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The hsp150Δ-carrier Confers Secretion Competence to the Rat Nerve Growth Factor Receptor Ectodomain in *Saccharomyces cerevisiae*

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When the extracellular domain of rat low-affinity nerve growth factor receptor ($NGFR_e$) was synthesized in *Saccharomyces cerevisiae* with the signal peptide of invertase, $NGFR_e$ was translocated to the endoplasmic reticulum (ER) and retained there. However, when $NGFR_e$ was fused to the C-terminus of the hsp150Δ-carrier, the hsp150Δ–$NGFR_e$ fusion protein was efficiently secreted to the growth medium with no apparent retention in the ER. The $NGFR_e$ portion was disulphide-bonded and its single N-glycosylation site was occupied. The hsp150Δ-carrier is an N-terminal signal peptide-containing fragment of a yeast secretory glycoprotein. Hsp150Δ–$NGFR_e$, harvested from the culture medium, inhibited the cross-linking of [^{125}I]NGF to authentic NGFR on the surface of human melanoma cells. Moreover, [^{125}I]NGF could be chemically cross-linked to secretory hsp150Δ–$NGFR_e$, suggesting that the $NGFR_e$ portion had adopted a ligand-binding conformation. However, inhibition of the cross-linking by unlabelled NGF was less effective than in the case of the authentic receptor. The hsp150Δ-carrier may have potential in the production of mammalian proteins, which require elaborate folding and disulphide formation in the ER.

KEY WORDS — yeast; NGFR; secretion; protein production; hsp150 protein

INTRODUCTION

A prime requirement for successful production of secretory mammalian proteins in *Saccharomyces cerevisiae* is that the heterologous protein folds properly in the endoplasmic reticulum (ER) to a biologically active and secretion-competent conformation. However, many heterologous proteins with authentic or yeast-derived signal peptides are inactive and fail to be secreted (Romanos *et al.*, 1992). Intracellular transport of a number of such proteins has been rescued by fusing them to the C-terminus of the prepro-region of yeast α-factor, or in some cases to that of killer toxin (Brake *et al.*, 1984; Hadfield *et al.*, 1993). Both homologous and heterologous secretory proteins are retained in the yeast ER when their conformation is distorted for example by *in vivo* reduction (Jämsä *et al.*, 1994; Simonen *et al.*, 1994).

We have recently shown that an N-terminal fragment of the yeast secretory glycoprotein hsp150 promoted efficient secretion of *Escherichia coli* β-lactamase to the culture medium of *S. cerevisiae*, allowing the β-lactamase portion to adopt an enzymatically active conformation in the ER (Simonen *et al.*, 1994). The hsp150Δ-carrier consists of a signal peptide, followed by subunit I that is cleaved off in the Golgi, and 11 *tandem* repeats of a peptide of 19 amino acids (Jämsä *et al.*, 1995). Here we studied the ability of the hsp150Δ-carrier to allow secretion and proper folding of the extracellular domain of rat low-affinity nerve growth factor receptor ($NGFR_e$). NGFR, also called $p75^{NGFR}$ and p75, binds all neurotrophic factors with low affinity and functions in neuronal development (Barbacid, 1995). Proper folding of $NGFR_e$ should pose a challenge for the folding machinery of the yeast ER, since most if not all of the 24 cysteines of authentic $NGFR_e$ form disulphide bonds (Banner *et al.*, 1993; Baldwin and Shooter, 1994). When fused to the C-terminus of the hsp150Δ-carrier, the $NGFR_e$ portion adopted a ligand-binding

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conformation, and the fusion protein was efficiently secreted in large quantities to the culture medium of *S. cerevisiae*.

MATERIALS AND METHODS

Strains and media

Plasmid constructions were performed using *E. coli* DH5α, grown in L-broth supplemented with 100 μg/ml of ampicillin. *S. cerevisiae* strains H23 (*Matα hsp150::URA3 ura3-1 his3-11,15 leu2-3,112 trp1-1 ade2-1 can1-100*), H1 (SEy2101a, *MAT***a**$_1$ *ura3-52 leu2-1,112 ade2-101 suc2Δ9 gal2*), and *sec18* (mBy12-6D, *Matα sec18-1 trp1-289 leu2-3,112 ura3-52 his*$^-$) were grown at 24°C in YPD-medium or synthetic complete (SC) medium.

Plasmid construction

The DNA fragment coding for NGFR$_e$ was synthesized with polymerase chain reaction using *Pfu* polymerase (Strategene). Rat p75 NGFR cDNA in pGEM-4Z vector (Promega) was used as a template, and oligonucleotides A (5′ ACATGGTACCAAGGAGACATGTTCCACAG) and B (5′ ACATAAGCTTAGAGGTTGTCGGTGGTGC) as primers. Oligonucleotide A created a *Kpn*I site to the 5′ end of NGFR$_e$, and oligonucleotide B an in-frame stop codon and a *Hin*dIII site to its 3′ end. The DNA fragment encoding residues 1–223 of mature NGFR was ligated between the repetitive region of *HSP150*, and the *ADC1* terminator (Russo *et al.*, 1992; Simonen *et al.*, 1994), using *Kpn*I and *Hin*dIII sites. The construct in the plasmid obtained, pKTH4594, was verified by sequencing. The fusion gene was transferred as a blunt-ended *Eco*RV-*Spe*I fragment to the *Sma*I site of the integrative shuttle vector pFL26 (Bonneaud *et al.*, 1991). The resulting plasmid, pKTH4600, was digested with *Alf*II at the *LEU2* locus, and introduced into strains H23 and *sec18* (Hill *et al.*, 1991), creating strains H426 and H458, respectively. The copy number and location of the fusion gene in the genome of H426 were studied by Southern analysis. To create a *SUC2–NGFR$_e$* construct, *HSP150Δ–NGFR$_e$* was transferred from pKTH4594 to pFL35 (Bonneaud *et al.*, 1991) and the *HSP150* portion was replaced by a 1 kb *Eco*RI-*Sal*I fragment from pMB2A (Bielefeld and Hollenberg, 1992), containing the *SUC2* promoter and signal sequence. To get the *SUC2* fragment and the *NGFR$_e$* portion in the same reading frame, the *Sal*I site at the 3′ end of the *SUC2* signal sequence was converted blunt-ended by digestion with mung bean nuclease (Boehringer Mannheim), and the *Asp*718 site at the 5′ end of the *NGFR$_e$* portion was filled in with the Klenow fragment of DNA polymerase I (Promega). The *SUC2–NGFR$_e$* junction in the resulting plasmid pKTH4613 was verified by sequencing. Then the 2 μ ori from YEp24 was inserted into the *Eco*RI site of the plasmid as a 2241 bp fragment. The obtained plasmid pKTH4616 was introduced into H23 to create H487. A multicopy plasmid was used here due to the low expression level driven by the *SUC2* promoter.

Metabolic labelling, immunoprecipitation and immunoblotting

Metabolic labelling of cells (2×10^8 cells/400 μl) with [^{35}S]methionine/cysteine (1000 Ci/mM, Amersham), lysis of cells, and immunoprecipitations with anti-hsp150 (1:100) (Russo *et al.*, 1992), anti-NGFR (1:100, polyclonal antibodies against the ligand-binding domain of NGFR), and anti-hsp150Δ–NGFR$_e$ (1:50) were as described (Jämsä *et al.*, 1994). Immunoblotting was performed as described (Simonen *et al.*, 1994) using anti-hsp150 (1:1000).

Purification of hsp150Δ–NGFR$_e$

The overnight culture supernatant was separated from cells by centrifugation, and dialysed at 4°C for 24 h against 10 mM-Tris–HCl, pH 8·0. The Q-Sepharose Fast Flow column (Pharmacia, 2·5 × 5·5 cm, flow rate 4 ml/min) was equilibrated with 10 mM-Tris–HCl, pH 8·0. Elution was with the same buffer containing 0·5 M-NaCl. 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. Desalting was carried out using a Bio-Gel P-6 (100–200 mesh) column (Bio-Rad, 0·5 × 10 cm, flow rate 0·2 ml/min), eluted with 20 mM-Tris–HCl pH 8·0. The MonoQ HR5/5 column (Pharmacia, flow rate 0·7 ml/min) was equilibrated with 20 mM-Tris–HCl pH 8·0, and eluted with a linear NaCl gradient (0–70% in 40 min) in the same buffer. Reversed-phase chromatography was performed on a TSK TMS 250 (0·21 × 4 cm, C1, 10 μm, 250 Å, TosoHaas) column using a linear gradient of acetonitrile (3–100% in 60 min)

in 0·1% trifluoroacetic acid at a flow rate of 0·25 ml/min. The fusion protein was detected by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) followed by Coomassie blue staining or immunoblotting using anti-hsp150. The purified fusion protein was used to raise antisera in rabbit, which recognized authentic NGFR and hsp150Δ–$NGFR_e$.

Chemical cross-linking

Human A875 melanoma cells were washed three times with Krebs–Ringer solution containing 1 mM-phenylmethylsulphonyl fluoride (Sigma), and incubated with [^{125}I]NGF (2000 Ci/mM, Amersham) for 30 min at 37°C in the same solution. Culture medium of H426 cells was dialysed overnight against PBS, and incubated similarly with [^{125}I]NGF. The amount of hsp150Δ–$NGFR_e$ in the preparation was estimated according to the yield of purified fusion protein, quantitated by N-terminal amino acid sequencing (see above). Chemical cross-linking of both preparations was performed with 30 mM-1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC; Pierce) for 5 min on ice. The reaction was quenched by addition of Tris–HCl pH 7·5, to a concentration of 52 mM. The A875 cells were washed three times with PBS supplemented with 52 mM-Tris–HCl, pH 7·5, prior to solubilization with Laemmli sample buffer. The fusion protein samples were subjected to immunoprecipitation with anti-hsp150.

Other methods

Northern analysis using *HSP150* and $NGFR_e$ as probes (Russo *et al.*, 1993), and N-terminal amino acid sequencing (Russo *et al.*, 1992) were as described. SDS–PAGE was in 8% gels, unless otherwise stated. Protein precipitation was with 14% trichloroacetic acid for 1 h on ice. Quantitation of the cross-linked products was with PhosphorImager™ (Molecular Dynamics). Cycloheximide, NaN_3, tunicamycin and dithiothreitol (DTT) were from Sigma, and used at concentrations of 100 μg/ml, 10 mM, 20 μg/ml and 20 mM, respectively. Bovine serum albumin was from Sigma, male mouse NGF from Harlan Bioproducts for Science, and restriction endonucleases from Promega, New England Biolabs and Boehringer Mannheim. Centricon-30 (Amicon) devices were used for ultrafiltration.

RESULTS

Construction of a yeast strain expressing an HSP150Δ–$NGFR_e$ fusion gene

The primary translation product of the *HSP150* gene consists of a signal peptide (Figure 1A, black area), subunit I (dotted area) and subunit II. Subunit II consists of 11 repeats of homologous peptides (diagonal stripes) and a unique C-terminus (white area). NGFR is a plasma membrane protein with one transmembrane segment (Figure 1B, cross-hatched area). The C-terminal 150 residues form a cytoplasmic domain (horizontal stripes), and 222 N-terminal residues are located on the extracellular face of the membrane (vertical stripes). The first 160 N-terminal residues form the cysteine-rich ligand-binding domains, linked to the transmembrane segment by a 62-residue stalk peptide (Radeke *et al.*, 1987; Baldwin *et al.*, 1992). A DNA fragment encoding the 223 N-terminal residues of mature NGFR was ligated downstream from the repetitive region of *HSP150*, creating *HSP150Δ–*$NGFR_e$ (Figure 1C), which was placed between the *HSP150* promoter and the *ADC1* terminator. The fusion gene was integrated into the genome of *S. cerevisiae* strain H23, carrying a disrupted *hsp150* gene (Russo *et al.*, 1992), creating strain H426. Integration was as a single copy into the *LEU2* locus (not shown). Northern analysis showed that strain H426 synthesized a new mRNA species of expected size, 1·9 kb, which hybridized with *HSP150* and *NGFR* probes, and was missing from the parental H23 strain (not shown).

Secretion and post-translational modification of HSP150Δ-$NGFR_e$

Expression of the *HSP150Δ–*$NGFR_e$ fusion gene was studied by labelling H426 cells with [^{35}S]methionine/cysteine for 1 h, followed by a chase for 20 min in the presence of cycloheximide to stop further protein synthesis. The culture medium and lysed cells were subjected to immunoprecipitation with anti-hsp150 and anti-NGFR, followed by SDS–PAGE analysis (Figure 2A). Anti-hsp150 precipitated from the culture medium (m) a 180 kDa protein (arrowhead), and molecules with slower electrophoretic mobility (lane 1). Similar proteins were recognized by anti-NGFR, albeit much more weakly (lane 2). Smaller amounts of 180 kDa and 130 kDa proteins were precipitated from the cell lysate (c) with anti-hsp150 (lane 3) and anti-NGFR (lane 4). Neither antiserum

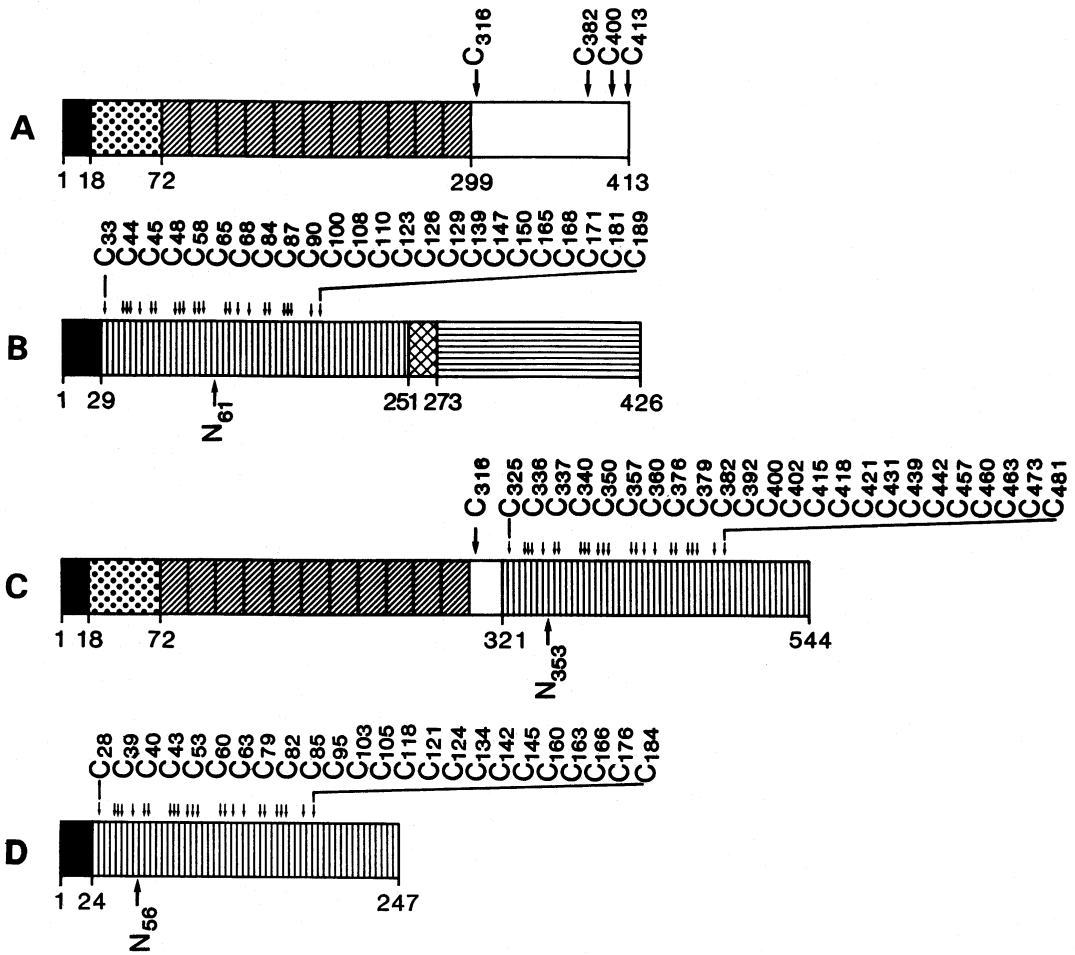

Figure 1. Schematic presentation of the primary translation products of the *HSP150* gene (A), the rat *NGFR* gene (B), the *HSP150*Δ–NGFR$_e$ fusion gene (C), and the *SUC2–NGFR$_e$* fusion gene (D). The cysteine residues (C) and the N-glycosylation sites (N) are indicated. The numbers below the figures refer to the last amino acid of each domain. Black area, signal peptide. Dotted area, subunit I of hsp150; diagonally striped area, repetitive region of subunit II (each diagonally striped block represents one copy of the homologous peptide); white area, C-terminal region of subunit II. The signal peptide is removed upon translocation of the protein to the ER, and subunit I is cleaved from subunit II during secretion to the culture medium, but remains non-covalently attached to it (Russo *et al.*, 1992). Vertically striped area, ectodomain of NGFR; cross-hatched area, transmembrane segment; horizontally striped area, cytoplasmic domain. Suc-NGFR$_e$ has 22 N-terminal amino acids of pre-invertase (signal peptide cleavage site is between residues 18 and 19). Linker (Gly-Thr) joins the invertase fragment to NGFR$_e$.

recognized proteins synthesized by the parental strain H23 (lanes 9–12). This shows that the fusion protein was efficiently secreted through the secretory organelles and the cell wall to the culture medium.

When H426 cells were labelled in the presence of tunicamycin to inhibit N-glycosylation, anti-hsp150 precipitated only the 180 kDa species from the culture medium (Figure 2A, lane 5). Small amounts of 180 kDa and 130 kDa proteins were precipitated from the cell lysate (lane 7). Both the secreted (lane 6) and the cell-associated (lane 8) proteins were recognized, although poorly, by anti-NGFR. Thus, a portion of the fusion protein molecules carried an N-glycan at the single N-glycosylation site of NGFR$_e$ (Figure 1C), but lack of the N-glycan did not compromise secretion. The hsp150Δ-carrier does not contain N-glycosylation sites, but is heavily O-glycosylated, migrating in SDS–PAGE gels much slower than expected from its deduced amino acid sequence (Jämsä *et al.*, 1995). The cell-associated

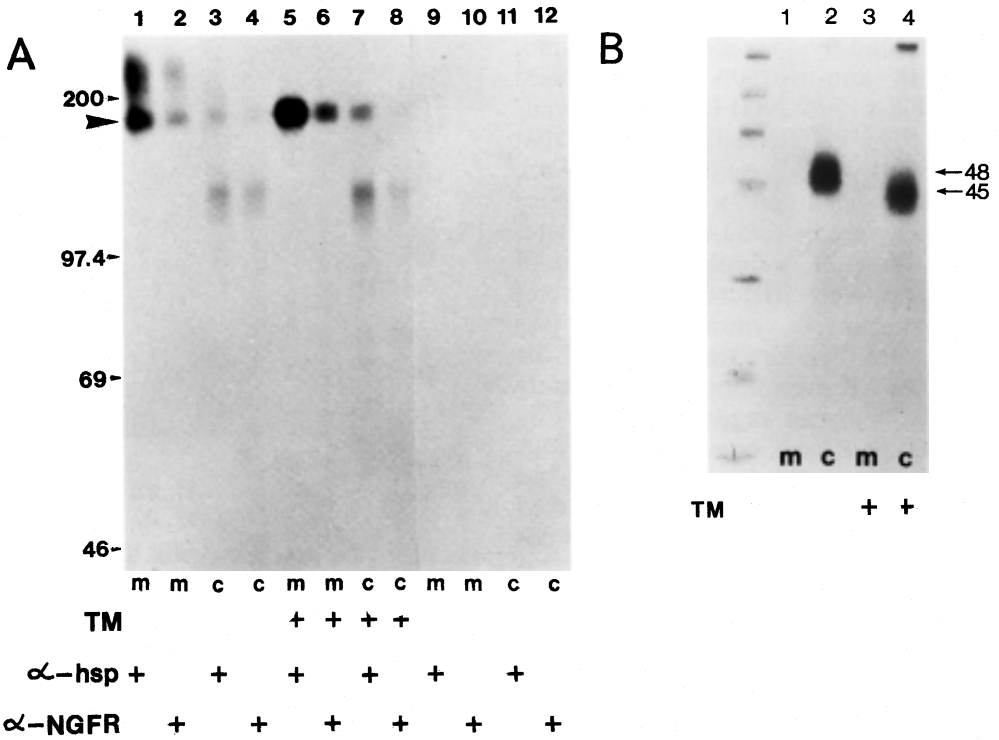

Figure 2. (A) Secretion of hsp150Δ–NGFR$_e$. H426 cells (lanes 1–8), and H23 cells (lanes 9–12) were labelled with [^{32}S]methionine/cysteine for 1 h at 37°C, in the absence (lanes 1–4 and 9–12), or presence of tunicamycin (TM+, lanes 5–8). Cycloheximide was added, and the incubations were continued for 20 min. Labelling was terminated by adding NaN_3, and the cells were separated from the supernatants, and lysed. The culture medium (m) and cell lysate samples (c) were subjected to immunoprecipitation with anti-hsp150 or anti-NGFR, as indicated. The precipitates were analysed by SDS–PAGE, followed by fluorography. Molecular weight markers (kDa) are on the left. (B) Secretion of NGFR$_e$ in the absence of the carrier. H487 cells were labelled with [^{35}S]methionine/cysteine for 1 h at 30°C in SC medium lacking tryptophan and containing 0.1% glucose, in the absence (lanes 1 and 2) or presence (TM+, lanes 3 and 4) of tunicamycin. After addition of cycloheximide, the incubation was continued for 30 min. Culture media (m) and cell lysate (c) samples were immunoprecipitated with anti-hsp150Δ–NGFR$_e$ and analysed by SDS–PAGE (12% gels) and fluorography. Molecular weight markers on the left are 200, 97·4, 69, 46, 30, 21·5 and 14·3 kDa. Figures on the right show the apparent molecular masses (kDa) of the indicated proteins.

130 kDa form (Figure 2A, lane 3), which was detected also in the presence of tunicamycin (lane 7), probably represented an immaturely O-glycosylated precursor. Thus, the increase in the apparent molecular mass to 180 kDa during secretion was due to extension of O-glycans. N-terminal amino acid sequencing (see below) confirmed that the signal peptide and subunit I had been cleaved during secretion (see Figure 1C). When the fusion protein was synthesized in the presence of the reducing agent DTT, it was not secreted (not shown). Thus, the NGFR$_e$ portion was disulphide-bonded under normal conditions (Jämsä *et al.*, 1994).

Fate of NGFR$_e$ in the absence of the hsp150Δ-carrier

To study secretion of NGFR$_e$ without the hsp150Δ-carrier, it was fused to the signal peptide of invertase, a secretory protein of yeast, and expressed under the control of the invertase (*SUC2*) promoter (Figure 1D). The *SUC2–NGFR$_e$* construct was introduced into strain H23, yielding strain H487. Upon derepression of the *SUC2* promoter, a new RNA species of 0·9 kb, hybridizing with the *NGFR$_e$* probe, could be detected (not shown). H487 cells were labelled with [^{35}S]methionine/cysteine for 1 h in the absence or presence of tunicamycin, and the label was chased

for 30 min in the presence of cycloheximide. The culture medium and lysed cells were immunoprecipitated with anti-hsp150Δ–$NGFR_e$, raised against purified hsp150Δ–$NGFR_e$ (see Materials and Methods). SDS–PAGE analysis showed that no labelled proteins were detectable in the culture media (Figure 2B, lanes 1 and 3), whereas proteins of 48 and 45 kDa were detected in the cell lysates in the absence (lane 2) or presence (lane 4) of tunicamycin, respectively. Reduction of the size of $NGFR_e$ by tunicamycin shows that it was N-glycosylated, and therefore translocated to the luminal side of the ER membrane. The apparently small size of the N-glycan suggests that $NGFR_e$ remained in the pre-Golgi compartment. Thus, when expressed without the hsp150Δ-carrier, but with a functional signal peptide, $NGFR_e$ entered the ER, but remained cell-associated and was not secreted to the culture medium.

Purification of hsp150Δ–$NGFR_e$

Strain H426 was grown in SC medium to different cell densities, and 1-ml samples of cell-free medium were subjected to SDS–PAGE, followed by Coomassie blue staining. The fusion protein was the most abundant protein in the culture supernatant (Figure 3, lanes 4–9). The culture medium of the parental H23 strain lacked this protein (lane 10). By comparing the staining of the fusion protein and serum albumin (lanes 1–3), one litre of culture medium, after 26 h of growth, was estimated to contain hsp150Δ–$NGFR_e$ in the milligram scale.

To purify hsp150Δ–$NGFR_e$, culture medium of strain H426 was dialysed and subjected to ion-exchange chromatography on Q-Sepharose. The fusion protein, bound to the column, was eluted with 0·5 M-NaCl, concentrated by ultrafiltration, and subjected to gel filtration on a Superdex-75 column. It eluted in the void volume fractions, which were pooled, and designated as semipurified hsp150Δ–$NGFR_e$. This preparation was desalted and subjected to anion-exchange chromatography on a MonoQ column. The fusion protein was detected in fractions 18·5–27 min (Figure 4A, bar), which were pooled, and subjected to reversed-phase chromatography. The fusion protein eluted in fractions 14–20 min, which were pooled (Figure 4B, bar). N-terminal amino acid sequence analysis of this material gave dual signals in each Edman degradation cycle, corresponding to about 75% (molar quantity) of the N-terminus of subunit II of

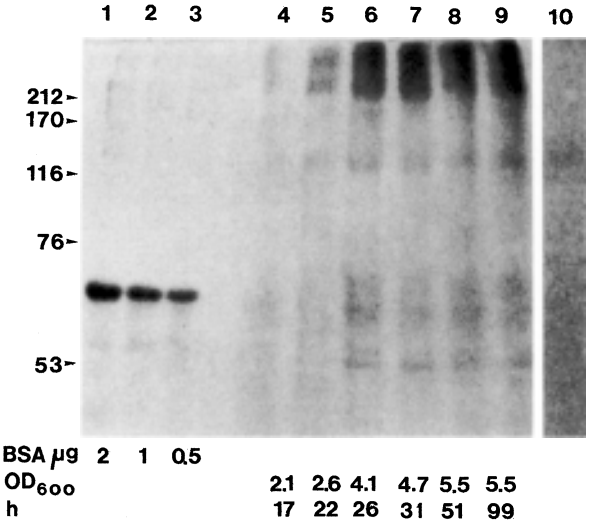

Figure 3. The yield of hsp150Δ–$NGFR_e$ in the culture medium. Strain H426 was grown for the indicated times in SC medium to the indicated cell densities. The culture supernatants of 1-ml samples were lyophilized and subjected to SDS–PAGE (lanes 4–9). In lane 10, a 1-ml sample of the culture supernatant of the parental strain H23, grown in YPD medium to a density of $OD_{600}=5$, was analysed similarly. The indicated amounts of bovine serum albumin served as standards (lanes 1–3). The gel was stained with Coomassie brilliant blue. The molecular weight markers (kDa) are on the left.

hsp150, and to about 25% of subunit II missing three amino acids at its N-terminus.

Ligand-binding activity of hsp150Δ–$NGFR_e$

Next we studied the ability of the $NGFR_e$ portion of the fusion protein to bind NGF. An hsp150Δ–$NGFR_e$ preparation containing roughly $2{\cdot}5 \times 10^{11}$ fusion protein molecules was subjected to cross-linking with 1 nM-[^{125}I]NGF (3×10^{10} molecules) using EDC. The products were immunoprecipitated with anti-hsp150, and analysed in SDS–PAGE. The radiolabelled products appeared as heterogeneous high molecular weight proteins (Figure 5A, lane 1, black arrowhead).

Human A875 melanoma cells express the low-affinity NGFR as the sole NGF-binding protein on their surface. About $4{\cdot}2 \times 10^5$ intact A875 cells, containing roughly $2{\cdot}5 \times 10^{11}$ receptors (Fabricant *et al.*, 1977), were subjected to cross-linking with 1 nM-[^{125}I]NGF, in parallel with the hsp150Δ–$NGFR_e$ samples described above. SDS–PAGE analysis revealed an ^{125}I-labelled product of about 75 kDa (Figure 5A, lane 3, open arrowhead), as expected (Johnson *et al.*, 1986). The amount of

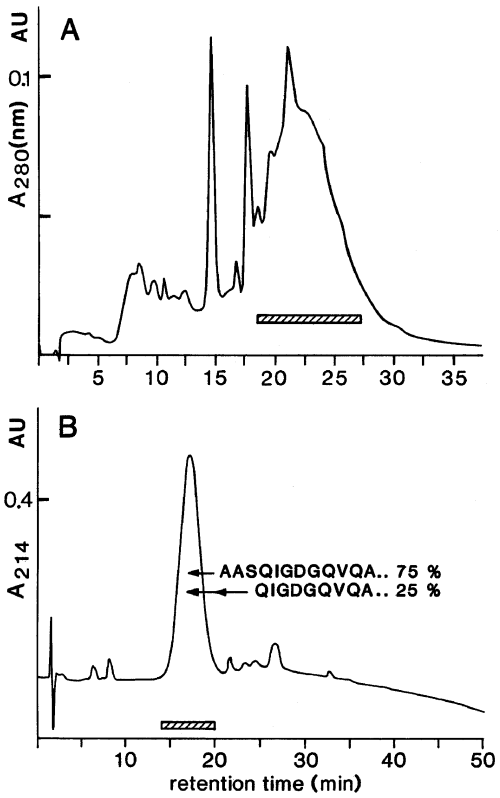

Figure 4. Purification of hsp150Δ–$NGFR_e$. The culture medium (SC) of strain H426, grown at 24°C to an OD_{600} of 4, was subjected to chromatography successively through Q-Sepharose and Superdex-75 columns, to yield a preparation designated as semipurified hsp150Δ–$NGFR_e$. (A) The semipurified hsp150Δ–$NGFR_e$ preparation was desalted, and subjected to anion-exchange chromatography on MonoQ. Fractions 18·5–27 min (bar) were pooled. (B) The pooled material was subjected to reversed-phase chromatography. Fractions 14–20 min were pooled (bar). The N-terminal amino acid sequences of the proteins were analysed, and their relative molar abundancies are indicated. Absorbance is plotted against retention time.

[^{125}I]NGF cross-linked to the A875 cell sample (lane 3) and to hsp150Δ–$NGFR_e$ (lane 1) appeared rather similar. Addition of 1000-fold excess of unlabelled NGF to the reaction mixture efficiently inhibited the cross-linking of [^{125}I]NGF to the A875 cells (lane 4), whereas it decreased, but did not completely abolish the binding of [^{125}I]NGF to the fusion protein (lane 2). To exclude that [^{125}I]NGF was non-specifically cross-linked to the hsp150Δ-carrier, the culture supernatant of H1 cells, with roughly $2{\cdot}5 \times 10^{11}$ hsp150 molecules and no hsp150Δ–$NGFR_e$, was subjected to cross-linking. No cross-linking of [^{125}I]NGF to hsp150 could be detected (Figure 5A, lane 5).

Since a 1000-fold excess of unlabelled NGF was unable to completely inhibit binding of [^{125}I]NGF to the fusion protein, we studied this in more detail. Hsp150Δ–$NGFR_e$ was produced to the culture medium in the presence of tunicamycin to obtain a more homogeneous protein. It was concentrated by ultrafiltration, and approximately $1{\cdot}2 \times 10^{12}$ molecules were incubated with 2 nM-[^{125}I]NGF ($1{\cdot}2 \times 10^{11}$ molecules) as above, but in the presence of 0–200 nM of unlabelled NGF. The proteins were cross-linked with EDC, followed by immunoprecipitation with anti-hsp150 antiserum, SDS–PAGE and autoradiography (Figure 5B). The cross-linked products (black arrowhead) were quantitated using PhosphorImager™. A ten-fold excess of unlabelled NGF inhibited 48% of the binding of [^{125}I]NGF to the fusion protein, whereas up to a 1000-fold excess had little additional effect (52%; Figure 5B, lanes 4 and 5).

We then studied the ability of hsp150Δ–$NGFR_e$ to compete with authentic NGFR on human melanoma cells for binding of [^{125}I]NGF. About 10^6 A875 cells, containing approximately 6×10^{11} receptor molecules, were subjected to cross-linking with 1·5 nM-[^{125}I]NGF, and analysed by SDS–PAGE. In addition to the 75 kDa receptor, a small amount of a dimeric NGFR–[^{125}I]NGF complex of 170 kDa (Grob *et al.*, 1985) was detected (Figure 5C, lane 1, open arrowheads). Addition of a 100-fold excess of semipurified hsp150Δ–$NGFR_e$ (see above) to the reaction mixture inhibited cross-linking significantly, but not completely (lane 3). Cross-linking of [^{125}I]NGF to authentic NGFR was inhibited almost completely by a 1000–fold excess of unlabelled NGF in the reaction mixture (lane 2). The data suggest that the $NGFR_e$ portion adopted a ligand-binding conformation. However, the binding characteristics, at least of the whole population of the fusion protein molecules, were not identical to those of authentic NGFR.

DISCUSSION

To evaluate whether the hsp150Δ-carrier could provide secretion competence to elaborately folded and disulphide-bonded mammalian proteins, we chose the ectodomain of rat low-affinity nerve growth factor receptor, $NGFR_e$, as a marker. To this end, $NGFR_e$ was fused to the C-terminus of the hsp150Δ-carrier, to yield hsp150Δ–$NGFR_e$ (see Figure 1). Pulse-chase experiments showed that hsp150Δ–$NGFR_e$ was efficiently transported through the secretory pathway and secreted across

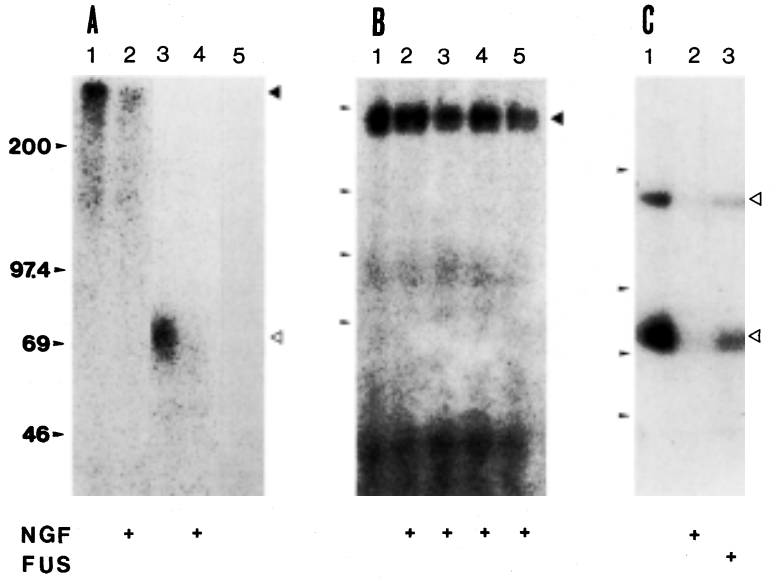

Figure 5. Cross-linking of hsp150Δ–NGFR$_e$ to [^{125}I]NGF. (A) Dialysed culture supernatant of strain H426 (lanes 1 and 2), strain H1 (lane 5), or intact A875 melanoma cells (lanes 3 and 4), were cross-linked with 1 nM-[^{125}I]NGF in the absence (lanes 1 and 3) or presence (NGF+, lanes 2 and 4) of 1 μM unlabelled NGF. (B) Ultrafiltrated culture supernatant of strain H426, produced in the presence of tunicamycin, was cross-linked to 2 nM-[^{125}I]NGF in the absence (lane 1) or presence of 2 nM (lane 2), 20 nM (lane 3), 200 nM (lane 4) or 2 μM (lane 5) unlabelled NGF. (C) A875 cells (6×10^{11} NGFR molecules) were cross-linked to 1·5 nM-[^{125}I]NGF in the absence (lane 1) or presence of 1·5 μM unlabelled NGF (lane 2), or in the presence of 6×10^{13} hsp150Δ–NGFR$_e$ molecules (FUS+, lane 3). The A875 cells were washed, and the yeast culture medium samples were immunoprecipitated with anti-hsp150, prior to SDS–PAGE in 6–15% (A and C) or 7·5–15% (B) gels and autoradiography. The molecular weight markers are on the left.

the cell wall to the culture medium. The single N-glycosylation site of NGFR$_e$ was occupied by large and heterogeneous glycans in part of the molecules, but inhibition of N-glycosylation did not affect secretion.

When expressed without the carrier with the signal peptide of invertase, NGFR$_e$ was translocated to the ER and N-glycosylated, but not secreted. The small size of the N-glycan suggests that it was retained in the ER. Thus, the hsp150Δ-carrier had a crucial role in promoting secretion of NGFR$_e$, apparently its exit from the ER. The mechanism by which the prepro-region of α-factor and the hsp150Δ-carrier confer secretion competence to heterologous protein portions is not known. The carrier may assist proper folding of the heterologous protein, allowing it to pass the quality control machinery of the ER (Hammond and Helenius, 1995), or perhaps provide a positive secretion signal (Balch *et al.*, 1994). We have shown by nuclear magnetic resonance and CD spectroscopy that the repetitive region of the hsp150Δ-carrier is unstructured, whereas subunit I and/or the C-terminal region following the repetitive region do adopt some secondary structure (see Figure 1A; Jämsä *et al.*, 1995). In the absence of a tendency to fold itself, the repetitive carrier may allow independent and productive folding of the heterologous protein. When β-lactamase was fused to subunit I or almost the whole hsp150 protein, the chimeras were translocated to the ER, but they were apparently misfolded since they were enzymatically inactive or secretion incompetent (Simonen *et al.*, 1994).

Many if not all of the 24 cysteines of NGFR are thought to be disulphide-bonded (Banner *et al.*, 1993; Grob *et al.*, 1985; Baldwin and Shooter, 1994). The NGFR$_e$ portion of hsp150Δ–NGFR$_e$ synthesized in yeast also acquired disulphide bonds, since the fusion protein was not secreted in the presence of DTT. We have shown that *in vivo* reduction of normally disulphide-bonded proteins

leads to their retention in the ER, though the secretion apparatus remains fully functional (Jämsä *et al.*, 1994). To examine whether the $NGFR_e$ portion was properly folded, we studied its ligand-binding ability. Hsp150Δ–$NGFR_e$ competed for binding of [^{125}I]NGF with the authentic receptor located on the surface of human A875 melanoma cells. [^{125}I]NGF could be chemically cross-linked to hsp150Δ–$NGFR_e$ with apparently similar efficiency as to the receptor on the melanoma cells, whereas no cross-linking to hsp150 could be detected. Many mutations altering the cysteine-repeats and disulphide-bonding of authentic NGFR result in loss of NGF-binding (Yan and Chao, 1991; Baldwin *et al.*, 1992; Baldwin and Shooter, 1994). Finally, unlabelled NGF decreased cross-linking of [^{125}I]NGF to hsp150Δ–$NGFR_e$. However, not more than half of the binding could be inhibited even with a 1000-fold excess of NGF, suggesting that the conformation of the $NGFR_e$ portion was non-native, albeit ligand-binding, e.g. due to linkage to the carrier.

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