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Different degradation pathways for heterologous glycoproteins in yeast

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Abstract Rat nerve growth factor receptor ectodomain ($NGFR_e$) and *Escherichia coli* β-lactamase were translocated into the yeast endoplasmic reticulum (ER), glycosylated, misfolded and rapidly degraded. $NGFR_e$ underwent ATP-dependent thermosensitive degradation independently of vesicular transport. Since no evidence for degradation by the cytoplasmic 26S proteosome complex could be obtained, $NGFR_e$ appeared to be degraded in the ER. β-Lactamase exited the ER by vesicular traffic and was transported from the Golgi via the Vps10 receptor pathway to the vacuole for degradation. Machineries in the ER and the Golgi appear to recognize distinct structural features on misfolded heterologous proteins and guide them to different degradation pathways.

Key words: Protein folding; Protein degradation; Chaperone; *Saccharomyces cerevisiae*

1. Introduction

Proteins start their journey on the secretory pathway by translocating through the Sec61 translocon complex into the endoplasmic reticulum (ER), the folding compartment for exocytotic proteins. The ER possesses a quality control machinery, which has been thought to ensure that only correctly folded and modified proteins proceed on the secretory route. Proteins that fail to acquire a proper conformation are retained in the ER and often eliminated [1–3]. In mammalian and yeast cells, degradation of some mutated yeast proteins has been shown to occur by the cytosolic proteasome complex [4–7]. Translocation to the cytosol appears to occur through the Sec61 translocon [8,9], and has been suggested to require the ER chaperone BiP (Kar2p) and its co-chaperone Sec63p [10]. In yeast, some homologous and heterologous mutant proteins are targeted via the Golgi to the vacuole for degradation [11–13]. Here we show that in *Saccharomyces cerevisiae* heterologous proteins can be rapidly degraded after ER translocation by different mechanisms.

2. Materials and methods

S. cerevisiae strains (Table 1) were grown at 24°C like before [14]. Plasmid pKTH4683 was constructed by ligating the *HSP150*Δ-β-*lactamase* gene to the *Bam*HI site of pFL26 [17]. Strain H825 was obtained by integrating pKTH4683 into the *LEU2* locus of strain H4. Plasmid pKTH4661 was created by replacing the *TRP1* gene of pKTH4616, containing the rat nerve growth factor receptor ectodomain ($NGFR_e$) gene [15], by *LEU2* acquired from pFL26 as a *Bgl*II fragment. Transformations were according to [18]. Cells (5×10^8/ml) were metabolically labeled with 50 μCi/ml of [^{35}S]methionine/cysteine (Amersham, UK) in SC medium lacking methionine and cysteine after a preincubation of 10–15 min at the labeling temperature. Derepression of $NGFR_e$ genes was in 0.1% glucose medium [15]. Cell lysis and immunoprecipitation with anti-Hsp150 (1:100), anti-Hsp150Δ-$NGFR_e$ (1:50), anti-β-lactamase (1:100) and anti-ubiquitin (1:100; Sigma) antisera were as described [19]. β-Lactamase activity determinations were as described [14]. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was in 12% gels under reducing conditions if not otherwise stated. The concentrations of NaN_3, cycloheximide, dithiothreitol and *N*-ethylmaleimide (NEM) were 10 mM, 100 μg/ml, 20 mM and 10 mM, respectively, and all were from Sigma.

3. Results

3.1. Rapid ER-associated elimination of $NGFR_e$

We have shown before that $NGFR_e$ was translocated into the ER by the aid of the invertase signal peptide, but remained intracellular [15]. To study the fate of $NGFR_e$, wild type cells were labeled for 5 min at 24°C with [^{35}S]methionine/cysteine. Cycloheximide (CHX) was added and the cells were incubated at 24°C for different times, lysed and subjected to immunoprecipitation with anti-Hsp150Δ-$NGFR_e$ antiserum. SDS-PAGE analysis of the precipitates revealed $NGFR_e$, migrating like a 48 kDa protein (Fig. 1A, lane 1), known to carry one *N*-glycan of about 3 kDa [16]. However, during the chase $NGFR_e$ disappeared with a half-life of about 7 min (Fig. 1A, lanes 2–5, Table 2). When sodium azide was added to the chase medium, $NGFR_e$ persisted (Fig. 1B). When the experiment of Fig. 1A was repeated at 37°C, $NGFR_e$ persisted (Fig. 1C), and none of it could be detected in the medium (not shown). Thus, $NGFR_e$ was rapidly degraded after translocation in an ATP-dependent and temperature-sensitive fashion.

A mutant form of pro-CPY is degraded at 24°C by the 26S proteasome complex after retrotranslocation to the cytoplasm, and the degradation is inhibited at 37°C [20–22]. To examine whether $NGFR_e$ was degraded similarly, it was expressed in strains defective in a proteasomal chymotrypsin-like activity (*pre1-1* and *pre2-1*) [23], proteasomal activation (*sen3-1*) [24] or deubiquitination needed for ubiquitin-dependent proteasomal degradation (Δ*doa4*) [25]. None of these mutations significantly inhibited the degradation of $NGFR_e$ (Table 2). To study whether $NGFR_e$ was ubiquinated, the pulse chase experiment of Fig. 1A was repeated using anti-ubiquitin antibody for immunoprecipitation. Several unknown proteins were precipitated, but not $NGFR_e$ (not shown). Neither was $NGFR_e$ degraded in the vacuole. It disappeared like in wild type cells in a mutant where vacuolar proteases are inactive (Table 2) [26].

3.2. $NGFR_e$ degradation is independent of vesicular traffic

Authentic $NGFR_e$ has 12 disulfide bonds [27]. To study disulfide bonding of $NGFR_e$ in yeast, wild type cells were ^{35}S-labeled at 24°C in the absence (Fig. 2A, lane 1) or presence (lane 2) of dithiothreitol (DTT), which prevents co-translocational disulfide bond formation [28]. The lysates were

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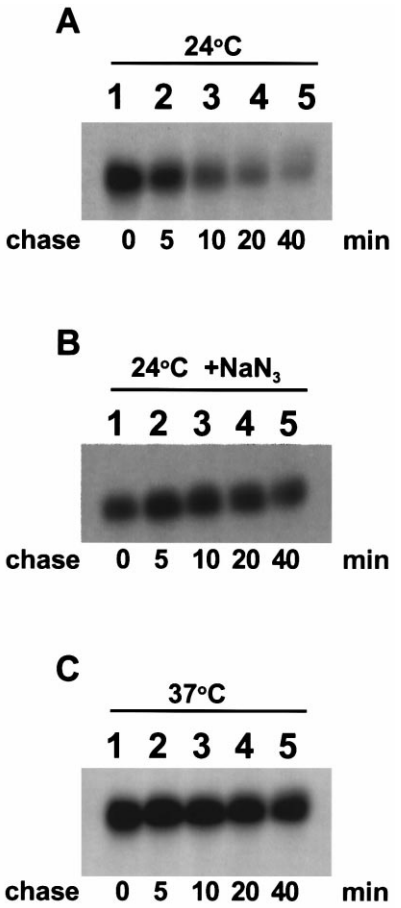

Fig. 1. Thermosensitive and ATP-dependent degradation of $NGFR_e$. Wild type cells (H487) were ^{35}S-labeled for 5 min and chased in the presence of CHX for the indicated times at 24°C (A and B) or 37°C (C). In B, NaN_3 was present during the chase. After lysis the cell samples were subjected to immunoprecipitation and SDS-PAGE analysis.

treated with NEM to block sulfhydryls, immunoprecipitated and subjected to non-reducing SDS-PAGE. The electrophoretic migration of in vivo reduced $NGFR_e$ was retarded (lane 2) as compared to the native molecules (lane 1) demonstrating that normally $NGFR_e$ was disulfide-bonded. A parallel cell sample labeled in the presence of DTT was washed and chased with CHX in the absence of DTT to allow restoration of oxidating conditions in the ER. $NGFR_e$ did not co-migrate with the native molecules, but slightly more slowly than the reduced ones (lane 3), indicating that the sulfhydryls had not undergone reoxidation. The intensity of the signal was decreased suggesting degradation (lane 3). As a positive control for reoxidation, this experiment was conducted in parallel for Hsp150Δ-$NGFR_e$ (Fig. 2B). We have shown before that $NGFR_e$, when fused to the Hsp150Δ fragment, is disulfide-bonded and folds to a ligand-binding and secretion-competent conformation in the yeast ER [15]. Hsp150Δ is an N-terminal fragment (signal peptide plus 303 amino acids) of the yeast secretory glycoproteins Hsp150 [15]. To keep Hsp150Δ-$NGFR_e$ in the pre-Golgi compartment, we used *sec18-1* cells, where ER-derived vesicles do not fuse with the Golgi at 37°C [16]. DTT treatment resulted in retardation of electrophoretic migration of Hsp150Δ-$NGFR_e$ (lane 2) as compared to native molecules (lane 1). However, removal of DTT allowed reoxidation of the sulfhydryls, since the fusion protein now comigrated with the native molecules (lane 3). We conclude that the conformation of $NGFR_e$ was different from that of the $NGFR_e$ portion in the Hsp150Δ-$NGFR_e$ fusion protein. The cysteine residues on $NGFR_e$ were apparently incorrectly juxtaposed and not able to bond after removal of DTT.

Lack of reoxidation of $NGFR_e$ expressed in the absence of the Hsp150Δ fragment could be explained by localization elsewhere than in the ER. Thus, the experiment of Fig. 2A was repeated in *sec18-1* cells at 37°C (Fig. 2C). Again, DTT in the labeling medium retarded the electrophoretic migration of $NGFR_e$ (lanes 1 and 2), and chase in the absence of DTT did not result in reoxidation of the sulfhydryls but degradation (lanes 3–5). In the absence of DTT, $NGFR_e$ persisted at 37°C in *sec18-1* cells (Fig. 2D). Degradation thus did not require exit from the pre-Golgi compartment by vesicular traffic. Though at 37°C degradation of oxidized $NGFR_e$ was inhibited or was very slow, the reduced molecules were rapidly eliminated even at this temperature. Persistence of $NGFR_e$ at 37°C might be due to elevated chaperone levels at this temperature.

3.3. β-Lactamase is degraded in the vacuole

Next we studied the fate of *Escherichia coli* β-lactamase, which also was earlier found to be poorly secreted in *S. cerevisiae* [14]. In these studies β-lactamase was fused to the

Table 1
Saccharomyces cerevisiae strains used in this study

Yeast strain	Reporter protein	Genotype	Reference
H393	Hsp150Δ-β-lactamase	*MATα his⁻ leu2-3,112 trp1-289 ura3-52 URA3::HSP150Δ-bla sec18-1*	[14]
H395	β-lactamase	*MAT***a** *ade2-101 gal2 suc2-9 leu2-3,112 ura3-52 URA3::HSP150*-bla*	[14]
H421	β-lactamase	*MATα his⁻ leu2-3,112 trp1-289 ura3-52 URA3::HSP150*-bla sec18-1*	[14]
H487	$NGFR_e$	*MATα hsp150::URA3 ade2-1 hsi3-11 leu2-3,112 trp1-1 ura3-1 can1-100* [pKTH 4616]	[15]
H538	Hsp150Δ-$NGFR_e$	*MATα his⁻ leu2-3,112 trp1-289 ura3-52 URA3::Hsp150Δ-NGFR$_e$ sec18-1*	[16]
H640	$NGFR_e$	*MATα hsp150::URA3 his3-11 leu2-3,112 trp1-289 ura3-52 sec18-1* [pKTH 4616]	[16]
H677	Hsp150Δ-β-lactamase	*MATα his⁻ leu2-3,112 trp1-289 ura3-52 LEU2::HSP150δ-bla sec18-1*	This study
H691	$NGFR_e$	*MAT***a** *can1 gal2 his3-200 lys2-801 trp1-101 ura3-52 Δpep4::HIS3 prb1-1.6R* [pKTH 4616]	This study
H693	$NGFR_e$	*MATα his3-200 leu2-3,112 lys2-801 trp1-1 ura352 Δdoa4::LEU2* [pKTH 4616]	This study
H710	$NGFR_e$	*MAT***a** *ade2-101 his3-200 leu2-1 trp1-1 ura3-1::TRP1 sen3-1* [pKTH4661]	This study
H712	$NGFR_e$	*MATα his3-11,15 leu2-3,112 ura3 pre1-1* [pKTH 4661]	This study
H713	$NGFR_e$	*MATα his3-11,15 leu2-3,112 ura3 pre1-1 pre2-1* [pKTH 4661]	This study
H817	β-lactamase	*MAT***a** *can1 gal2 his3-200 lys2-801 trp1-101 ura3-52 URA3::HSP150*-bla Δpep4::HIS3 prb1-1.6R*	This study
H818	β-lactamase	*MAT***a** *his3-200 leu2-3,112 lys2-801 suc2-9 trp1-901 ura3-52 URA3::HSP150*-bla Δvps10::HIS3*	This study
H825	β-lactamase	*MATα his⁻ leu2-3,112 trp1-289 ura3-52 LEU2::HSP150*-bla sec18-1*	This study

C-terminus of a short N-terminal fragment of Hsp150 (see Table 1, strain H393, *HSP150*-bla*). The fragment consists of the signal peptide plus 45 amino acids providing *O*-glycosylation sites for distinction of ER-located molecules from those that had reached the Golgi. Wild type cells were pulse-labeled for 10 min and chased at 37°C (Fig. 3A). Immunoprecipitation of the lysates with anti-β-lactamase antiserum and SDS-PAGE analysis revealed the 44 kDa primary *O*-glycosylated ER form [14], and the fully *O*-glycosylated mature 48 kDa form (Fig. 3A, lane 6). During chase, both disappeared with a half-life of about 9 min (lanes 7–10). Since the protein did not appear in the medium (lanes 1–5), it must have been degraded. In *sec18-1* cells, the 44 kDa form persisted (Fig. 3B). In the mutant defective for vacuolar proteolysis, the ER form was converted to the mature form without loss of signal, as determined with phosphorimaging (Fig. 3C). This suggested that β-lactamase was transported by vesicular traffic to the vacuole and degraded there. To confirm this, we expressed β-lactamase in a mutant which lacks the transmembrane receptor Vps10. In the absence of Vps10, native pro-CPY is not delivered to the vacuole but secreted [30]. When the Δvps10 mutant was ^{35}S-labeled for 5 min, the cell lysate contained the 44 kDa ER form (Fig. 3D, lane 4), and no protein could be detected in the medium (lane 1). During chase the intracellular form disappeared (lanes 5 and 6), with concomitant appearance of the mature protein in the

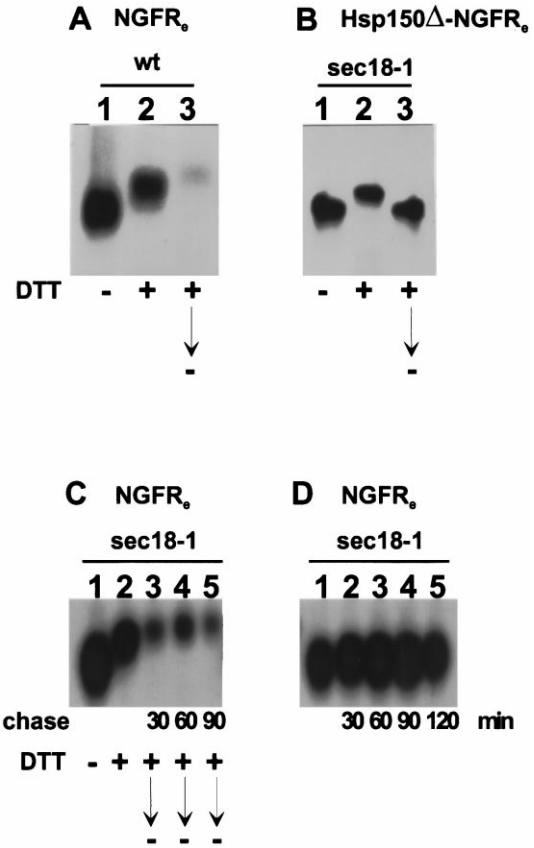

Fig. 2. Disulfide bonding of NGFR$_e$. A: Strain H487 (NGFR$_e$, wild type) was ^{35}S-labeled at 24°C for 1 h in the absence (lane 1) or presence (lanes 2 and 3) of DTT. One DTT-treated sample (lane 3) was pelleted and resuspended in fresh medium lacking DTT, and incubated for 30 min with of CHX at 24°C. B: Strain H538 (Hsp150Δ-NGFR$_e$, *sec18-1*) was labeled as in A but at 37°C. C: Strain H640 (NGFR$_e$, *sec18-1*) was pulse-labeled for 10 min at 37°C in the absence (lane 1) or presence of DTT (lanes 2–5). DTT was removed, CHX added and the incubations continued for indicated times at 37°C (lanes 3–5). D: Strain H640 was pulse-labeled in the absence of DTT as in C (lane 1), after which the cells continued with CHX for the indicated times (lanes 2–5). The cells were treated with 10 mM NEM on ice for 5 min, lysed, immunoprecipitated and subjected to non-reducing SDS-PAGE in 12% (A, C and D) or 8% (B) gels.

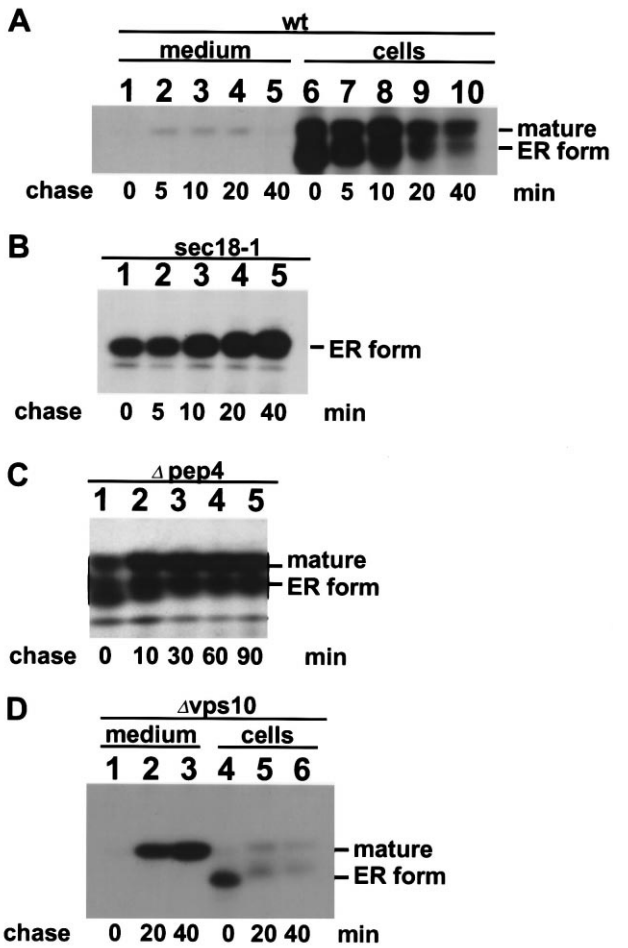

Fig. 3. Degradation of β-lactamase. Wild type (H395) (A), *sec18-1* (H421) (B), Δ*pep4* (H817) (C) and Δ*vps10* (H818) (D) cells were ^{35}S-labeled for 5 min at 37°C and chased with CHX for the indicated times. The medium samples and lysed cell samples were immunoprecipitated and subjected to 8% SDS-PAGE.

Table 2
Half-life of NGFR$_e$ degradation in proteosomal degradation mutants

Strain designation	Mutation	Half-life of NGFR$_e$ degradation (min)
H487	–	6.8
H712	*pre1-1*	5.9
H713	*pre1-1 2-1*	6.8
H710	*sen3-1*	6.7
H693	Δ*doa4*	7.4
H691	Δ*pep4*	8.6

The cells were ^{35}S-labeled at 24°C for 5 min, chased with CHX for 10, 30, 60 and 90 min, lysed, immunoprecipitated with anti-Hsp150Δ-NGFR$_e$ antiserum and subjected to SDS-PAGE analysis. The relative amounts of ^{35}S-NGFR$_e$ were quantitated by phosphorimaging.

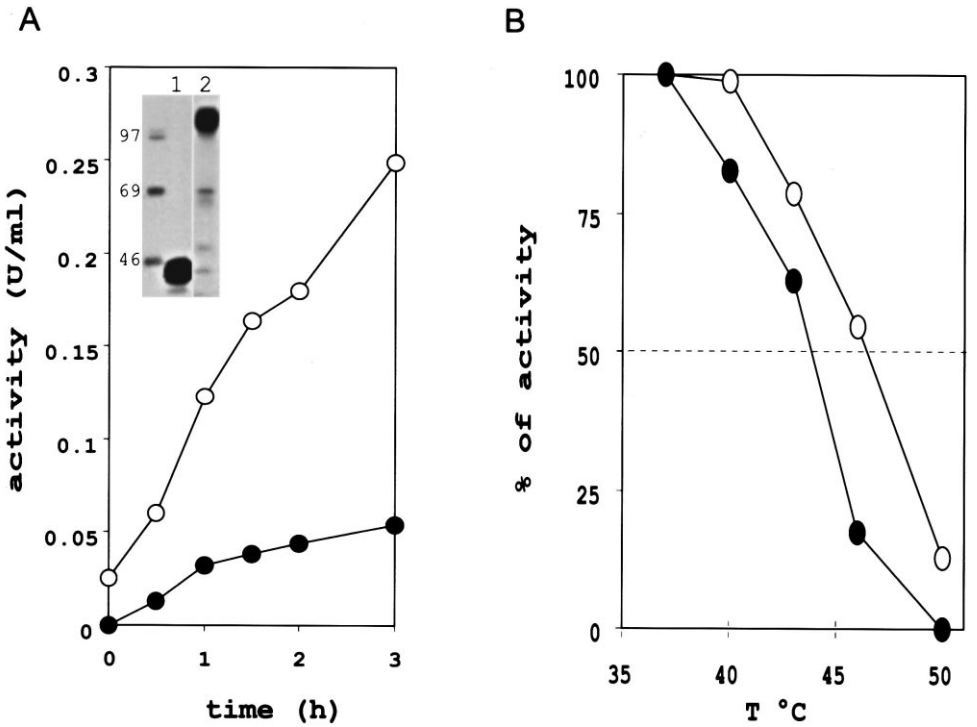

Fig. 4. A: Enzymatic activity of ER-trapped β-lactamase and Hsp150Δ-β-lactamase. *Sec18-1* cells expressing β-lactamase (H825; closed circles) or Hsp150Δ-β-lactamase (H677; open circles) were incubated at 37°C, and intracellular β-lactamase activity was measured and plotted against time. Insert: Strains H825 (lane 1) and H677 (lane 2) were ^{35}S-labeled for 1 h at 37°C, lysed, immunoprecipitated and subjected to SDS-PAGE analysis in an 8% gel. Figures on the left indicate molecular weight markers (kDa). B: T_m values for β-lactamase and Hsp150Δ-β-lactamase. *Sec18-1* cells expressing β-lactamase (H421; closed circles) or Hsp150Δ-β-lactamase (H393; open circles) were incubated at 37°C for 1.5 h. NaN_3 was added and the cells incubated for 20 min at the indicated temperatures. Intracellular β-lactamase activity was measured and plotted against temperature (B).

medium (lanes 2 and 3). Thus, β-lactamase was targeted by the Vps10 receptor to the vacuole for degradation.

3.4. β-Lactamase is misfolded

β-Lactamase adopts an enzymatically active and secretion-competent conformation in the yeast ER, when it is fused to the Hsp150Δ fragment (see above). The K_m value for nitrocefin of Hsp150Δ-β-lactamase is similar to that of authentic *E. coli* β-lactamase [31]. To study whether β-lactamase was targeted to the vacuole because of misfolding, we compared its enzymatic activity to that of Hsp150Δ-β-lactamase. Both proteins were collected in the ER of *sec18-1* cells at 37°C. 20% of activity was detected in the case of β-lactamase (Fig. 4A, closed circles) as compared to Hsp150Δ-β-lactamase (open circles). According to ^{35}S-labeling and immunoprecipitation, similar amounts of both proteins accumulated in the ER (Fig. 4A, insert). Both contain nine methionines, and β-lactamase two cysteines and Hsp150Δ-β-lactamase three. Finally, both proteins were accumulated in the ER of *sec18-1* cells at 37°C, and incubated briefly at up to 50°C. Determination of intracellular activity showed that the temperature of 50% thermal denaturation (T_m) was 44°C for β-lactamase and 47°C for Hsp150Δ-β-lactamase (Fig. 4B), confirming that β-lactamase expressed without the Hsp150Δ fragment was misfolded.

4. Discussion

Rat $NGFR_e$ was misfolded upon translocation into the yeast ER and rapidly degraded. Vesicular traffic was not required and neither was the vacuolar proteolytic machinery. Using mutants defective in various steps of the proteosomal degradation pathway (see Table 2), no evidence for involvement of the 26S proteasome complex in the degradation was obtained. Thus, destruction of $NGFR_e$ in the ER lumen appears likely, but was not conclusively proven. Soluble human α-1-protease inhibitor has been suggested to be degraded in the ER [32], but no conclusive evidence for a degradation machinery functioning in the yeast ER lumen is available. *E. coli* β-lactamase exited the ER in spite of being misfolded. However, non-native structural features were recognized in the Golgi by the Vps10 receptor system, resulting in targeting to the vacuole for degradation, like in the case of a misfolded mutated domain of λ-repressor protein [13]. Vps10 is not specialized for misfolded proteins, since native pro-CPY interacts with it directly via the vacuolar sorting signal QRPL [30]. Whether misfolded β-lactamase binds to Vps10 directly, and what structural features are recognized, remains unknown. Like the λ-repressor domain [33], authentic β-lactamase is a tight globule and has no QRPL sequence [34]. In mammalian cells aggregation in the Golgi of the unprocessed transmembrane endoprotease furin has been proposed to trigger lysosomal targeting, whereas processed molecules are externalized [35]. Both in the case of $NGFR_e$ and β-lactamase, misfolding appeared to cause degradation. Both proteins fold properly in the yeast ER when fused to the C-terminus of Hsp150Δ, an N-terminal fragment of the yeast secretory protein Hsp150. Hsp150Δ-$NGFR_e$ and Hsp150Δ-β-lactamase were stable and secreted to the culture medium [14,15]. The Hsp150Δ fragment

adopts no regular secondary structure but occurs as a random coil [29], and appears to assist proper folding of fusion partners in a chaperone-like manner.

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