

Structural Features of a Polypeptide Carrier Promoting Secretion of a β -Lactamase Fusion Protein in Yeast

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Escherichia coli β -lactamase was secreted into the culture medium of *Saccharomyces cerevisiae* in biologically active form, when fused to the C-terminus of the hsp150 Δ -carrier. The hsp150 Δ -carrier is an N-terminal fragment of the yeast hsp150 protein, having a signal peptide and consisting mostly of a 19 amino acid peptide repeated 11 times *in tandem*. Here we expressed the hsp150 Δ -carrier fragment alone in *S. cerevisiae*. Apparently due to a positional effect of the gene insertion, large amounts of the hsp150 Δ -carrier were synthesized. About half of the *de novo* synthesized carrier molecules were secreted into the culture medium, the rest remaining mostly in the pre-Golgi compartment. The extensively O-glycosylated carrier fragment was purified from the culture medium under non-denaturing conditions. Circular dichroism spectroscopy showed that it had no regular secondary structure. Nuclear magnetic resonance spectroscopy showed that a non-glycosylated synthetic peptide, the consensus sequence of the repetitive 19 amino acid peptide, also lacked secondary structure. The unstructured carrier polypeptide may facilitate proper folding and secretion of heterologous proteins attached to it.

KEY WORDS — secretion; yeast; glycosylation; β -lactamase; fusion protein

INTRODUCTION

Saccharomyces cerevisiae is regarded as a suitable host for the production of secretory mammalian proteins, since it is capable of modifying them properly, unlike prokaryotic cells. However, many secretory proteins are not transported to the culture medium, unless fused to a carrier polypeptide. The prepro-region of the yeast pheromone α -factor has been used to secrete successfully tens of heterologous proteins (Brake *et al.*, 1984; Romanos *et al.*, 1992; Hadfield *et al.*, 1993). The secondary structure of the pro-region is not known. The same is true for the prepro-region of killer toxin, which has been used to secrete some foreign proteins to the culture medium (Baldari *et al.*, 1987; Parentesis *et al.*, 1988; Hadfield *et al.*, 1993). Difficulties in using other carriers are exemplified by experiments with invertase fusion proteins. When β -galactosidase was fused to different N-terminal fragments of the mature invertase protein, the fusion proteins were trapped early in the secretory

route, apparently in the endoplasmic reticulum (ER) (Emr *et al.*, 1984).

The hsp150 protein is one of the few proteins of *S. cerevisiae* that are secreted to the culture medium (Tanner and Lehle, 1987; de Nobel and Barnett, 1991). The expression of this secretory glycoprotein is up-regulated by heat stress, but its function is as yet unknown (Russo *et al.*, 1992, 1993). However, it turned out that an N-terminal fragment of the *HSP150* gene product could be used to direct foreign proteins to the culture medium. The primary translation product of the *HSP150* gene contains a signal peptide, followed by subunit I, which is joined via a kex2 protease recognition site to subunit II. Subunit II is composed of a region with high internal periodicity, where a 19 amino acid peptide is repeated 11 times, and of a unique C-terminal region (Russo *et al.*, 1992). When *Escherichia coli* β -lactamase or the extracellular domain of rat nerve growth factor receptor (p75) were fused to the C-terminus of the repetitive region of subunit II, the hsp150 Δ -carrier, the heterologous protein portions adopted

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biologically active conformations, and the fusion proteins were efficiently secreted to the culture medium (Simonen *et al.*, 1994; Simonen *et al.*, submitted). In order to understand the properties of the hsp150 Δ -carrier, we studied its structure, post-translational modifications and secretion, and compared them with those of authentic hsp150.

MATERIALS AND METHODS

Strains and media

Plasmid constructions were performed using *E. coli* DH5 α , grown in L-broth supplemented with 100 μ g/ml of ampicillin. *S. cerevisiae* strains H23 (*Mata hsp150::URA3 ura3-1 his3-11,15 leu2-3,112 trp1-1 ade2-1 can1-100*), H431 (H23 transformed with truncated *HSP150*, see below), H433 (mBY12-6D, *sec18-1 trp1-289 leu2-3,112 ura2-53 his⁻*, transformed with truncated *HSP150*), and SF821-8A (*Mata sec7-1 his4-580 ura3-52 leu2-3,112 trp1-289*) were grown at 24°C in YPD-medium or synthetic complete (SC) medium as before (Simonen *et al.*, 1994).

Plasmid construction

A DNA fragment containing the *HSP150* structural gene, devoid of its four last codons, and about 2 kb of upstream sequence was placed upstream from the *ADCI* terminator in Bluescript SK⁻ vector (Stratagene) as described (Simonen *et al.*, 1994). Plasmid pKTH4553 was obtained when the missing four codons plus a stop codon were inserted as a synthetic oligonucleotide (5' CGATT TGATAGACTGTTA 3', and 5' AGCTTAACAG TCTATCAAAT 3') between the *ClaI* and *HindIII* sites (Russo *et al.*, 1992; Simonen *et al.*, 1994). The 3' sequence of the *HSP150* gene was removed as follows. The *HSP150* sequence was cleaved with *KpnI*, 63 bp downstream from the last repetitive unit, and digested with mung bean nuclease (Boehringer Mannheim). The 3' portion was removed by *ClaI* digestion. By filling in with the Klenow fragment of DNA polymerase I (Promega) and ligation, the 5' sequence of *HSP150* was joined to the 3' sequence in the wrong reading frame, creating two extra codons plus two consecutive stop codons after codon 315 of *HSP150*. The resulting construct (pKTH4568) was verified by sequencing. The truncated *HSP150* fragment was transferred as a 3.9 kb *BamHI* fragment to pFL26 (Bonneaud *et al.*, 1991). The resulting

plasmid, pKTH4605, was linearized with *BstEI* at the *LEU2* locus, and introduced into strain H23 (Becker and Guarente, 1991), creating strain H431.

Metabolic labeling, immunoprecipitation and SDS-PAGE

Cells were labeled with 100 μ Ci/ml (analytical experiments) or 300 μ Ci/ml (preparative labeling) of 2-[³H]mannose (11.5 Ci/mM; Amersham) in YPD-medium containing 0.1% glucose, after a preincubation of 8 min in the same medium. Immunoprecipitation and SDS-PAGE in 8% gels were as described (Jämsä *et al.*, 1994).

Protein purification

To purify the hsp150 Δ -carrier, the culture medium of strain H431, grown overnight in SC-medium at 24°C, was concentrated by hollow fibre filtration (PM5 membrane, Romicon) and membrane filtration (PM10 membrane, Amicon). The concentrate was subjected to Bio-Gel P-100 gel filtration with 20 mM-Tris-HCl, pH 8.0. Gel filtration on a Superdex-75 HR10/30 column (Pharmacia) was performed in 50 mM-sodium phosphate pH 7.4, 150 mM-NaCl (PBS), at a flow rate of 0.8 ml/min. The MonoQ HR5/5 (Pharmacia) column was equilibrated with 20 mM-Tris-buffer, pH 8.0. Elution was with a linear gradient of 1 M-NaCl (0–70% in 40 min) in the same buffer, and detection at 280 nm. Hsp150 was produced to the culture medium of SF821-8A cells as described (Russo *et al.*, 1992). The culture medium was adjusted to pH 8.0, and passed over a DEAE cellulose (DE 52, Whatman) column (50 ml/l of culture medium), equilibrated with 20 mM-Tris-HCl, pH 8.0. Dialysis was for 24 h against 10 mM-Tris-HCl, pH 8.0. Chromatography over a Q-Sepharose (Pharmacia) column (12 ml/l of culture medium) was with 10 mM-Tris-buffer, and elution was carried out with 20 mM-Tris-buffer containing 1 M-NaCl. Bio-Gel P-100 and MonoQ chromatography were as above. Reversed-phase HPLC was carried out for both proteins in a TSK TMS 250 column (0.3 \times 3 cm, TosohHaas), eluted with a linear gradient (3–100% in 60 min) of acetonitrile in 0.1% trifluoroacetic acid, with detection at 218 nm. The proteins were visualized by immunoblotting with anti-hsp150 antiserum (Russo *et al.*, 1992), except that the hsp150 Δ -carrier was blotted on a double layer, due to its facile penetration through the nitrocellulose filter.

Glycan analysis

SF821-8A cells were labeled with 2-[³H]mannose for 1 h at 37°C. The proteins of the culture medium were precipitated with 14% trichloroacetic acid for 30 min on ice, and subjected to preparative SDS-PAGE. ³H-Labeled subunit II of hsp150 was electroeluted from gel slices (Allington *et al.*, 1978), and SDS was removed (Henderson *et al.*, 1979). The preparation was desalted by passing it over a Bio-Gel P-6 column (1.5 × 40 cm, Bio-Rad). β-Elimination was performed in 0.1 M-NaOH/1 M-NaBH₄ for 17 h at 40°C (Nakajima and Ballou, 1974; Salovuori *et al.*, 1987). The desalted glycan preparation was purified further by ion exchange chromatography using Dowex 50x8 and Dowex 1x8 columns (0.7 × 4 cm, 200–400 mesh, Fluka). Thin-layer chromatography (TLC) was as described (Salovuori *et al.*, 1987), with (Man)₄GlcNAc, (Man)₃GlcNAc and (Man)₂GlcNAc, lactose and mannose as markers.

CD spectroscopy

Purified protein samples were subjected to CD spectroscopy at 5°C, 27°C and 37°C, using a JACSO J-720 spectropolarimeter, in PBS (hsp150Δ-carrier, 1.5 μM), or in 10 mM-acetate buffer, containing 50 mM-NaCl, pH 5.0 (hsp150, 17 μM). The spectra were recorded in the far UV region (190–250 nm) in a thermostatted rectangular cell. Each spectrum was the mean of four scans obtained with a time constant of 4 s and a scan speed of 10 nm/min. The solvent dichroic absorbance was subtracted using the JACSO software.

Nuclear magnetic resonance (NMR) spectroscopy

The synthetic peptide (Multiple Peptide Systems, San Diego, U.S.A.) was dissolved in H₂O/10% D₂O. The peptide solution (pH 5.0) was subjected to NMR spectroscopy at 5°C, using a General Electric Omega instrument working at 500.13 MHz. The data were processed using the GE Omega software on Sun workstations. One TOCSY spectrum (Braunschweiler and Ernst, 1983) and one ROESY spectrum (Bax and Davis, 1985) were recorded using the hypercomplex method (States *et al.*, 1982), with the carrier frequency placed on the solvent resonance. A spectral width of 5 kHz was employed with a relaxation delay of 1.5 s incorporated into each sequence. The TOCSY and ROESY experiments were recorded with mixing times of 120 ms and 200 ms, respectively. Sixty-four transients were acquired for each

free induction decay and the data size was 512 × 2048 complex points for all two-dimensional experiments. Prior to Fourier transformation in two dimensions, the free induction decays were multiplied by a phase-shifted squared sine bell window function. Zero filling in the evolution dimension provided final two-dimensional spectra of 1024–2048 complex points.

Other methods

Northern analysis was performed using *HSP150* and *ACT1* probes as described (Russo *et al.*, 1993). An RNA ladder (Gibco-BRL) was used for size determination. N-terminal amino acid sequencing was performed using a gas phase sequencer, equipped with an on-line PTH amino acid analyser (Kalkkinen and Tilgman, 1988). Ultrafiltration was with Centricon ultrafilters (Amicon) at 5000 g. Cycloheximide and NaN₃ were from Sigma, and were used at concentrations of 100 μg/ml and 10 mM, respectively. Restriction endonucleases were from Promega, New England Biolabs and Boehringer Mannheim.

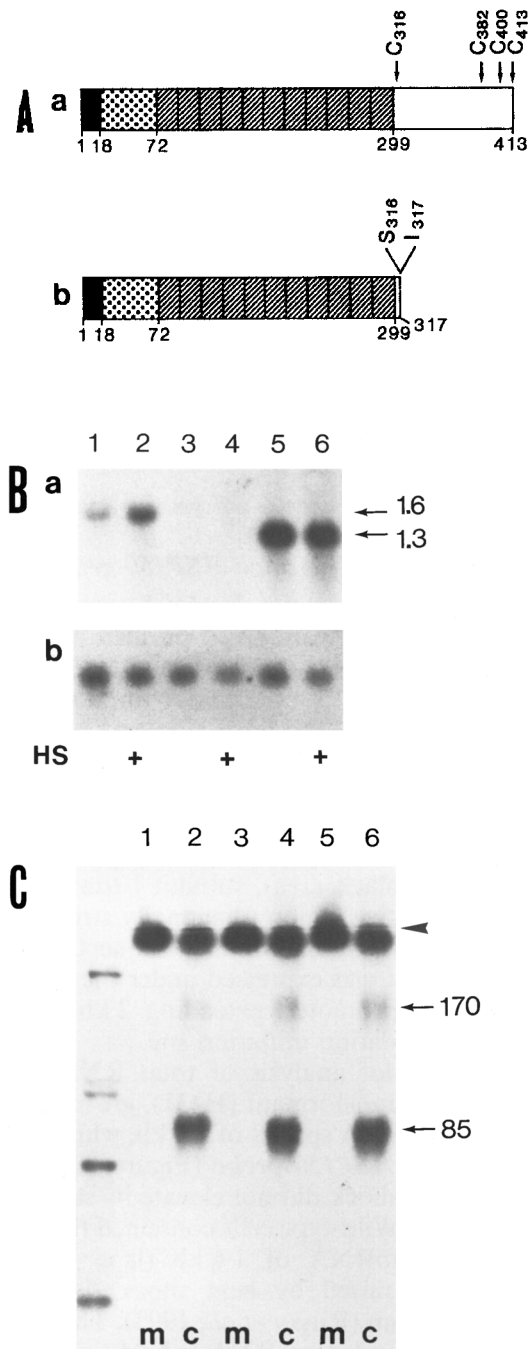
RESULTS

Expression of the truncated *HSP150* gene

To study the repetitive hsp150Δ-carrier, we expressed it in *S. cerevisiae* and purified it from the culture medium. To this end, a DNA fragment coding for the 315 N-terminal amino acids of hsp150, plus Ser₃₁₆ and Ile₃₁₇ (Figure 1A, panel b), was targeted to the *leu2* locus of strain H23, which lacks a functional *HSP150* gene (Russo *et al.*, 1992). The truncated *HSP150* gene contained the nucleotides encoding the signal peptide (Figure 1A, panel a; black area), subunit I (dotted area), and the repetitive region (diagonally striped area), but lacked the codons for the unique C-terminus (white area). It was expressed under the control of the *HSP150* promoter, extending 2 kb upstream from the translation initiation site.

Northern blot analysis of total RNA showed that the new transformant (H431), grown at 24°C, expressed an RNA species of 1.3 kb, which hybridized with the *HSP150* probe (Figure 1B, panel a, lane 5). Heat shock did not elevate its steady-state level (lane 6). Wild-type cells contained the authentic *HSP150* mRNA of 1.6 kb (lane 1), whose amount was raised by heat shock (lane 2), as described before (Russo *et al.*, 1993). The parental strain H23 revealed no RNA hybridizing with the

HSP150 probe (lanes 3 and 4). *ACT1* mRNA served as an internal control (panel b). Thus, though placed under the *HSP150* promoter, the truncated gene was not under heat regulation, and it produced a much higher constitutive level of mRNA than authentic *HSP150*.



Secretion of the *hsp150A*-carrier

To study the expression and secretion of the product of the truncated *HSP150* gene, the *hsp150A*-carrier, strain H431 was labeled with [³H]mannose for 1 h (the carrier lacks Met and Cys). Cycloheximide was added, and the incubation was continued for 30 min. The culture medium (m) and cell lysate (c) samples were subjected to immunoprecipitation with anti-*hsp150*. SDS-PAGE analysis showed that the *hsp150A*-carrier was secreted into the medium (Figure 1C, lane 1). Since it lacks N-glycosylation sites, [³H]mannose was incorporated into O-glycans. The electrophoretic migration of the carrier fragment was anomalous, as it hardly penetrated the 8% gel. The band in lane 1 represents the glycosylated repetitive fragment only (amino acids 73–317 in Figure 1A, panel b), since the signal peptide and subunit I were lost during secretion (see below). The polypeptide backbone of 23 747 daltons has 56 potential O-glycosylation sites. If all the sites were occupied with similar O-glycans, as in full-length subunit II (see below), the molecular mass of the carrier would still be only 50 kDa.

Figure 1. (A) Primary translation products of *HSP150* and truncated *HSP150*. (Panel a) The product of the authentic *HSP150* gene consists of a signal peptide of 18 amino acids (black area), subunit I (amino acids 19–72; dotted area) and subunit II (amino acids 73–413; diagonal stripes plus white area). Subunit II consists of 11 repeats of homologous peptides (amino acids 73–299; see Figure 6A for sequence), and a unique C-terminus with four cysteine residues (amino acids 300–413). (Panel b) The product of the truncated *HSP150* gene, the *hsp150A*-carrier, lacks amino acids 316–413 of *hsp150*, and has the C-terminal residues Ser₃₁₆ and Ile₃₁₇. (B) Northern analysis of the transcript of truncated *HSP150*. Wild-type cells (H1; lanes 1 and 2), strain H23 devoid of a functional *HSP150* gene (lanes 3 and 4), and strain H431, where the truncated *HSP150* gene was introduced into strain H23 (lanes 5 and 6), were grown overnight at 24°C. The cultures were split into two, one half continuing for 30 min at 24°C (lanes 1, 3 and 5), and the other at 37°C (HS+; lanes 2, 4 and 6). Total RNA was extracted and subjected to Northern analysis using *HSP150* as a probe (panel a). The membrane was washed and reprobed with *ACT1* DNA (panel b). Figures on the right are the sizes (kb) of the indicated RNA molecules. (C) Secretion of the *hsp150A*-carrier. H431 cells were labeled with [³H]mannose for 1 h and chased in the presence of cycloheximide for 30 min at 24°C (lanes 1 and 2), 60 min (lanes 3 and 4), or 90 min (lanes 5 and 6). NaN₃ was added, and the culture media (m) and lysed cell samples (c) were immunoprecipitated with anti-*hsp150*, and analysed by SDS-PAGE and fluorography. The molecular weight markers on the left are 200, 97.4, 69 and 46 kDa. The arrowhead indicates the start of the separating gel. Apparent molecular masses (kDa) of indicated proteins are on the right.

Authentic hsp150 is secreted into the culture medium without significant retention in secretory organelles or the cell wall (Russo *et al.*, 1992; Jämsä *et al.*, 1994). The hsp150 Δ -carrier was secreted less efficiently. Polypeptides with an apparent molecular weight of about 85 kDa, and evidently glycosylated molecules could be detected in the cell lysate (lane 2). The 85 kDa polypeptide was probably the ER-form with unextended O-glycans. When the carrier fragment was retained in the pre-Golgi compartment at 37°C in the *sec18* mutant H433, only this species could be immunoprecipitated from the cell lysate with anti-hsp150 (not shown). The cell-associated mature form apparently resided in the Golgi or cell wall. In addition to the ER-form and the apparently mature form, a minor species, migrating like a 170 kDa protein, was detected only in the lysate. All three forms remained cell-associated after chase periods of 60 min (lanes 3 and 4) and 90 min (lanes 5 and 6). The relative amount of the ER-form was higher than that of the secreted protein, since the first residues of O-glycans are extended in the Golgi (Haselbeck and Tanner, 1983).

Purification of the hsp150A-carrier and full-length hsp150

To purify the hsp150 Δ -carrier fragment, strain H431 was grown overnight in SC-medium at 24°C. The culture medium was concentrated by ultrafiltration, and subjected to gel filtration in a Bio-Gel P-100 column (not shown). The pooled void volume fractions were subjected to MonoQ chromatography (Figure 2A). The fractions indicated by bars, containing the carrier fragment according to Western blotting, were pooled and subjected to gel filtration on a Superdex-75 column (Figure 2B). The fractions indicated by the bars, containing the carrier fragment, were pooled and subjected to CD spectroscopy (see below). The pooled preparation was analysed further by reversed-phase HPLC, which resolved it into three main species (Figure 2C). Since all of them had the N-terminus of subunit II, the heterogeneity revealed during the chromatographic steps was probably due to differences in glycosylation. No peak corresponding to subunit I could be detected.

We have earlier reported the N-terminal amino acid sequences of subunits I and II of hsp150 (Russo *et al.*, 1992). To obtain preparative amounts of authentic native hsp150 for CD analysis, it was purified as follows. The culture medium of SF821-8A cells was passed over a

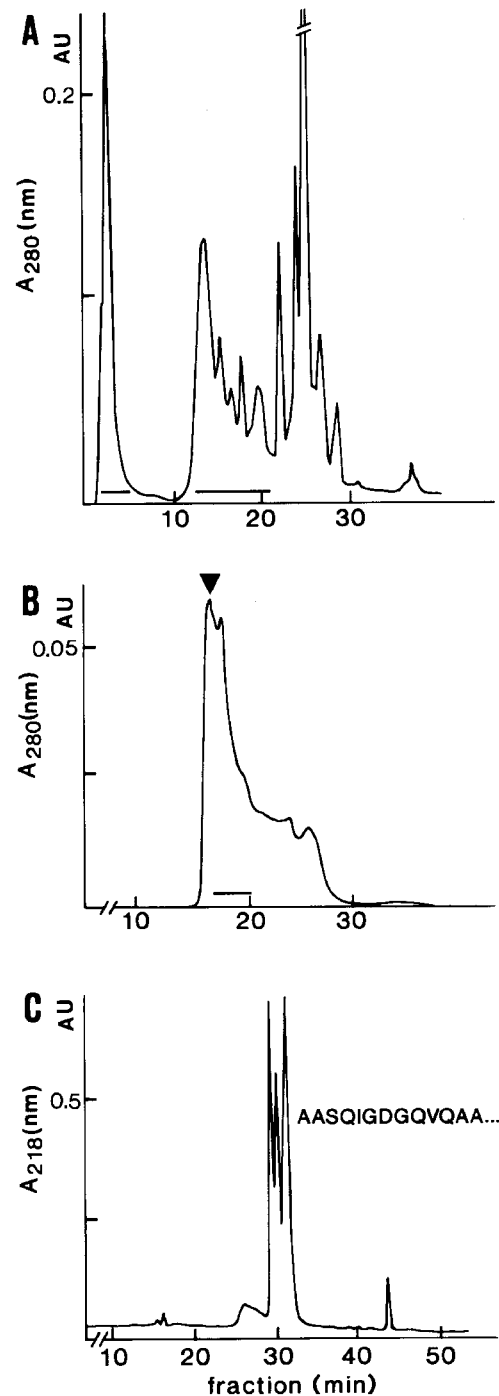


Figure 2. Purification of the hsp150 Δ -carrier. (A) MonoQ anion-exchange chromatography of the hsp150 Δ -carrier after gel filtration on Bio-Gel P-100. Fractions indicated by bars were pooled. (B) Gel filtration of 1385 the pooled fractions from panel A on a Superdex-75 column. Arrowhead indicates void volume. (C) The fractions indicated by a bar in panel B were pooled and subjected to reversed-phase chromatography. All three main species gave the same N-terminal sequence, as indicated.

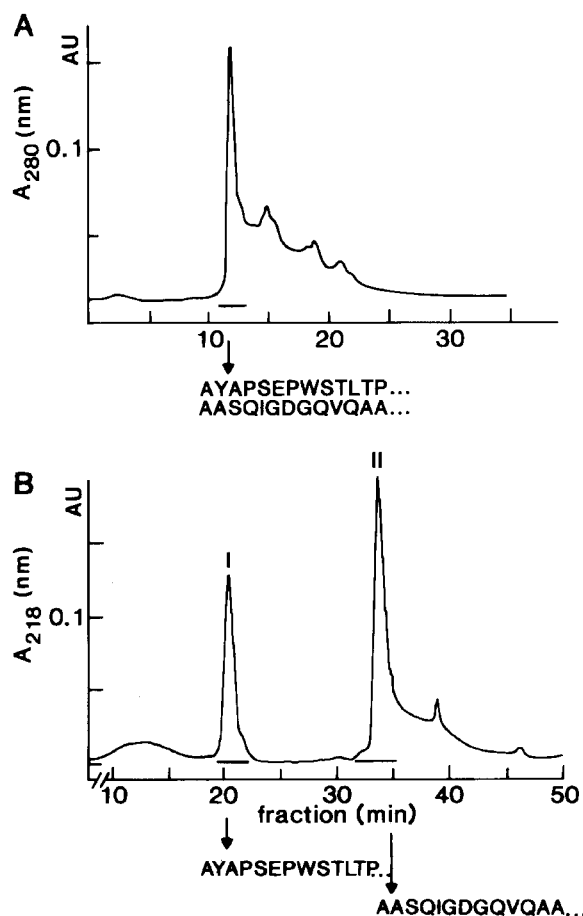


Figure 3. Purification of authentic hsp150. (A) MonoQ chromatogram of hsp150 (see text). The fraction indicated by the bar was subjected to N-terminal amino acid sequencing, which yielded double residues, as indicated. (B) The fraction from panel A (bar) was subjected to reversed-phase chromatography. The material in peaks I and II (bars) gave the indicated N-termini.

DEAE-cellulose column. The flow-through material was dialysed and subjected to chromatography on a Q-Sepharose column, from which hsp150 was eluted with 1 M-NaCl. The eluate was concentrated and subjected to gel filtration on a Bio-Gel P-100 column. The pooled void volume fractions were subjected to anion-exchange chromatography on MonoQ. The material of the major peak (Figure 3A, bar) was analysed by direct N-terminal amino acid sequencing, where it gave a double signal in about equal molar amounts at each degradation cycle, as indicated. This fraction was subjected to CD spectroscopy (see below). The fraction could be resolved into two species by reversed-phase HPLC (Figure 3B). The material of peak I gave the sequence of subunit I (see Figure

1A), and that of peak II gave the sequence of subunit II. Since subunit I was missing from the hsp150 Δ -carrier, it appears to be attached in authentic hsp150 to the C-terminal region of subunit II, which is missing from the hsp150 Δ -carrier.

Glycan analysis

Hsp150 has no N-glycosylation sites, but carries O-glycans, since its apparent molecular weight was reduced by hydrogen fluoride treatment (Russo *et al.*, 1992). To size the O-glycans of full-length subunit II, SF821-8A cells were labeled with [3 H]mannose, and the culture medium was subjected to preparative SDS-PAGE. 3 H-Labeled subunit II of hsp150 was eluted from gel slices (subunit I is released by denaturation), and the eluate was passed over a Bio-Gel P-6 column. The void volume fractions (Figure 4A, full circles) were lyophilized and subjected to β -elimination to release O-glycans. The hydrolysate was passed over a Bio-Gel P-6 column, and now 87% of the radioactivity eluted with the total volume (Figure 4A, open circles). TLC analysis showed that the glycans migrated like di-, tri-, tetra- and pentasaccharides (Figure 4B). Assuming the same specific radioactivity for each mannose residue, the average molar ratios of the mannosides of subunit II were 4:1:1:1, respectively (Figure 4C).

CD analysis of the hsp150 Δ -carrier and authentic hsp150

The hsp150 Δ -carrier, purified under non-denaturing conditions (see above), was analysed by CD spectroscopy (Figure 5A). The spectrum shows a minimum around 200 nm, and resembles that of an unfolded polypeptide lacking regular secondary structure. Equivalent spectra were obtained at 5°C and 37°C (not shown).

The CD spectrum of full-length hsp150, containing subunits I and II attached to each other non-covalently, is presented in Figure 5B. It shows superposition of random coil and β -sheet structure. Thus, the repetitive region of subunit II apparently lacked secondary structure even in native hsp150. The β -sheet structure must have been due to subunit I, the C-terminal region of subunit II, or both.

NMR analysis of the non-glycosylated repetitive peptide

A peptide was synthesized according to the consensus sequence of the homologous peptides of

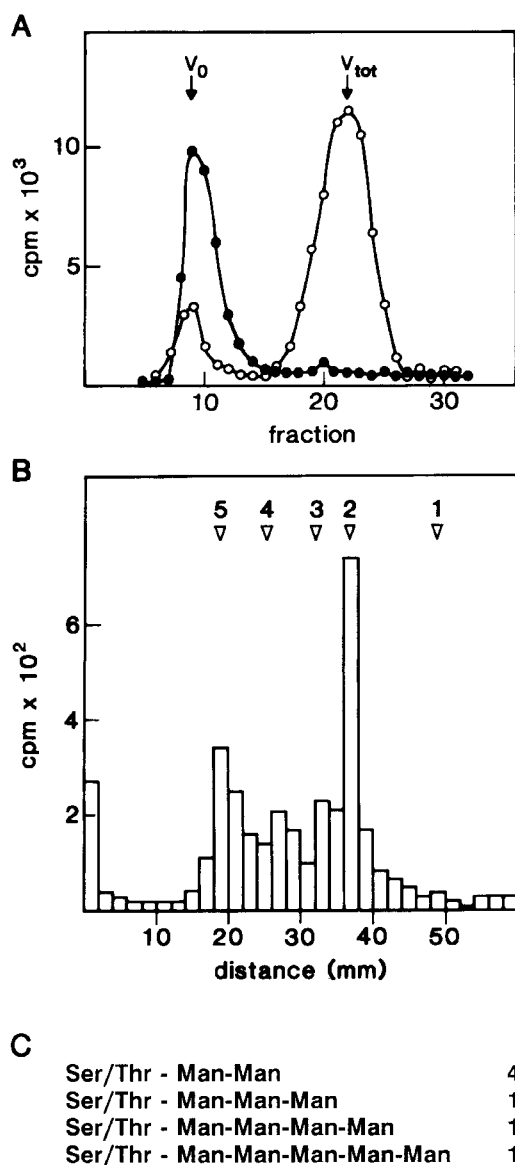


Figure 4. Glycan analysis of subunit II of hsp150. (A) β -Elimination. [^3H]Mannose labeled subunit II was passed over a Bio-Gel P-6 column before (filled circles) or after (open circles) β -elimination. Fractions 16–26 were pooled and desalted. The void volume (V_0) and total volume (V_{tot}) are indicated. (B) Sizing of the O-glycans. The desalted material was analysed by TLC. Arrows indicate the migration of (1) mono-, (2) di-, (3) tri-, (4) tetra- and (5) pentasaccharide markers. (C) O-Glycans of subunit II, and their relative ratios.

subunit II (Figure 6), and subjected to two-dimensional TOCSY and ROESY NMR spectroscopy. Complete assignments for all proton signals were provided from the TOCSY spectrum (Figure 7A). The amino acid residues were identified according to their characteristic pattern of chemical

shifts based on connectivity from scalar homo-nuclear couplings. Sequential assignment (Wüthrich, 1986) of the amino acid residues was carried out from the analysis of the ROESY spectrum (Figure 7B). The secondary structure determination was based on proton-proton distances derived from the relative strength of inter-residue nuclear Overhauser effects (NOE). The NOE connectivity between the C^αH -proton of a given residue i and the NH-proton of the following residue ($i+1$) is termed the $d_{\alpha\text{N}}(i,i+1)$ NOE connectivity. Similarly, $d_{\text{NN}}(i+1)$ denotes an NOE connectivity between the amino protons of adjacent amino acid residues. The ROESY spectrum shows that the $d_{\alpha\text{N}}(i,i)$ connectivities are much weaker than the sequential $d_{\alpha\text{N}}(i,i+1)$ connectivities. Neither were $(i,i+2)$, $(i,i+3)$, and so on, NOEs observed. Thus, the non-glycosylated peptide had no region of a preferred conformation (Dyson *et al.*, 1988).

DISCUSSION

We have previously shown that when *E. coli* β -lactamase was fused to the C-terminus of the hsp150 Δ -carrier, it acquired an enzymatically active conformation in the ER, and was efficiently secreted into the culture medium of *S. cerevisiae* (Simonen *et al.*, 1994). In contrast, fusion to subunit I, or to almost the entire hsp150 protein (see Figure 1A), resulted in inactive fusion proteins. The hsp150 Δ -carrier also promoted the secretion of the extracellular domain of rat low-affinity nerve growth factor receptor. This protein remained in the ER, when joined directly to the invertase signal peptide (M. Simonen, H. Vihinen, E. Jämsä, U. Arumäe, N. Kalkkinen and M. Makarow, submitted). The hsp150 Δ -carrier has also been successfully applied to secrete human pro-insulin (our unpublished data). Thus, the carrier fragment seems to have the ability to promote secretion of several heterologous proteins.

In order to understand the role of the carrier fragment, we wanted to study its secondary structure. A synthetic peptide of 19 amino acids with the same amino acid sequence as the consensus peptide of the repetitive region (Figure 6B) was subjected to NMR analysis. This peptide had no secondary structure. Since the repetitive peptides in the full-length subunit II, as well as in the hsp150 Δ -carrier were extensively O-glycosylated (Figure 4C), we subjected the two polypeptides to CD analysis. To this end, a truncated *HSP150*

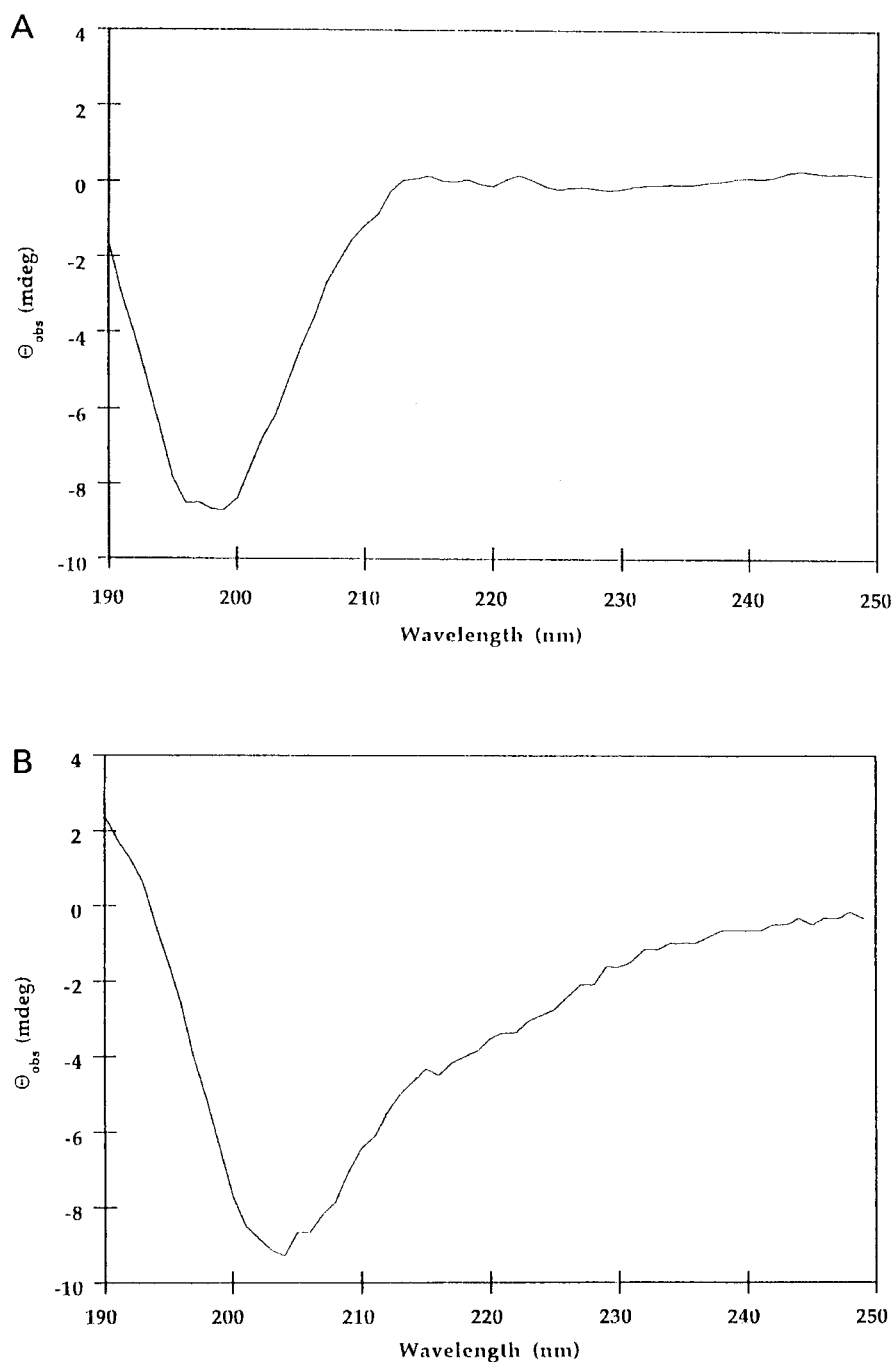


Figure 5. CD analysis of the hsp150 Δ -carrier and authentic hsp150. (A) The hsp150 Δ -carrier fragment (amino acids 73–317 in Figure 1Ab), and (B) the hsp150 protein (subunits I and II non-covalently attached to each other), were purified under non-denaturing conditions and subjected to CD spectroscopy.

gene, encoding the hsp150 Δ -carrier fragment, was expressed in a *S. cerevisiae* strain lacking the authentic *HSP150* gene, and the polypeptide was purified from the culture medium. CD spectro-

scopy showed that the carrier fragment, without subunit I, also lacked regular secondary structure. The CD spectrum of purified authentic hsp150, a non-covalent dimer of subunits I and II, revealed

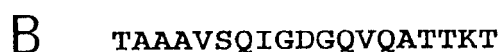
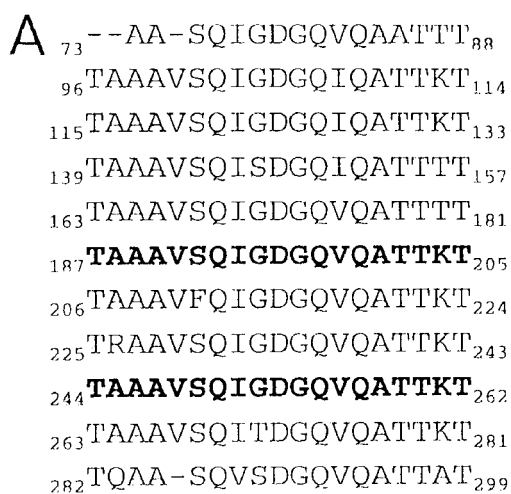
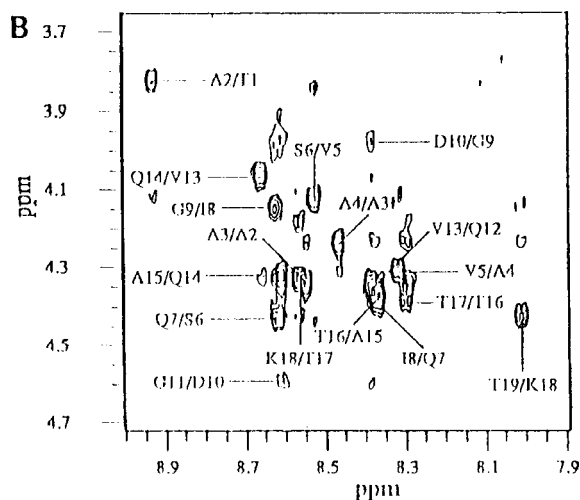
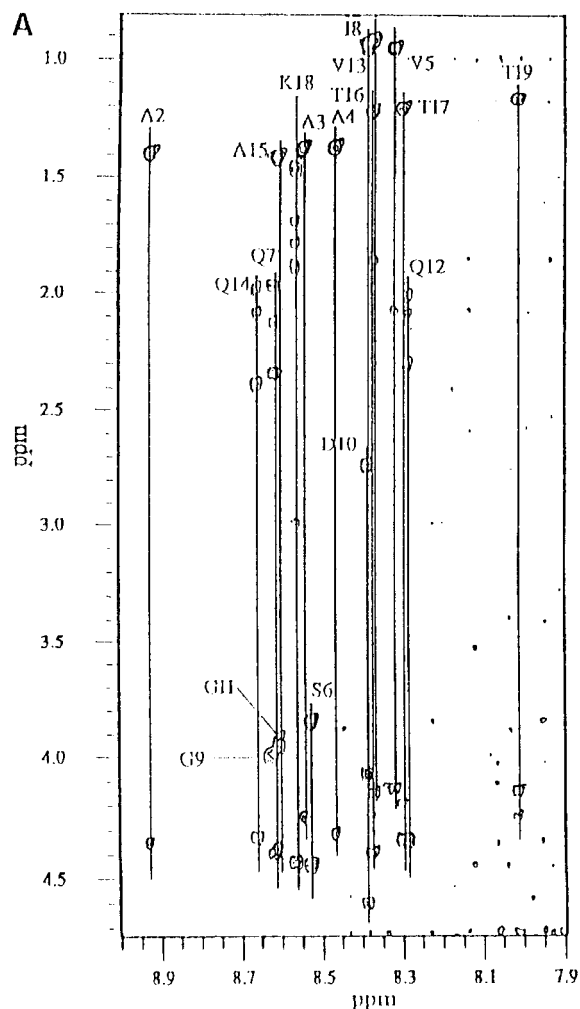


Figure 6. (A) Amino acid sequence of the repetitive region of hsp150 subunit II. (B) The synthetic peptide. A 19-mer peptide was synthesized for NMR spectroscopic analysis, according to the sequence highlighted in panel A.

superposition of random coil and some β -sheet, the latter evidently due to subunit I and the C-terminal region of subunit II.

The special features of the hsp150 Δ -carrier must be responsible for the ability to promote secretion of polypeptides fused to it. One tempting possibility is that it allows, or even facilitates the folding of the polypeptide portion attached to it. In mammalian cells, polypeptides that have not folded properly are retained in the ER by quality control mechanisms, including association with BiP (Rothman, 1989; Gething and Sambrook, 1992). We have shown a direct correlation between conformation and secretion competence of proteins in *S. cerevisiae*. Normally disulphide-bonded proteins, like authentic hsp150 and hsp150 Δ - β -lactamase, were retained in the ER, the latter in an enzymatically inactive form,

Figure 7. NMR analysis of the synthetic peptide. The synthetic peptide shown in Figure 6B was subjected to NMR spectroscopy. (A) TOCSY spectrum of the peptide. The displayed region shows cross peaks between backbone amide protons and side-chain protons. Spin systems of all amino acids, except the N-terminal threonine, are marked. (B) Part of the ROESY spectrum, showing strong sequential $d_{\alpha N}(i, i+1)$ NOE connectivities in the 19-mer peptide.



when the cells were treated with a reducing agent. After removal of the drug, the proteins were secreted. BiP appeared to be associated with reduced hsp150, but not with native hsp150 (Jämsä *et al.*, 1994). We cannot exclude the possibility that the hsp150 Δ -carrier promoted secretion by containing a positive secretion signal. Though protein secretion has been suggested to be based on bulk flow (Wieland *et al.*, 1987), evidence for export signals has been presented lately (Mizuno and Singer, 1993; Balch *et al.*, 1994). Paradoxically, the hsp150 Δ -carrier itself was incompletely secreted. Provided that it had the same amount of occupied glycosylation sites as the complete subunit II, more than half of *de novo* synthesized molecules remained in the pre-Golgi compartment. This estimation is based on the assumption that the ER-form of the carrier fragment was glycosylated with single mannose residues (Haselbeck and Tanner, 1983). The reasons for the retention of part of the carrier polypeptides are not yet known. One possibility is that they were proteolytically modified, e.g. to expose retention signals not present in the secreted molecules. Anyhow, the amount of the secreted hsp150 Δ -carrier was much higher than that of authentic hsp150.

An interesting observation was made concerning the expression of the hsp150 Δ -carrier. We have reported previously that the expression of the authentic hsp150 protein is heat regulated. Upon shift of cells from 24°C to 37°C, the amount of the *HSP150* mRNA, and the synthesis of the hsp150 protein are abruptly and substantially elevated. The promoter contains at least one DNA element, located close to the TATA box, which has been shown to be involved in the heat sensitivity (Russo *et al.*, 1992, 1993). When the truncated *HSP150* gene encoding the hsp150 Δ -carrier, with 2 kb of upstream sequence of the structural *HSP150* gene, was targeted to the *leu2* locus, the expression of *HSP150* Δ -carrier mRNA and the protein was constitutive. The expression levels were much higher than those of the authentic *HSP150* gene (see Figure 1B). One possibility is that the activation of transcription and loss of heat inducibility was due to a positional effect. In this case the heat-inducible DNA elements of the *HSP150* promoter would normally be influenced by DNA sequences beyond the 2 kb promoter region. In the *leu2* locus, a change in the putative DNA interaction would lead to increased transcription.

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