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Intracellular transport of a heterologous membrane protein, the human transferrin receptor, in *Saccharomyces cerevisiae*

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Abstract We have analyzed the intracellular behavior of the human transferrin receptor (TfR) in *Saccharomyces cerevisiae*. The major part of the heterologously expressed TfR, which has previously been used as a model for heterologous expression of membrane proteins in yeast, is localized in the endoplasmic reticulum (ER) membranes; a minor fraction is present in the plasma membrane (PM). The stability of the TfR depends on vacuolar proteases, implying that it is degraded in the vacuolar compartment. Degradation is further dependent on favorable transport conditions to this compartment. The main bottleneck of transport seems to be the transition from the ER to the PM. The chaperone Cne1p, which is involved in quality control in the ER, plays a role in regulating the amount of heterologous TfR, as deletion of *CNE1* leads to significant accumulation of the protein. This is the first demonstration of the involvement of *CNE1* in regulating the level of heterologous membrane proteins.

Keywords Intracellular transport · Heterologous membrane protein · Yeast · *CNE1* · Transferrin receptor

Introduction

The production of mammalian membrane proteins in a foreign host cell is not as efficient as that of soluble proteins [26]. Both the translocation to the endoplasmic reticulum (ER) membrane and the transport to their final destination are more complex than the synthesis

of soluble proteins in the cytosol. Heterologous expression of membrane proteins is highly desired to obtain sufficient material for structural and functional analyses [14, 23, 28]. Our group has expressed the human transferrin receptor (TfR), a type II membrane protein that mediates the uptake of cellular iron in human cells [4, 30], in the yeast *Saccharomyces cerevisiae*. The protein is functional in yeast and binds its natural ligand transferrin (Tf) in vitro [29]. Yeast cells expressing human TfR are used as a model for studies regarding the behavior of a heterologous membrane protein. Our group showed [29] that the TfR is seen in the plasma membrane (PM), but binding studies with its ligand Tf have shown that only 70 binding sites per cell are detectable on the surface of protoplasts, while 230 binding sites per cell are found in isolated membranes. Here we have used this model protein to establish its steady-state localization, to clarify the pathway of the TfR in yeast and to answer the question of how TfR is transported through the host cell. Furthermore, we describe ways to influence the transport and the amount of a heterologous membrane protein, by adapting cultivation conditions and deleting the chaperone Cne1p, a protein involved in quality control in the ER.

Materials and methods

Strains and culture conditions

S. cerevisiae strains GRF18 (*MAT α, leu2-3, 112, his3-11, 15, can1*) and YHH32 (*MAT α, pral::URA3, prb1ΔA5, leu2-3, 112, his3-11, 15, ura3Δ5*) were used throughout this study. Cells were grown overnight at 28°C in minimal medium WMIX supplemented with glucose, then harvested and transferred to WMIX with galactose as sole carbon source for induction of TfR expression. Incubation was performed at 24°C. WMIX medium is WMVIII [13] with the following modifications: 0.02 M potassium phosphate buffer, pH 6.5, was used instead of $\text{NH}_4\text{H}_2\text{PO}_4$ and KH_2PO_4 , and NH_4Cl was omitted. Glucose or galactose (both at 2%) and appropriate supplements (100 mg/l histidine) were added as required. TfR transcription was repressed by shifting cells from galactose- to glucose-containing medium.

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Plasmids and construction of *CNE1*-deletion strain

The plasmids used were the expression plasmid YEpGTfR (kindly provided by H.-J. Terng), a shuttle plasmid based on the 2 μ m DNA origin, the yeast *LEU2* gene as selection marker and the cDNA encoding the human TfR [29] under the control of the yeast *GALI/10*-promoter and the *FLP1*-terminator, and the control plasmid YEpGal without the TfR-cDNA. The *CNE1* deletion strain was constructed according to the method described by Güldner and colleagues [7]. The fragment used to generate the deletion was amplified by PCR from the template pUG6. The upstream oligonucleotide primer was 5'-ACG CAT TTC TAA TAT AGA TAA CGG CCA CAC AAA GTA GTA CCA GCT GAA GCT TCG TAC GC-3' and the downstream oligonucleotide primer was 5'-AAT ACT ACA CAA CAA AGA ACC GAC GTC GTC AAG AGC ACG AGC ATA GGC CAC TAG TGG ATC TG-3'. The fragment, comprising the kanamycin resistance gene as selectable marker, was used to transform strain GRF18 and disruption by homologous recombination was confirmed in strain GRF18cne (MAT α , *cne1::kan^r*, *leu2-3*, *112*, *his3-11*, *15*, *can1*) by Southern blot analysis (data not shown).

Membrane protein isolation

Cells (1×10^9) were harvested, washed and resuspended in 500 μ l phosphate-buffered saline (PBS) and 1 mM phenylmethylsulfonylfluoride (PMSF); an equal volume of glass beads (0.5 mm diameter) was added and cells were disrupted by vortexing 10 times for 1 min and incubating for 1 min on ice in between. Membranes and cell walls were isolated by spinning the lysate for 30 min at 10,000 *g*. The pellet was resuspended in 400 μ l PBS, 1 mM PMSF, 2% SDS to solubilize membrane proteins. After centrifugation for 5 min at 4,000 *g*, the supernatant was loaded onto an SDS-polyacrylamide gel. SDS-PAGE and immunoblotting were performed as described [29]. Antibodies against human TfR (monoclonal OKT9 and polyclonal) and human TfR from placenta were kindly provided by R. Geßner (Virchow Klinikum, Charite, Germany).

Subcellular fractionation

Fractionation was performed as described by Serrano et al. [25]. A total of 5×10^{10} cells were harvested, washed and resuspended in 7 ml H₂O. Glucose (2 ml of a 20% stock) was added for PM ATPase activation. After 10 min incubation at room temperature, 1 ml 1 mM Tris-HCl pH 8.0; 100 mM EDTA; 10 mM PMSF; 10 μ g/ml leupeptin was added and cells were disrupted in a Disintegrator-S (Biomatic) with an equal volume of glass beads (0.5 mm diameter). The lysate was mixed with 20 ml STED10 [STED10, STED20, STED36, STED53: containing 10, 20, 36, or 53% (w/w) sucrose, respectively, in 10 mM Tris-HCl pH 7.6; 1 mM EDTA; 1 mM DTT] and cleared at 700 *g*. Membranes were collected by centrifugation of the supernatant at 20,000 *g*. The pellet was resuspended in 1 ml STED10, including 1 mM PMSF and 10 μ g/ml leupeptin and loaded onto a continuous sucrose gradient preformed by laying 4 ml each of STED53, STED36 and STED20 into a tube, sealing it with Parafilm and keeping it in a horizontal position at 4°C. After 4 h, a continuous gradient was formed by setting the tube in an upright position. Gradients were spun for 16 h at 100,000 *g*. Fractions of 700 μ l were collected from the top to the bottom. Aliquots were subjected to enzyme assays or to SDS-PAGE. Western blot analysis was carried out using anti-Pma1p (PM ATPase) antibodies (kindly provided by R. Serrano, Polytechnic University, Valencia, Spain), anti-Tf (transferrin) antibodies (DAKO, Glostrup, Denmark), anti-Dpm1p (dolicholphosphatemannose-synthetase) and anti-CPY (carboxypeptidase Y) antibodies (Molecular Probes, Leiden, The Netherlands).

Enzyme assays

NADPH-cytochrome *c* oxidoreductase (ER marker) was measured in a reaction mixture consisting of 1 ml 50 mM KPi (potassium phosphate) buffer (pH 7.0); 1 mM KCN; 0.1 mM NADPH and 15 μ l of the gradient fractions. The reaction was started by adding 10 μ l cytochrome *c* (2.5 mM) and the increase in absorbance at 550 nm was monitored. Vacuolar α -mannosidase was assayed with *p*-nitrophenyl mannoside as the substrate and the generation of *p*-nitrophenol was followed. Appropriate dilutions of gradient fractions (100 μ l) were added to a 325 μ l reaction mixture (50 mM KPi buffer, pH 7.0; 0.1% Triton X-100; 1 mM *p*-nitrophenyl mannoside). The reaction was incubated for 1 h at 37°C and stopped by adding 650 μ l Na₂CO₃. Absorbance was measured at 400 nm. PM ATPase activity was measured by the formation of inorganic phosphate (P_i) from ATP. A 100 μ l aliquot of a gradient fraction was added to 325 μ l reaction mixture (50 mM MES-Tris pH 6.5; 5 mM MgSO₄; 50 mM KNO₃; 5 mM NaN₃; 0.2 mM ammoniummolybdate; 2 mM ATP). The reaction was incubated for 20 min at 30°C and stopped by adding 650 μ l 2% H₂SO₄; 0.5% ammoniummolybdate; 0.5% SDS; 0.1% ascorbic acid. Absorbance was measured at 690 nm after incubation for 5 min and the P_i concentration was calculated using a P_i-standard of 25–250 nmol. Activity was calculated according to [24].

Results

The heterologously expressed human TfR is predominantly localized in ER membranes

To investigate the steady-state localization of the heterologously expressed human TfR, yeast cell membranes were fractionated by sucrose gradient centrifugation. The TfR was mainly localized in intracellular membranes, presumably in membranes of the secretory pathway, such as ER or Golgi membranes (Fig. 1) as deduced from cofractionation of TfR with the ER-markers NADPH cytochrome *c* oxidoreductase (Fig. 1C) and dolichol-phosphatemannose-synthetase (Dpm1p; Fig. 1D). Only a small amount of the TfR is localized in the PM, as shown after concentrating the PM fractions (Fig. 1B). The protein level of the PM fractions was lower and therefore the amount of heterologously expressed TfR had to be concentrated to be above the detection limit.

Immunostaining was performed to confirm these data and revealed a dot-like staining in the cell (data not shown). The staining did not co-localize with the vacuolar membrane or with the nucleus, but localization within ER membranes or Golgi was seen. This is in good agreement with the fractionation data and suggests that the TfR is localized predominantly in the ER, although a small fraction is localized in the PM. This confirms the data of Terng et al. [29] and shows in addition that only a minor part of the TfR is present in the PM.

The degradation of the TfR in yeast takes place in the vacuole

The TfR was expressed under the control of the galactose-inducible *GALI/10* promoter, which is repressed by

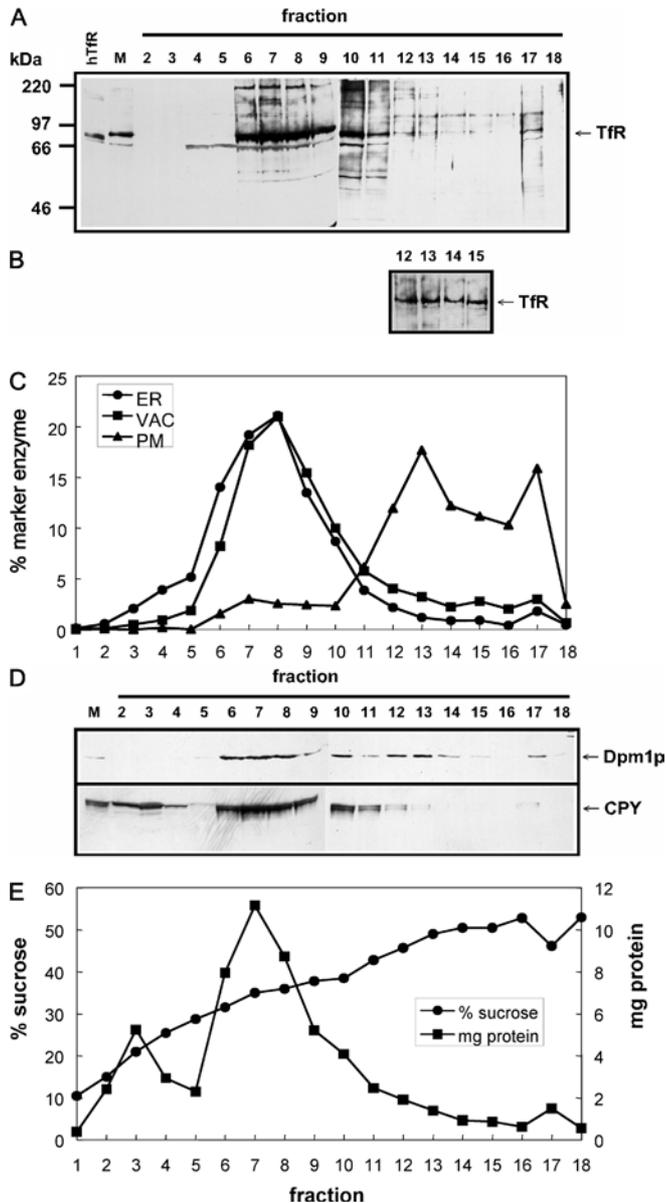


Fig. 1A–E Fractionation of the human transferrin receptor (TfR)-expressing strain. The strain YHH32/YEpGTfR was induced for TfR synthesis with galactose for 48 h. Cells were harvested and treated as described in Materials and methods. Fractions of 700 μ l were collected from top to bottom (lanes 1–18, respectively) and analyzed as follows. Aliquots (30 μ l) were separated on SDS-PAGE and analyzed by Western blotting with polyclonal anti-TfR antibodies (A, B), monoclonal anti-Dpm1p antibodies [endoplasmic reticulum (ER) marker] and monoclonal anti-CPY antibodies (vacuolar marker; D). *hTfR* Human TfR from placenta as positive control, *M* aliquot of the membrane before fractionation. Marker enzyme activity (C) was measured for each fraction and expressed as percentage of total enzyme activity in all fractions. *VAC* Vacuolar marker α -mannosidase, *ER* ER marker NADPH cytochrome *c* reductase, *PM* PM-ATPase as plasma membrane (PM) marker. Density (% sucrose) and protein concentration (mg) was also measured for each fraction (E). Aliquots (100 μ l) of fractions 12–15 were precipitated with trichloroacetic acid to increase the amount of protein and again subjected to SDS-PAGE analysis. TfR is unambiguously detectable in PM fractions when protein concentrations equivalent to concentrations in ER fractions were used (B)

glucose, so the degradation behavior could be assayed by repressing transcription. TfR synthesis was induced upon shifting the cells from glucose- to galactose-containing medium. Afterwards, the cells were transferred to glucose-containing medium to repress transcription of the TfR gene and to stop TfR synthesis. Thus, the degradation behavior of the expressed TfR could be followed. The TfR was extremely stable in yeast and could still be detected 6 h after repression of transcription (Fig. 2). The degradation behavior of the TfR was compared in strains GRF18, which is protease-competent, and YHH32, a vacuolar proteinase A- and B-deficient strain to test whether the turnover of the TfR took place in the vacuole. Samples were taken at different time points after repression of transcription and translation by shifting the cells to glucose in the presence of cycloheximide. Data revealed that the TfR could be detected 6 and 8 h after repression of transcription and translation in the protease-deficient strain, whereas it could no longer be detected in the protease-competent strain (Fig. 3). Cycloheximide is known to enhance protein turnover [33] and therefore the stability of TfR in the GRF18 strain is lower in the presence of cycloheximide than without cycloheximide. These data show that the receptor is more stable in the protease-deficient strain, and imply that the turnover of the receptor takes place in the vacuole and depends on vacuolar proteases.

Degradation of the TfR depends on favorable transport conditions

The degradation rate of the TfR was also reduced at low temperature (4°C) or in the absence of an energy source

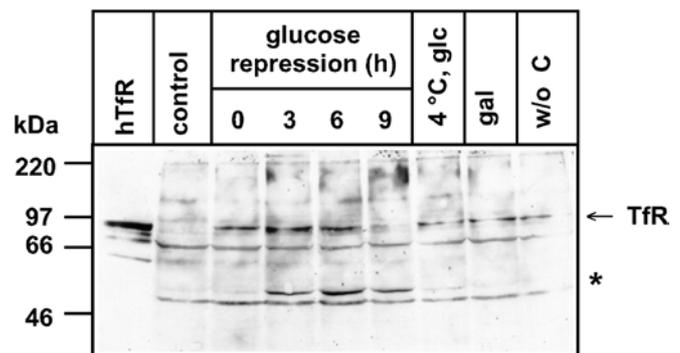


Fig. 2 Stability of TfR under diverse conditions. TfR expression was induced in *Saccharomyces cerevisiae* strain GRF18/YEpGTfR by incubating cells for 48 h in galactose. Afterwards, the cells were transferred to glucose-containing medium and incubated at 28°C for different times or at 4°C for 9 h (4°C, *glc*). Two more samples were taken and either incubated with galactose (*gal*) or without a carbon source (*w/o C*) for 9 h. Cells not expressing TfR (GRF18/YEpGal) were used as a negative control. Membrane proteins were isolated and 200 μ g protein was loaded on SDS-PAGE. hTfR (1 μ g) from placenta was used as a positive control. * Specific degradation product of TfR. Detection was performed using polyclonal anti-TfR antibodies

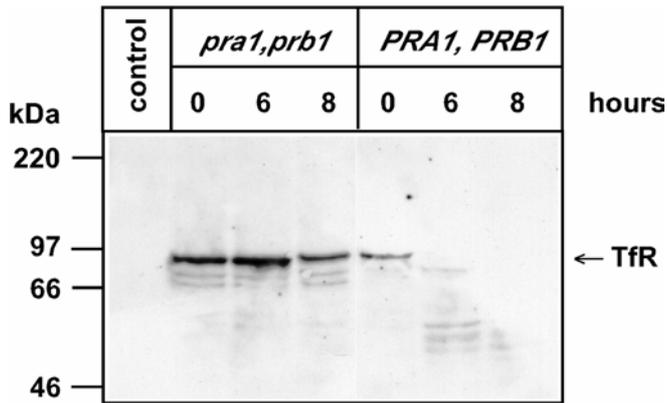


Fig. 3 Stability of Tfr in a protease-deficient strain. Tfr expression was induced in a protease-deficient (YHH32/YEpGTfr: *pra1, prb1*) and a protease-competent (GRF18/YEpGTfr: *PRA1, PRB1*) strain by incubating cells for 48 h in galactose. Afterwards, the cells were transferred to glucose-containing medium with cycloheximide (100 μ g/ml) to completely stop synthesis and samples were taken at different time points to follow the Tfr degradation. Membrane proteins were isolated and 300 μ g protein was loaded in each lane. Membrane proteins from the strain YHH32/YEpGal after 48 h in galactose were loaded as a negative control. Tfr was detected using monoclonal anti-Tfr antibodies (OKT9)

(glucose), conditions both known to retard transport processes. In either case, a specific degradation product of the Tfr, which was always seen in cells treated with glucose, could not be detected (see Fig. 2). Cells grown in galactose showed a Tfr degradation pattern similar to that seen in cells grown without an energy source or at low temperature. Again, the degradation band could not be detected. This suggested that galactose is not as good a source of energy as glucose under our cultivation conditions. The transport of the Tfr from the ER to the vacuole via the PM can thus be enhanced by supplying a good energy source, and it can be reduced by inhibiting transport processes, e.g., at low temperature.

The ligand Tf is taken up nonspecifically in yeast

In human cells, the Tfr is partly localized in the PM [27], where it binds its ligand Tf and takes it up by receptor-mediated endocytosis. Tfr heterologously expressed in yeast is able to bind its natural ligand Tf in vitro as shown by Terng et al. [29]. Studies on Tf uptake were performed to investigate if the Tfr in yeast was able to bind Tf in vivo. Yeast cells induced for the expression of the receptor were incubated with Tf in glucose-containing medium. Yeast cell extracts were then fractionated on a sucrose gradient. The ligand Tf was detected in PM fractions as deduced from co-fractionation of Tf and PM ATPase (Fig. 4). This implies that the ligand Tf reaches the yeast PM after crossing the cell wall and is thus able to bind the Tfr on the PM. However, Tf could also be seen in the PM fractions of the negative control strain, which does not express the Tfr. This suggests that Tf is trapped in the

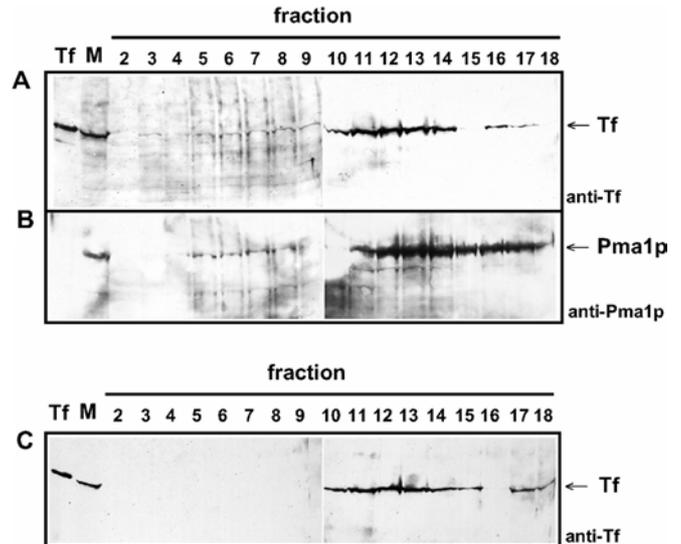


Fig. 4A–C Transferrin uptake. Tfr expression was induced in the strain YHH32/YEpGTfr as described in Fig. 1. After incubation in glucose-containing medium with 20 μ g/ml ferri-transferrin for 6 h, cells were harvested and membranes were fractionated as described in the legend to Fig. 1. Aliquots of fractions (60 μ l) were subjected to electrophoresis and analyzed by western blotting with polyclonal anti-Tf antibodies (A) and polyclonal anti-Pma1p antibodies (B). The control strain YHH32/YEpGal without the Tfr gene was treated likewise and also analyzed for transferrin uptake with anti-Tf antibodies (C)

periplasmic space after crossing the cell wall and is enclosed in PM vesicles by fluid phase endocytosis. It is, however, remarkable that the uptake of the ligand is energy-dependent and not forced by diffusion, as cells incubated without a carbon source or at low temperature for 12 h do not show any uptake of Tf into membrane fractions (data not shown). Thus, Tf might be internalized by both fluid phase endocytosis and endocytosis of Tfr-Tf complexes. Tf transport is therefore unspecific and cannot be used to follow the behavior of the Tfr.

Influencing quality control in the ER by deleting the chaperone Cne1p

As the Tfr was predominantly found in the ER membrane, the question arose if modifying quality control in the ER could influence Tfr transport to the PM. The quality control machinery might identify the Tfr as a foreign protein and retain it in the ER. The yeast chaperone *CNE1* has homology to the mammalian chaperones calnexin and calreticulin [32]. Deletion of *CNE1* in yeast increases the secretion of heterologously expressed soluble protein [17]. To investigate whether the deletion of the *CNE1* gene influences the transport of a heterologous membrane protein, we deleted this gene in strain GRF18. A time-course profile showed that the amount of Tfr was higher in the *CNE1*-deletion strain compared to the reference strain (Fig. 5). Tfr

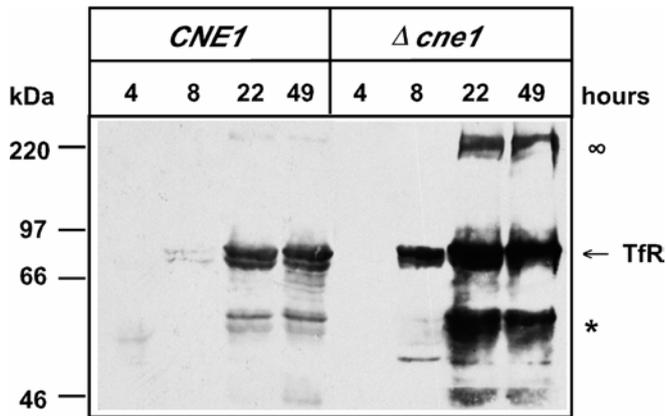


Fig. 5 Expression profile of the Tfr in a *CNE1* deletion strain. The strains GRF18/YEpGTfr and GRF18cne/YEpGTfr were induced with galactose for Tfr expression. After 4, 8, 22 and 49 h of induction, aliquots of *CNE1* competent cells (*CNE1*) and *CNE1* deleted cells ($\Delta cne1$) were harvested, membranes were isolated and proteins (100 μ g) were separated by SDS-PAGE. * Specific Tfr degradation product, ∞ putative dimer. The receptor was detected using polyclonal anti-Tfr antibodies

expression could also be detected earlier (after 8 h) than in the reference strain (after 22 h). Membrane fractionation of the *CNE1* deletion strain revealed that the Tfr was detectable in the ER-containing fractions as well as in PM fractions (Fig. 6). A large amount of the Tfr still accumulated in the ER, but Tfr was localized in the PM to a higher degree than in the reference strain. This is seen when comparing the amount of Tfr in ER-specific fractions and PM-specific fractions. The Tfr bands on the blot were measured by density scan for quantification. The ratio of the Tfr in three ER-containing fractions (fraction 9, 10, 11) to three PM-containing fractions (fraction 13, 14, 15) is 1.5 in the *CNE1* deletion strain vs 3 in a control strain without the *CNE1* deletion.

Discussion

Human Tfr heterologously expressed in the yeast *S. cerevisiae* is localized predominantly in the ER membrane; only a small amount is localized in the PM. The Tfr is degraded in the vacuole, as shown by its enhanced stability in a vacuolar protease-deficient strain. Our data support the hypothesis that, in yeast, the heterologously expressed Tfr is translocated into the ER membrane, travels from the ER through the secretory pathway to the PM and is then taken up and degraded in the vacuole. The degradation of the Tfr depends on its transport to the degradation compartment and the long half-life implies that the amount of Tfr reaching this compartment is low. The bottleneck in transport through the cell seems to be the route from ER to PM, as a large part of the Tfr accumulates in the ER. Its long half-life also indicates that the Tfr is not degraded by ER-associated degradation (see [3, 34] for review of ER-associated degradation).

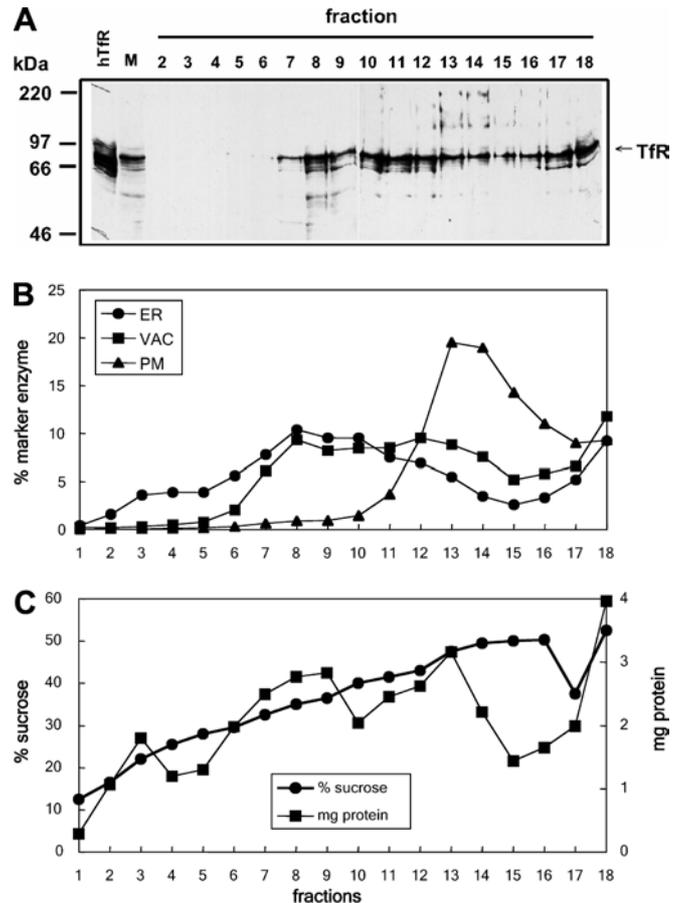


Fig. 6A–C Fractionation of the *CNE1* deletion strain. The strain GRF18cne/YEpGTfr was induced for Tfr synthesis with galactose for 48 h. Cells were harvested and treated as described in Materials and methods. Fractions of 700 μ l were collected and analyzed as described in Fig. 1. Aliquots (60 μ l) were separated by SDS-PAGE and analyzed by Western blotting with polyclonal anti-Tfr antibodies (A). *hTfr* Human placental Tfr as positive control, *M* aliquot of the membrane before fractionation. Marker enzyme activity (B) was measured for each fraction and expressed as percentage of total enzyme activity in all fractions. *VAC* Vacuolar marker α -mannosidase, *ER* ER marker NADPH cytochrome *c* reductase, *PM* PM-ATPase as PM marker. Density (% sucrose) and protein concentration (mg) was also measured for each fraction (C)

As shown in this paper, favorable transport conditions are necessary to efficiently transport the Tfr to the vacuole. In our studies, galactose does not seem to be as good a source of energy as glucose. A typical degradation product of the Tfr is seen in cells incubated with glucose, but not in cells treated with galactose. In addition, cells with a reduced transport activity due to low temperature or incubation without carbon source do not show this degradation band. In these cases, degradation of Tfr is slowed. Thus, it seems that the transport to the vacuole is reduced on galactose. Peñalver and colleagues [20] also described that the protein turnover of membrane proteins depends on the carbon source. Although sucrose, mannose and fructose are good energy sources for turnover, galactose requires a long

adaptation time and does not achieve the protein turnover rates of the other carbon sources.

It has previously been reported that some overexpressed membrane proteins accumulate in the ER and do not reach their destination, the PM [2, 8, 9, 10, 11, 12, 18, 31]. Limitations of either the transport machinery from ER to PM or the capacity of the PM surface for the uptake of membrane proteins might cause this accumulation. The bottleneck in transportation of homologous overexpressed proteins is the amount of protein to be transported. Heterologous overexpressed proteins encounter an additional bottleneck, as they have to use a transport system that is not designed for them (see [6, 19] for discussion of transport machinery).

The ER is also the site of quality control [5]; a foreign protein has to traverse this quality control. The Cne1 protein has been described as a homologue of the mammalian chaperones calnexin and calreticulin [32]. Deleting the chaperone Cne1p does not have any influence on the transport of homologous soluble proteins such as the α -factor and acid phosphatase, but it increases the secretion of heterologous soluble proteins such as α 1-antitrypsin [17] and a fusion protein composed of a hydrophobic peptide and lysozyme [1]. Parlati and colleagues [17] suggested that the deletion of *CNE1* might also affect homologous membrane proteins. These data suggest that the chaperone Cne1p is involved in quality control in yeast, especially in influencing the transport of heterologous or mutated proteins by retaining them in the ER and diminishing their transport to the PM. To date, no data have been reported on the influence of the *CNE1* deletion on a heterologous membrane protein in yeast. Our data show that TfR accumulation is significantly increased in a Δ *cne1*-strain, implying that the chaperone Cne1p acting in the quality control machinery reduces the production or the accumulation of the heterologous membrane protein. Cne1p seems to be involved in ER-associated degradation [15]. Cne1p might recognize the heterologous protein and direct it to the ER degradation machinery. Overexpression might lead to a mixture of correctly and incorrectly folded TfR. While the incorrectly folded TfR is delivered to ER degradation in the reference strain, both the correctly and the incorrectly folded TfR accumulate in the ER of the Δ *cne1*-strain. The high amount of overexpressed membrane protein might also give a feedback signal in the reference strain and thus result in decreased synthesis or translocation of the heterologous protein. Cne1p might be involved in such regulation by sensing the amount of protein in the ER. This regulation would be turned off in the deletion strain, so that synthesis is not decreased following the accumulation of proteins in the ER. Furthermore, protein import into, and export from, the ER share some translocation proteins [21, 22], and both these processes, translocation and ER-associated degradation, compete for limiting components [16]. Cne1p, which is involved in quality control and is therefore coupled to the ER degradation

machinery, might recruit such components so that the translocation process is limited and accumulation in the ER is reduced. The deletion strain, however, would not be limited in translocation and thus would allow high accumulation of the heterologous protein.

Our report describes the intracellular transport of a heterologous membrane protein and highlights the bottleneck of transport from ER to PM. Influencing quality control by genetic modification seems to be a promising tool to enhance transport and yield of heterologous membrane proteins in yeast. The TfR accumulating in the ER can be used as a model in the further improvement of transport, by genetic engineering of components of the transport machinery.

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