Abstract
Background: Secretion of recombinant proteins in yeast can be affected by their improper folding in the endoplasmic reticulum and subsequent elimination of the misfolded molecules via the endoplasmic reticulum associated protein degradation pathway. Recombinant proteins can also be degraded by the vacuolar protease complex. Human urokinase type plasminogen activator (uPA) is poorly secreted by yeast but the mechanisms interfering with its secretion are largely unknown.

Results: We show that in Hansenula polymorpha overexpression worsens uPA secretion and stimulates its intracellular aggregation. The absence of the Golgi modifications in accumulated uPA suggests that aggregation occurs within the endoplasmic reticulum. Deletion analysis has shown that the N-terminal domains were responsible for poor uPA secretion and propensity to aggregate. Mutation abolishing N-glycosylation decreased the efficiency of uPA secretion and increased its aggregation degree. Retention of uPA in the endoplasmic reticulum stimulates its aggregation.

Conclusions: The data obtained demonstrate that defect of uPA secretion in yeast is related to its retention in the endoplasmic reticulum. Accumulation of uPA within the endoplasmic reticulum disturbs its proper folding and leads to formation of high molecular weight aggregates.

Background
Though the secretory pathway is organized similarly in yeast and in other eukaryotic organisms, not all proteins of higher eukaryotes can be efficiently secreted from yeast cells. Secretion of some recombinant or mutant proteins is affected by their improper folding in the yeast endoplasmic reticulum (ER) [1,2] and rapid degradation of misfolded molecules by cytosolic proteasome complex after retrograde translocation from the lumenal to cytoplasmic face of the ER (ER associated protein degradation, ERAD) [3,4]. Alternatively, misfolded proteins can be sorted from the late Golgi to vacuole, yeast homologue of lysosome, and degraded by the vacuolar protease complex [5,6]. The recognition and retention of misfolded proteins in the ER is carried out by the "ER quality control" (ERQC) machinery. Saccharomyces cerevisiae differs by the organization of ERQC not only from higher eukaryotes, but also from Schizosaccharomyces pombe. However, in spite of these species-specific differences, the ERQC involves processing of the N-linked oligosaccharides in all eukaryotes reflecting importance of N-glycosylation for protein folding in vivo [for review, see [7]].
Human urokinase type plasminogen activator (uPA) is poorly secreted by yeast. Although three mutations improving uPA secretion in yeast have been characterized [8–10], the bottleneck for uPA secretion remains uncertain. There are some data indicating that the efficiency of uPA secretion in yeast depends on the ERQC machinery. Disruption of the S. cerevisiae PMR1 gene, encoding the Golgi membrane Ca\(^{2+}\) ion pump, improves uPA secretion, decreases protein glycosylation [8] and causes a defect of the ERAD [11]. The Hansenula polymorpha opu24 mutation with the uPA supersecretion phenotype also impairs protein glycosylation and possibly causes defect of the ERAD, since, similarly to pmr1, the opu24 mutant is also hypersensitive to dithiothreitol and tunicamycin, drugs perturbing protein folding in the ER [10]. However, the S. cerevisiae ssu21 mutation improving uPA secretion, does not alter neither glycosylation of secretory proteins [9], nor sensitivity to diithiothreitol and tunicamycin (M. Agaphonov, unpublished).

In this study we demonstrate that inefficient uPA secretion in yeast is related to its retention in the ER that is conditioned by the uPA N-terminal domains. Accumulation of uPA within the ER affects its folding and leads to formation of high molecular weight aggregates.

**Results**

**Overexpression of the uPA impairs its secretion and causes accumulation in an aggregated form**

We have constructed several H. polymorpha strains bearing different copy numbers of the uPA expression cassette. This cassette consisted of the uPA gene under the control of strong regulatable promoter of the H. polymorpha MOX gene, which encodes a key enzyme of the methanol utilization pathway, alcohol oxidase. In all cases *max*-negative recipients were used for obtaining these transformants (see Materials and methods). Among them three transformants possessed a single copy of the expression cassette integrated into different genomic loci. The productivity of extracellular uPA in single copy integrants varied depending on the integration locus but was always higher than in strains with multiple copies of the integrated cassette (Table 1). Immunoblot analysis of cell lysates revealed that the amount of intracellular uPA grew drastically with the increase of dosage of the expression cassette, while uPA activity dropped. This indicates that intracellular uPA was accumulated mostly in an inactive form.

Further analysis has shown that intracellular uPA accumulated in a form of high molecular weight aggregates, since it could be precipitated by centrifugation of cell lysates at 10,000 g for 10 min in the presence of a detergent, solubilizing membrane associated proteins (Figure 1B). uPA from culture supernatants of the single copy integrants migrated on the SDS PAGE as a broad smear (Figure 2, lane 3), which could be converted into the distinct ~30 kDa band by treatment with endoglycosidase H (EndoH) (Figure 2, lane 4). In human cells uPA is synthesized as a 48 kDa zymogen consisting of three domains: two N-terminal domains, not essential for the enzymatic activity, and the C-terminal serine protease domain (Figure 1A). uPA zymogen is activated by hydrolysis of the K\(^{158}\)-I\(^{159}\) peptide bond. The accuracy of the cleavage is essential for uPA activation, since hydrolysis of the R\(^{156}\)-F\(^{157}\) peptide bond by thrombin produces inactive protein [for review, see [12]]. EndoH-treated extracellular uPA migrated faster than its N-terminally truncated variant lacking the N-glycosylation site (Figure 2, lane 5), indicating that it is a product of the uPA cleavage at the activation site, because cleavage at other sites would result in uPA derivatives showing either different molecular weight or no activity.
In contrast to the extracellular form, intracellular uPA was core glycosylated, since it migrated on SDS gel as a distinct band and its electrophoretic mobility only slightly increased after EndoH treatment (Figure 2, lanes 1 and 2). This means that the N-linked oligosaccharide chain of intracellular uPA had not received the Golgi modifications, and, therefore, uPA aggregates were formed within the ER. This conclusion was further confirmed (see below).

**Secretion of uPA depends on the presence of N-glycosylation site and N-terminal domains**

In eukaryotic cells uPA is modified by an attachment of the glycoside chain to the N$_{302}$ residue. Substitution of this residue for another one abolishes the N-glycosylation of uPA [13]. uPA variants with N or Q residue at the position 302 in the full-length enzyme (uPA and uPA-Q) or in its N-terminally truncated form (uPA-C and uPA-CQ) (Figure 1A) were expressed in *H. polymorpha* and their amounts in culture medium were compared with the use of fibrinolytic activity assay and immunoblotting. These analyses have shown that both alteration of the N-glycosylation site and presence of the N-terminal domains decreases efficiency of uPA secretion (Table 2). The lower difference in activity of glycosylated and unglycosylated variants, than it may be deduced from the difference in amounts of immunoreactive protein can be explained by the inhibitory effect of the yeast N-glycoside chain on the specific activity of uPA towards the high molecular weight substrates [14].

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**Figure 1**

Schematic representation of the uPA variants (A) and Western blot analysis of uPA in subcellular fractions of transformants expressing different uPA variants (B). Arrows indicate the position of activation cleavage site. "Q$^{302}$" indicates the mutation abolishing the N-glycosylation of uPA. Transformants DLU, DLC, DLQ and DLCQ, expressing uPA, uPA-C, uPA-Q and uPA-CQ, respectively, are described in Materials and methods. P and S, pellet and supernatant fractions, respectively. Pellets were dissolved in a 2-fold volume of starting lysate.

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**Figure 2**

Western blot analysis of uPA in intracellular aggregates and culture medium. Lanes 1 and 2, pellet fraction of cell lysate of the DLU strain expressing full-length uPA before and after treatment with EndoH, respectively; lanes 3 and 4, culture medium of the same strain before and after EndoH treatment, respectively; lane 5, pellet fraction of cell lysate of the DLCQ strain expressing uPA-CQ.
Similar pattern of secretion of different uPA variants was revealed in the *S. cerevisiae* integrants carrying a single copy of the uPA expression cassette (Figure 3), indicating applicability of the obtained results to different yeast species. However, in contrast to *H. polymorpha*, the transformants of *S. cerevisiae* bearing the expression cassettes integrated in a single copy did not accumulate uPA intracellularly. On the other hand, such accumulation took place in transformants with the multicopy uPA expression vector (data not shown). Above we demonstrated that in *H. polymorpha* high levels of uPA expression, inhibiting secretion, are always accompanied with its intracellular accumulation (Table 1). Basing on this, we concluded that uPA expression levels in *S. cerevisiae* transformants with a single copy of the uPA expression cassette were lower than those, which might inhibit its secretion.

**Aggregation of uPA is defined by accumulation within the ER**

Distribution of different uPA variants between the soluble and pellet fractions obtained by centrifugation of cell lysates (Figure 1) depended on their secretion potential. The highest proportion of soluble to aggregated form was observed for the best secreted uPA variant (uPA-C), whereas the lowest one was in the case of the worst secreted variant (uPA-Q). Since the major bands of the soluble fractions of the uPA and uPA-C variants corresponded to the core-glycosylated protein, they might represent a pool of newly translocated molecules, whose fate had not been determined yet, and properly folded molecules, which are ready to leave the ER or already entered the Golgi apparatus, but have not received modifications altering their electrophoretic mobility.

The amount of uPA in the soluble fraction should depend on the rates of uPA synthesis, aggregation and exit from the ER. To prevent the latter process, the uPA and uPA-C variants were modified by the substitution of 12 C-terminal amino acid residues for the KDEL sequence, representing the ER retention signal. This should retain in the ER correctly folded uPA, which is normally secreted, and thus increase proportion of soluble to aggregated uPA in cell lysates. Indeed, such modification abolished the exit of both uPA variants into culture medium (data not shown) and led to the increase of their amounts in cell lysates, though this effect was much more pronounced for uPA-C (Figure 4). Surprisingly, this did not increase the amount of soluble form of both uPA variants in cell lysates. Since

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**Table 2: Comparison of secretion efficiencies of different uPA variants by *H. polymorpha***

| uPA variant | Fibrinolytic activity assay (%) | Western blotting* (%) |
|-------------|--------------------------------|-----------------------|
| uPA         | 100                            | 100                   |
| uPA-C       | 1850                           | 1500                  |
| uPA-Q       | 30                             | 14                    |
| uPA-CQ      | 770                            | 330                   |

*Calculated by comparison of intensities of the 30 kDa band revealed by Western blotting of serially diluted culture supernatants. uPA in culture medium was treated with EndoH prior to the analysis.

**Figure 3**

Halo-forming ability of *S. cerevisiae* (Sc) and *H. polymorpha* (Hp) transformants expressing different uPA variants. Yeast transformants were patched on fibrin-containing plates and incubated for 20 h (*H. polymorpha*) or 40 h (*S. cerevisiae*).
full-length uPA showed a high degree of aggregation on its own, the addition of the ER retention signal only slightly increased its aggregation degree. In contrast, the retention in the ER of uPA-C, which normally is less prone to aggregate, led to a drastic increase of amount of aggregated form. At the same time amount of the soluble form of uPA dropped (Figure 4). This indicates that accumulation of uPA in the ER causes its aggregation.

Accumulation of the wild type uPA in the *H. polymorpha* ER may result from its retention in this compartment due to incorrect folding in the heterologous host. Normally misfolded proteins in the ER are eliminated by the ERAD pathway. The data presented in this study suggest that uPA can partially degrade within the yeast cell. In fact, the rate of uPA synthesis in the transformant DLU/pAM226, bearing two copies of the expression cassette, must not exceed more than two-fold the rate of uPA synthesis in the DLU/L integrant with a single copy of this cassette. However, the difference in amount of intracellular uPA in these transformants appeared to be much bigger (Table 1). The discrepancy between the expected and observed difference in intracellular accumulation of uPA cannot be explained by worsening uPA secretion, since in the DLU/L strain the amount of extracellular enzyme constituted less than 10% of that of its intracellular form (0.11 μg of extracellular uPA vs. 1.6 μg of intracellular uPA per 1 mg of total cellular protein). Thus, the observed difference in amount of intracellular uPA probably was due to the degradation of significant portion of uPA in the transformants with lower expression levels and rescue of misfolded uPA by aggregation upon the higher expression levels.

Above it was shown that accumulation of uPA within the ER causes its aggregation. This means that defect of degradation of uPA in this compartment should increase its amount and, therefore, the aggregation degree. This suggestion was supported by the results of analysis of the aggregation pattern of uPA-KDEL expressed in the *H. polymorpha* opu24 mutant. The uPA supersecretion opu24 mutation has been previously shown to cause phenotypes characteristic of the ERAD lesion [10]. Impairment of the ERAD should save misfolded uPA from degradation, thus increasing its amount within the ER. The amount of aggregated but not soluble form of uPA-KDEL appeared to be much higher in the mutant than in the wild type cells confirming that accumulation of uPA in the ER is accompanied by its aggregation (Figure 5). Interestingly, this mutation did not noticeably influence the aggregation pattern of the full-length uPA lacking the ER retention signal (data not shown). This indicates that accumulation of wild type uPA within the ER of the mutant cells was not
accelerated though degradation of misfolded protein was impaired. Thus, it is possible to suggest that this mutation facilitates exit of uPA from the ER probably due to the activation of mechanisms improving efficiency of its folding.

**Discussion**

The data obtained show that efficient uPA secretion demands an optimal level of its expression: both low and increased expression did not result in the maximal levels of uPA secretion. Indeed, we observed that the best uPA secretion did not result in the maximal levels of uPA expression. Thus, it is possible that to some extent, the aggregation of uPA even improves its secretion: accumulation in aggregates may decrease uPA crowding in the ER, thus facilitating its proper folding and subsequent secretion.

The increased aggregation of uPA-KDEL in the *H. polymorpha* opu24 mutant provides an additional evidence for the interference of uPA accumulation within the ER with its folding. We suggest that the degradation rate of uPA-KDEL in this mutant was reduced due to the ERAD lesion. In contrast, the *opu24* mutation did not stimulate aggregation of uPA lacking the ER retention signal. This indicates that a significant portion of uPA escaped from the ER, probably due to improved folding, which may be the reason of the "supersecretion" phenotype of this mutant.

**Conclusions**

In this work we demonstrate that in *H. polymorpha* overexpression poisons uPA secretion and stimulates its intracellular aggregation. The absence of the Golgi modifications in accumulated uPA suggests that the aggregation occurs within the ER. Deletion analysis has shown that the N-terminal domains of uPA were responsible for its poor secretion and propensity to aggregate. Mutation abolishing N-glycosylation decreased the efficiency of uPA secretion and increased its aggregation degree. Retention of uPA in the ER by means of the KDEL signal also led to its accumulation in an aggregated, but not in soluble form, and the amount of uPA-KDEL aggregates increased in the *opu24* mutant with defects in the ER-associated degradation of misfolded proteins. These experiments demonstrate that the increase of uPA content in the yeast ER interferes with its folding. The data obtained in this work allow us to suggest that uPA is poorly secreted by yeast, because its folding requires milieu of the ER of higher eukaryotes. The unfavorable milieu in the yeast ER causes incorrect folding of significant portion of uPA, which is retained in the ER and undergoes either degradation or aggregation.

**Methods**

**Yeast strains**

The *H. polymorpha* strains DL1-L (leu2) derived from DL-1 (ATCC 26012), 8 V (leu2) and 24–8 V (opu24 leu2) derived from CBS4732 (ATCC 34438) were described earlier [10,16,17]. The *trp3* mutant DLT2 (leu2 max, trp3::LEU2) was obtained by transformation of the DL1-L strain with the plasmid pMLTA, digested with XhoI and *Ecl*III digested plasmid pSMΔ. The cassettes expressing different variants of uPA were introduced into the
H. polymorpha genome either by replacement of the MOX gene [18], or via single copy integration of the expression vectors possessing the HplEU2-d selectable marker into the LEU2 locus [19]. The uPA expressing transformants are listed in Table 3. Integration into the MOX locus was performed via transformation of the DLT2 strain with the plasmids pSM1, pUR2SM1, pSMWC or pSMWCQ, digested with XhoI and EcoRI. Integration into the LEU2 locus was performed via transformation of the LEU2 strains with the plasmids pAM226, pAM410, pNR4 or pNR5, digested with EcoRI and XhoI. The S. cerevisiae strains expressing different uPA variants were obtained via integration of the EcoRV-digested expression vectors pNR9, pNR23, pNR26 and pNR27 into the URA3 locus of the YPH499 strain [20].

Plasmids

The plasmids used in this study are listed in Table 4. The uPA expression vectors pAM219, pAM226 and pAM281 were constructed by the insertion of sequence encoding uPA fused to the signal peptide of the Kluyveromyces lactis killer toxin [21] under control of the MOX promoter into the BamHI and HindIII sites of the AMIpL1, AMIpLD1 and AMIpSL shuttle vectors [19], respectively. This insert consisted of a 1.8 kb Xhol-BamHI fragment of the pSM1 plasmid [18] and an adapter, which was obtained by annealing the oligonucleotides 5’GATCCGCAGTCACACCAAGGAAGAATGGCCTGG 3’ and 5’GATCAAGGACGAGCTGT3’. To allow ligation of the BamHI cohesive end of these vectors and the XhoI cohesive end of the insert, two bp of their overhangs were filled in by the Klenow enzyme. The expression vectors pAM410 and pSMWC encoding the N-terminally truncated uPA were constructed by insertion of a 0.8 kb BglII-BamHI fragment of pWC28 (kindly given by M. Minashkin), carrying the sequence encoding uPA protease domain, into the KpnI and BamHI sites of the pAM226 and pSM1 plasmids, respectively (3’-terminal overhangs of KpnI and BglII were removed by the Klenow enzyme). The plasmid pSMWCQ was constructed by replacement of the 0.7 kb BamHI-EcoRI fragment of pSMWC for the corresponding fragment of pUR2SM [10]. The expression vectors pNR4 and pNR5 encoding uPA variants with the KDEL ER retention signal were constructed by the insertion of an adapter, which was obtained by annealing the oligonucleotides 5’GATCAAGGACGAGCTGT3’ and 5’AGCIAACGCTGCTTCTT3’, between the BamHI and HindIII sites of pAM226 and pAM410.

The S. cerevisiae uPA expression vectors were based on the YEpSEC-NR8 integrative shuttle vector, which was obtained from the YEpSEC1 plasmid [21] by deletion of the SnaBI-AatII fragment, carrying the 2 μm DNA sequence and the LEU2 gene. The wild type uPA expression vector pNR9 was constructed by insertion of the 1.2 kb KpnI-HindIII fragment of the pAM226 plasmid into the corresponding sites of the YlpSEC-NR8 plasmid. The uPA-C expression vector pNR23 was obtained by replacement of the KpnI-BamHI fragment in the pNR9 plasmid for the BglII-BamHI fragment of the pWC28 plasmid (3’-terminal overhangs of KpnI and BglII were removed by the Klenow enzyme). The uPA-Q and uPA-CQ expression vectors pNR26 and pNR27 were obtained by replacement of the EcoRI-BamHI fragment in the plasmids pNR9 and pNR23 for the corresponding fragment of pUR2SM.

Culture conditions for the H. polymorpha strains and conditions for the induction of uPA expression in the CBS4732 derivatives were described previously [19]. To induce uPA expression in transformants of the DL1-L strain, overnightYPD cultures were 6-fold diluted with YPM medium (2% Peptone, 1% Yeast extract, 2.5% methanol, 150 mM NaCl) and incubated at 37°C for 50–60 h.

Analyses of uPA expression

To prepare lysates, cells were disrupted with glass beads in TBST buffer (30 mM tris-HCl pH7.5, 150 mM NaCl, 2% Triton × 100), containing protease inhibitors, 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.1 mM benzamidine, 0.1 mM sodium metabisulphite, 0.5 μg/ml TPCK, 0.5 μg/ml TLCK, 2.5 μg/ml antipain, 0.5 μg/ml leupeptin, 1.0 μg/ml pepstatin A, 2.0 μg/ml aproline. The protease inhibitors were omitted if lysates were analyzed for the uPA activity. Amounts of active uPA were determined by fibrinolytic activity assay [22]. Total amounts of uPA in cell lysates and culture medium of different transformants were compared by probing appropriately diluted lysates and culture supernatants with the anti-uPA antibody specific to the uPA protease domain [23]. The amounts of uPA in culture medium were normalized to the levels of total cellular protein [24], whereas amounts of uPA from cell lysates were normalized to the levels of soluble cellular protein in a sample assayed as described [25]. To study uPA aggregation, cell debris was removed from lysates by centrifugation at 300 g for 10 min and lysates were centrifuged again at 10,000 g for 10 min. Obtained pellet and supernatant fractions were analyzed by Western blotting.

A qualitative test for the ability of yeast transformants to secrete uPA was performed by examination of their capacity to create haloes during growth on fibrin-containing media, as described previously [9,18].

Transformation of H. polymorpha and Escherichia coli cells

H. polymorpha was transformed by the modified lithium acetate method [26]. E. coli was transformed as described [27].
Additional material

Additional File 1

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