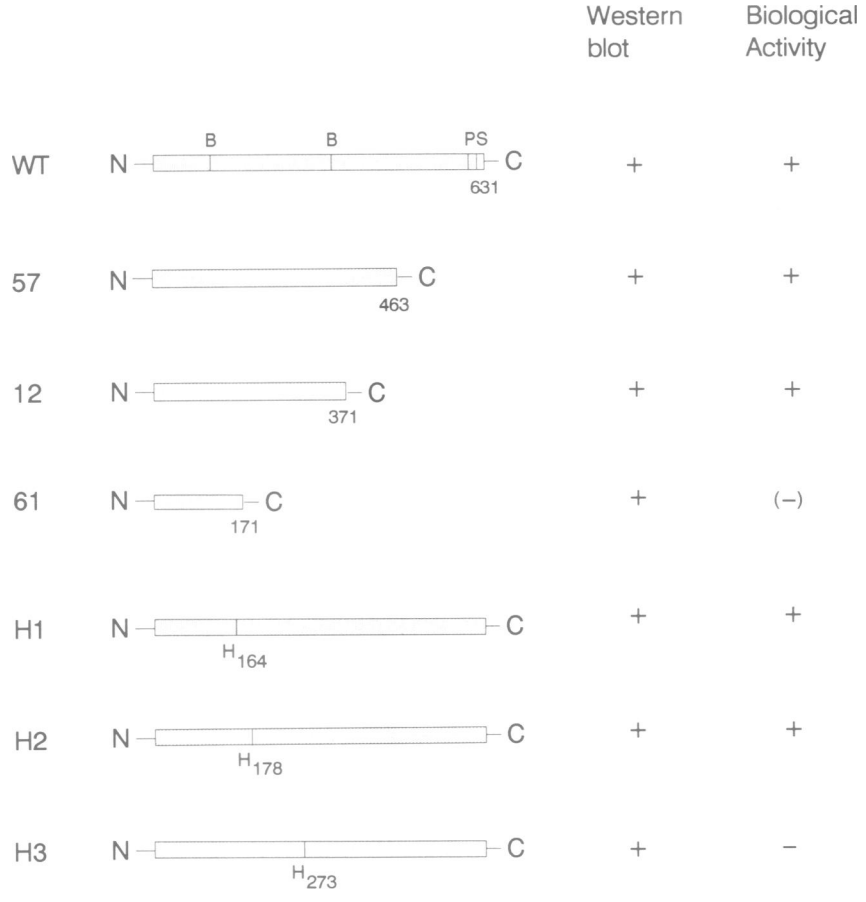

Fig. 7. *AGα1*– mutant analysis. C-terminal deletions and site directed mutagenesis of the *AGα1* gene were made as described in Materials and methods. Both types of mutated DNA were transformed into an *AGα1* disrupted MATα strain (DBY 746-3) and the transformants were analysed. All transformants express the *AGα1* gene product in the corresponding size as tested by Western blot analysis. Biological activity was measured as described by Hauser and Tanner (1989). Mutants 12 and 61 do not agglutinate, but whereas the gene product of mutant 12 has biological activity, that of mutant 61 has not, (since the protein of mutant 61 was only detected in membrane fractions, however, it was not sure whether a sufficient amount of it was solubilized plus stayed in solution during the agglutination test). Mutants H1 and H2 show wild-type agglutination ability, but mutant H3 only agglutinates weakly after a 10-fold longer incubation time than H1 and H2 mutants. The biological activity of the isolated H3 gene product was no longer detectable. WT, wild-type; 57, 12 and 61, C-terminal deleted mutants; H1, H2 and H3, site directed mutants; the histidines indicated are exchanged by arginines (see Materials and methods). Restriction sites: B, *Bam*HI; P, *Pvu*II; S, *Sac*I.

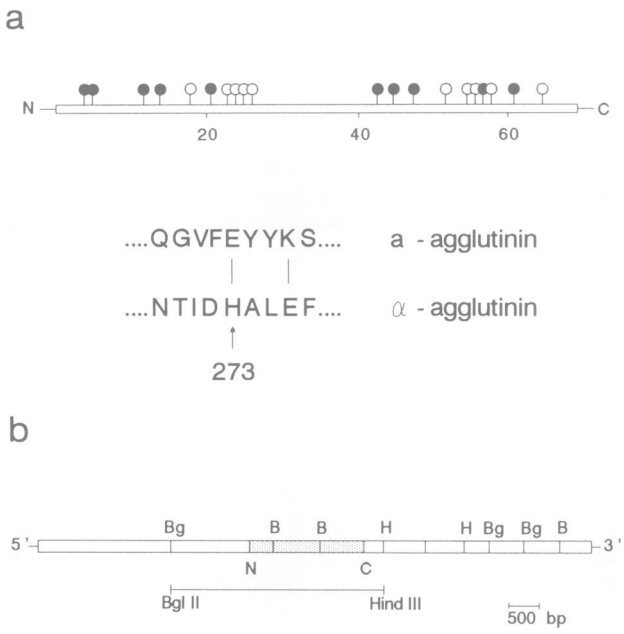

Fig. 8. (**a**) O-glycosylation patterns of *a*-agglutinin and region assumed to interact with α-agglutinin. Closed symbols, Ser/Thr mannosylated; open symbols, potential O-glycosylation sites (see Discussion). (**b**) Restriction map of clone 11 isolated previously (Hauser and Tanner, 1989). Bg, *Bgl*II; B, *Bam*HI; H, *Hin*dIII; N, N-terminus; C, C-terminus.

were removed with α-mannosidase or by β-elimination under conditions where most of the protein remained intact (data not shown). *a*-Agglutinin demannosylated in various ways also still inhibits the agglutination of *a* with α cells (data not shown). However, in the whole cell test a 5-fold higher concentration of the carbohydrate-free *a*-agglutinin had to be used to achieve the same long lasting degree of inhibition (the inhibition with *a*-agglutinin normally fades away after several hours). It is assumed, therefore, that *S.cerevisiae* mating type recognition and agglutination is brought about by a protein−protein interaction, but that the complex formed between the two agglutinins is stabilized in some way by the O-linked sugars of *a*-agglutinin.

Discussion

The *S.cerevisiae a* cell agglutinin consists of two components: the 7.5 kDa binding fragment, the gene product of *AGA2* described in this paper, and a 725 amino acid large cell wall protein constituting the attachment site of the binding fragment. The gene for the attachment subunit *AGA1* has been cloned and sequenced by Roy *et al.* (1991). The cystein of position 7 or that of position 50 (or both) of the binding fragment should form the SS link to the attachment protein, since the binding fragment is easily removed from intact cells by SH reagents (Orlean *et al.*, 1986).

In a number of different yeast species analogous agglutination systems have been described (Crandall and Brock, 1968; Yen and Ballou, 1974; Burke *et al.*, 1980; Pierce and Ballou, 1983). In each species there exists a small fragment, SH-bound to the cell surface, which resembles the *S.cerevisiae a*-agglutinin binding fragment, and a large glycoprotein on the partner cell, resembling the *S.cerevisiae* α-agglutinin.

The *a*-agglutinin binding fragment shows a number of unusual features. Thus it retains its full biological activity after boiling (Orlean *et al.*, 1986; Sijmons *et al.*, 1987) and also after separation on SDS−PAGE (Watzele *et al.*, 1988). The protein is highly O-glycosylated with one to three mannosyl residues attached to serine and threonine residues (Watzele *et al.*, 1988). Ten hydroxy amino acids were shown to be linked to sugars by peptide sequencing. However, another 10 residues are potential sugar attachment sites; they were either not sequenced as peptides or found non-glycosylated due to partial demannosylation during peptide preparation. It seems safe to assume, therefore, that a variable amount of 10−20 sites are modified in each individual molecule, thus giving rise to the typical diffuse band on SDS-gel.

The two glycosylated cell surface proteins responsible for sexual agglutination are highly induced by the mating pheromone peptides at least in *a* and α cells of the strain X2180 and in *a* cells of the strain 4277-7. The interaction of the two purified proteins has been achieved *in vitro* giving rise to a one-to-one complex. The sugars of the α-agglutinin (~200 kDa) are not required for binding the *a*-agglutinin (Terrance *et al.*, 1987; Hauser and Tanner, 1989). Also the O-linked short mannosyl oligosaccharides of the *a*-agglutinin are not required for interaction with the α-agglutinin (Figure 5c); the complex formed with sugar-free *a*-agglutinin, however, seems to be less stable, as judged from whole cell experiments. O-glycosylated proteins often seem to constitute rather stiff rods (Jentoft, 1990) and it may very well be, therefore, that the *a*-agglutinin becomes more flexible after deglycosylation, leading to a destabilization of the α−*a*-agglutinin complex.

As demonstrated in this paper the histidyl residue 273 of α-agglutinin is essential for binding. It seems unlikely that the default of the corresponding mutant is due to an indirect, e.g. conformational effect, since the *in vitro* interaction of the two proteins is completely inhibited by DEPC and the presence of *a*-agglutinin results in a substrate-like protection against this inhibitor (Figure 6). The pH optimum for agglutination of *a* with α cells is 5.5−6.5 (K.Hauser, unpublished), which would agree with a protonated histidyl residue taking part in the binding of *a*-agglutinin.

The amino acid sequence of *a*-agglutinin obtained from the peptides and including the part predicted from the DNA sequence, shows a 16 amino acid central non-glycosylated stretch (J27-K42, Figure 8a). Since the α-agglutinin binds to *a*-agglutinin by protein−protein interaction, it is tempting to speculate that the α-agglutinin recognizes the only sugar-free sequence of the *a*-agglutinin. This region possesses a pair of charges ..EXXK.., which may interact with the opposite pair H273XXE in α-agglutinin (Figure 8a). For the homophilic interaction of the *Dictyostelium* agglutinin also two pairs of charged amino acids are thought to be important (Kamboj *et al.*, 1989). Future experiments with synthetic peptides and specific mutations in *a*-agglutinin will support or dismiss our hypothesis.

Materials and methods

Yeast and Escherichia coli strains, media, growth conditions

S.cerevisiae X2180-1A(MAT*a*), X2180-1B(MATα), 4277-7 (MAT*a*, *bar1-1*) (Chan and Otte, 1982), DBY 746 (MATα, *His3-Δ1, leu2-3, leu2-112, ura3-52, trp1-289*) and DBY 747 (MAT*a*, *his3-Δ1, leu2-3, leu2-112, ura3-52, trp1-289*) (Botstein *et al.*, 1979) were grown in YPD medium (1% yeast extract, 2% bacto peptone, 2% glucose) overnight at 29°C. A minimal medium 0.67% yeast nitrogen base without amino acids

(Difco) was used and supplemented with 20 mg/l histidine, 30 mg/l leucine, 20 mg/l uracil, 20 mg/l tryptophan and 2% glucose.

BMH71/18 (Messing *et al.*, 1977) and JM109 (Yanisch-Perron *et al.*, 1985) were selected for F′ plasmid in minimal medium M9 (Maniatis *et al.*, 1982). For transformation and plasmid preparation cells were grown in LB.

Induction of yeast cells by pheromone (tester strains)

S.cerevisiae 4277-7 (MAT*a*) was grown in YPD to OC_{578} = 2.5. Then synthetic α factor (Bachem, Bubendorf, Switzerland) was added to a final concentration of 10 nM. After 2 h of shaking at 30°C induction was stopped by adding *p*-dinitrophenol (1 mM). *S.cerevisiae* α cells (X2180-1B and DBY 746) were induced with *a*-medium: 50 ml of an overnight culture of α cells with an OD_{578} = 2.0 were centrifuged and resuspended in 50 ml *a*-medium. This medium was obtained by removing X2180-1A cells of an overnight culture (OD_{578} = 2.0) by centrifugation and filtration (nitrocellulose, pore size 0.45 μm). The α cells were grown in this medium for 90 min at 30°C. Induction was stopped by cycloheximide (20 μg/ml). Cells prepared in this way were kept in the refrigerator and could be used as tester strains for 2−3 weeks.

In vivo assay for agglutinin activity and agglutination assay

Biological activity of agglutinins was assayed by their ability to inhibit aggregation of pheromone induced MAT*a* and MATα cells as described previously (Orlean *et al.*, 1986; Hauser and Tanner, 1989).

The agglutination ability of MATα cells was assayed by mixing 50 μl induced 4277-7 (MAT*a*) cells (7.5×10^6 cells), 50 μl induced α cells (7.5×10^6 cells) and 150 μl assay buffer (100 mM sodium phosphate, pH 6.3) in a flat bottom microtiter plate (15 × 17 mm wells). The mixture was gently shaken and agglutination observed. Quantitative assays for inhibitory activity of extracted agglutinins were carried out by 1:2 dilution series as described previously (Orlean *et al.*, 1986).

Purification of agglutinins

α-Agglutinin was extracted and purified as described previously (Hauser and Tanner, 1989). *a*-Agglutinin was purified according to Watzele *et al.* (1988) with modifications. The HPLC gel filtration step was replaced by ion exchange chromatography (Mono Q-FPLC HR 5/5). The sample was applied to the column in 25 mM Tris−HCl buffer pH 9.0 and eluted with a linear gradient of NaCl (25 mM Tris−HCl, pH 9.0, 1 M NaCl). Proteins on SDS−PAGE (Laemmli, 1970) were detected by silver staining (Morrissey, 1981).

Deglycosylation of agglutinins

Digestion of α-agglutinin with endoglycosidase F (Boehringer, Mannheim) was carried out as described previously (Hauser and Tanner, 1989). O-glycosidically linked sugars of *a*-agglutinin were removed by anhydrous HF cleavage (Mort and Lamport, 1977). The sample was dried over P_2O_5 and then treated with HF for 1 h at 0°C. HF was removed in a N_2 stream and the resulting pellet was neutralized with a saturated Na_2CO_3 solution.

α-Mannosidase digest. Digestion was carried out in a dialysis cup (50 mM sodium citrate, pH 4.3) for 58 h at room temperature. Enzyme (Sigma, Müchen) was added four times (~100 mU/μg protein).

Northern analysis

RNA was isolated following the method of Domdey *et al.* (1984). Total RNA (50 μg) was electrophoresed on 0.8% formaldehyde−agarose gels and transferred to nitrocellulose (Maniatis *et al.*, 1982). Oligonucleotides and DNA probes were labelled with ^{32}P (Maniatis *et al.*, 1982).

Cloning of C-terminal part of AGA2

RNA was isolated from α factor induced *a* cells as indicated above and transcribed into cDNA (Maniatis *et al.*, 1982). Two oligonucleotides were synthesized (TIB MOLBIOL, Berlin): the first one deduced from the N-terminal sequence of the mature protein, the other one directed against the poly(A)tail [oligo (dT)]. For PCR 5 ng cDNA and 0.7 μM primer were used and buffers and concentrations were as indicated in Perkin Elmer Cetus Gene Amp™ DNA amplification kit. The cycling procedure was modified by a 50°C step after annealing and the annealing temperature was increased after the first eight cycles (for a further 32 cycles) from 38 to 43°C (programme: 40 s, 95°C; 1 min, 39°/43°C; 40 s, 50°C; 1.5 min, 72°C). The resulting 350 bp fragment was cloned in the *Cla*I site of PIC20H (Marsh *et al.*, 1984).

Cloning of N-terminal part of AGA2

Genomic yeast DNA (Cryer *et al.*, 1975) was digested with *Alu*I and polyadenylated with dATP addition catalysed by the terminal transferase kit (Boehringer, Mannheim).

For PCR three oligonucleotides were designed: two of them were neighbouring sequences of the known cDNA sequence (01: 5′CAACT-CGAGAACCGCAATTACTGACAAAC-3′ and 02: 5′-GCGGATCCCG-TTGGCCAAAATAGTAGTCGTT-3′). The third one was oligo$(dT)_{30}$. All oligonucleotides contained restriction sites at their 5′-ends. With these oligonucleotides a nested primed PCR was performed (Leu, 1990): 1/100 of the first round (40 s, 95°C; 1 min, 39°C; 1.5 min, 72°C; 20 cycles) was re-amplified (40 s, 95°C; 1.5 min, 53°C; 2 min, 72°C; 25 cycles). The band obtained was cloned in PUC18 using JM109.

Sequencing

DNA sequencing was carried out by the dideoxy method (Sanger *et al.*, 1977) using the T7 polymerase kit (US Biochemical, Cleveland, OH); at least three independent PCR reactions were sequenced.

Gene disruptions

AGα1. The *Bgl*II−*Hin*dIII fragment of clone 11 (Hauser and Tanner, 1989; see Figure 8b) containing the *AGα1* gene was subcloned into pUC19. The resulting plasmid was pHB15. The *URA3*-containing *Hin*dIII fragment from YEp24 was subcloned into *Bam*HI cleaved pHB15; the *URA3* fragment and pHB15 were blunted previously by the Klenow fragment. The resulting plasmid was pHBurall. The *Kpn*I−*Hin*dIII−*agα1::URA3* fragment of pHBurall was used to make a gene disruption in DBY 746 by the technique of Rothstein (1983). The disruptions were tested by Southern blot analysis, Western blot analysis and agglutination test (data not shown). This disruption mutant was called DBY 746-3.

AGA2: The gene fragment obtained by PCR with cDNA in PIC20H was partially digested with *Ssp*I. In a blunt end ligation the *URA*3 gene (*Hin*dIII fragment) of YEp24 was inserted (Rothstein, 1983) and the isolated linear construct introduced into a *ura*$^-$ DBY747 strain. The mutation was checked by an *in vivo* test for agglutination, by immuno- and Southern blots.

Site directed mutagenesis

Site directed mutagenesis was carried out with synthetic oligonucleotides and PCR using the method of Ho *et al.* (1989). Three mutants were constructed, each with one histidine replaced by arginine. Every replacement resulted in one nucleotide exchange: His164 CAC → Arg CGC, His 178 CAT → Arg CGT, His 273 CAT → Arg CGT. The *Bgl*I−*Hin*dIII fragment of clone 11 (Figure 8b) was cloned into YEp 351 containing *LEU2* (Hill *et al.*, 1986). The resulting plasmid was pMG317. Mutagenesis was carried out with the internal *Bam*HI fragment of the *AGα1* gene which was cloned into pUC18, using the mutating oligonucleotides and the pUC/M13 (Promega) forward primer (24mer) and reverse primer (22mer) as primers for PCR. The mutated PCR fragments were sequenced and ligated back into *Bam*HI cleaved pMG317. Mutated plasmids were detected by Southern blot analysis with the oligonucleotides used for mutating as probes and by sequencing. They were transformed into DBY 746-3. Leu$^+$ transformants were tested for agglutination and by Western blot analysis.

C-terminal deletions

The *Pvu*II and *Sac*I sites near the 3′-end of the coding sequence (Figure 7) of the *AGα*1 gene were used to make deletions in the N-terminal direction with exonuclease III (Henikoff, 1984). The deleted fragments were cloned into YEp 351 and transformed into DBY 746-3. Leu$^+$ transformants were tested for agglutination, for agglutination inhibitory activity of extracts and by Western blot analysis.

Antibody preparation

Antibodies were obtained from New Zeeland rabbits as described by Hurn and Chantler (1980). Purified α-agglutinin was deglycosylated by endoglucosidase F and repurified once more by separating the products on an LKB UltroPac TSK G 2000 SW column. The active fractions were pooled and used for immunization. The antibodies were preadsorbed onto *a* cells before use. Anti-*a*-antibody was raised as described by Watzele *et al.* (1988). Antibody against deglycosylated *a*-agglutinin was obtained by deglycosylating purified *a*-agglutinin by HF cleavage as described in this paper. After separation by 15% SDS−PAGE the protein was eluted, dialysed and injected. The antibody was preadsorbed to α-cells before use.

Staining of yeast cells with fluorescing antibody

Anti-*a*-agglutinin antibody was labelled with FITC following the method of Hurn and Chantler (1980). Staining of zygotes was carried out as described by Watzele *et al.* (1988) with slight modifications. Cells 3×10^7 of the 'zygote mixture' (30−40% zygotes) were centrifuged and washed three times with PBS. Then the cells were incubated: (i) 20 μl PBS + 1 μl anti-α-agglutinin serum; 3× wash with PBS. (ii) 10 μl PBS + 1 μl rhodamine (TRITC) labelled anti-rabbit IgG (Sigma); 3× wash with PBS. (iii) 10 ml

PBS + 20 μl preserum; 2× wash with PBS; (iv) 30 μl FITC labelled anti-*a*-agglutinin antibody (see above); 3× wash with PBS. All incubations were carried out at room temperature and in darkness in an Eppendorf shaker for 45 min. After the last washing step the pellet was resuspended in 20 μl H_2O containing 1 mg/ml *p*-phenylenediamine and kept in darkness and on ice. Photographs (Agfa RS 1000 film) were taken using a fluorescence phase microscope (Zeiss Standard 16 with objective Neofluar 100/1.3). The film was exposed twice, first for FITC with excitation light of 450–490 nm and light emission >520 nm and second for TRITC with excitation light of 560 –580 nm and light emission >590 nm.

Glutaraldehyde crosslinking and Western analysis

Both agglutinins (purified or crude extracts) were dialysed against 50 mM sodium phosphate buffer pH 6.3. α-agglutinin (25–30 U) and 5–25 U *a*-agglutinin (for definition of units see Watzele *et al.*, 1988) were mixed in a volume of 30 μl and incubated for 10 min at room temperature. Then glutaraldehyde was added to a final concentration of 0.2%. After 30 min crosslinking was stopped by adding an excess of lysine or 6× concentrated Laemmli sample buffer. After SDS-gel electrophoresis proteins were transferred to nitrocellulose (Dunn, 1986) and incubated with anti-*a*-antibodies. Bound anti-*a*-antibodies were detected by alkaline phosphatase coupled to anti-rabbit antibodies (Turner, 1986).

Diethylpyrocarbonate treatment

Diethylpyrocarbonate (DEPC) (Sigma, München) was diluted in ethanol just before use and added to the sample (0.3 μg *a*- or/and 1.5 μg α-agglutinin in 30 μl sodium phosphate buffer, 50 mM, pH 6.3) to give a final concentration of 5 mM. After 10 min DEPC was neutralized with an excess of histidine or removed by Speed Vac evaporation. The treated proteins were resuspended in a solution containing the partner agglutinin or only buffer and crosslinked as indicated above.

Acknowledgements

We are grateful to Drs R.Deutzmann, Regensburg, and J.Hoppe, Würzburg, for sequencing peptides and to Drs W.Duntze and R.Betz, Bochum, for purified *a* factor. This work has been supported by the Deutsche Forschungsgemeinschaft (SFB 43) and by the Fonds der Chemischen Industrie.

References

Achstetter,T. (1989) *Mol. Cell. Biol.*, **9**, 4507–4514.
Betz,R., Duntze,W. and Manney,T.R. (1978) *FEMS Microbiol. Lett.*, **4**, 107–110.
Botstein,D., Falco,S.C., Steward,S.E., Brennan,M., Scherer,S., Stinchcomb,D.T., Struhl,K. and Davis,R.W. (1979) *Gene*, **8**, 17–24.
Burke,D., Mendonça-Previato,L. and Ballou,C.E. (1980) *Proc. Natl. Acad. Sci. USA*, **77**, 318–322.
Chan,R.K. and Otte,C.A. (1982) *Mol. Cell. Biol.*, **2**, 11–20.
Crandall,M. and Brock,T.D. (1968) *Bacteriol. Rev.*, **32**, 139–163.
Cryer,D.R., Eccleshall,R. and Marmur,J. (1975) In Prescott,D.M. (ed.), *Methods in Cell Biology*. Academic Press, New York, Vol. 12, pp. 39–44.
Doi,S., Suzuki,Y. and Yoshimura,M. (1979) *Biochem. Biophys. Res. Commun.*, **91**, 849–853.
Domdey,H., Apostol,B., Lin,R.-J., Newman,A., Brody,E. and Abelson,J. (1984) *Cell*, **39**, 611–621.
Dunn,S.D. (1986) *Anal. Biochem.*, **157**, 144–153.
Fehrenbacher,G., Perry,K. and Thorner,J. (1978) *J. Bacteriol.*, **134**, 893–901.
Hagiya,M., Yoshida,K. and Yanagishima,N. (1977) *Exp. Cell Res.*, **104**, 263–272.
Hauser,K. and Tanner,W. (1989) *FEBS Lett.*, **255**, 290–294.
Heijne,G. von (1985) *J. Mol. Biol.*, **184**, 99–105.
Henikoff,S. (1984) *Gene*, **28**, 351–359.
Hill,J.E., Meyers,A.M., Koerner,T.J. and Tzagoloff,A. (1986) *Yeast*, **2**, 163–167.
Ho,S.N., Hunt,H.D., Horton,R.M., Pullen,J.K. and Pease,L.R. (1989) *Gene*, **77**, 51–59.
Hurn,B.A.L. and Chantler,S.M. (1980) *Methods Enzymol.*, **70**, 104–142.
Jentoft,N. (1990) *Trends Biochem. Sci.*, **15**, 291–294.
Kamboj,R.K., Gariepy,J. and Siu,C.-H. (1989) *Cell*, **59**, 615–625.
Laemmli,U.K. (1970) *Nature*, **227**, 680–685.
Leu,T.M.J. (1990) Ph.D Thesis, University of Zürich.
Lipke,P.N., Wojciechowicz,D. and Kurjan,J. (1989) *Mol. Cell. Biol.*, **9**, 3155–3165.
Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning. A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
March,J.L., Erfle,M. and Wykes,E.J. (1984) *Gene*, **32**, 481–485.
Messing,J., Gronenborn,B., Müller-Hill,B. and Hofschneider,H.P. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 3642–3646.
Miles,E.W. (1977) *Methods Enzymol.*, **47**, 431–442.
Morrissey,J.H. (1981) *Anal. Biochem.*, **117**, 307–310.
Mort,A.J. and Lamport,D.T.A. (1977) *Anal. Biochem.*, **82**, 289–309.
Orlean,P., Ammer,H., Watzele,M. and Tanner,W. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 6263–6266.
Pierce,M. and Ballou,C.E. (1983) *J. Biol. Chem.*, **258**, 3576–3582.
Rothstein,R.J. (1983) *Methods Enzymol.*, **101**, 202–211.
Roy,A., Lu,C.F., Marykwas,D.L., Lipke,P. and Kurjan,J. (1991) *Mol. Cell. Biol.*, **11**, 4196–4206.
Sanger,F., Nicklen,S. and Coulson,A.R. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 5463–5467.
Sijmons,P.C., Nederbraft,A.J.A., Klis,F.M. and Van Den Ende,H. (1987) *Arch. Microbiol.*, **148**, 200–212.
Strahl-Bolsinger,S. and Tanner,W. (1991) *Eur. J. Biochem.*, **196**, 185–190.
Strazdis,J.R. and MacKay,V.L. (1983) *Nature*, **305**, 543–545.
Terrance,K. and Lipke,P.N. (1981) *J. Bacteriol.*, **148**, 889–896.
Terrance,K., Heller,P., Wu,Y.-S. and Lipke,P.N. (1987) *J. Bacteriol.*, **169**, 475–482.
Turner,B.M. (1986) *Methods Enzymol.*, **121**, 848–855.
Watzele,M., Klis,F. and Tanner,W. (1988) *EMBO J.*, **7**, 1483–1488.
Yanisch-Perron,C., Vieira,J. and Messing,J. (1985) *Gene*, **33**, 103–119.
Yen,P.H. and Ballou,C.E. (1974) *Biochemistry*, **13**, 2428–2437.

Received on July 8, 1991; revised on September 18, 1991

Note added in proof

The sequence data reported here are available from the EMBL/GenBank/DDBJ databases under accession number X62877.