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Defect of vacuolar protein sorting stimulates proteolytic processing of human urokinase-type plasminogen activator in the yeast *Hansenula polymorpha*

Michael Agaphonov [a,*], Nina Romanova [a], Sviatoslav Sokolov [b], Anna Iline [a], Tatyana Kalebina [b], Gerd Gellissen [c,1], Michael Ter-Avanesyan [a]

[a] *Institute of Experimental Cardiology, Cardiology Research Center, 3rd Cherepkovskaya Str. 15A, Moscow 121552, Russia*
[b] *Molecular Biology Department, Faculty of Biology, Lomonosov Moscow State University, Vorobiovy Gory, Moscow 119899, Russia*
[c] *Rhein Biotech GmbH, Eichsfelder Str. 11, 40595 Dusseldorf, Germany*

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Abstract

Human urokinase-type plasminogen activator (uPA) is poorly secreted by yeast cells. Here, we have selected *Hansenula polymorpha* mutants with increased productivity of active extracellular uPA. Several of the obtained mutants also demonstrated a defect of sorting of carboxypeptidase Y to the vacuole and the mutant loci have been identified in six of them. All these mutations damaged genes involved in protein traffic between the Golgi apparatus and the vacuole, namely *PEP3*, *VPS8*, *VPS10*, *VPS17*, and *VPS35*. We have shown that inactivation of the *VPS10* gene encoding the vacuolar protein sorting receptor does not increase uPA secretion but stimulates its proteolytic processing.
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Keywords: *Hansenula polymorpha*; Protein secretion; Urokinase; Vacuolar protein sorting; Yeast

1. Introduction

Yeasts, as single-cell eukaryotic organisms, are attractive hosts for the production of secretory proteins of higher eukaryotes. However, despite the similarities of the secretory pathway organization in different eukaryotes, many secretory proteins of higher eukaryotes are inefficiently secreted by yeast cells. This can be due to the inability of such proteins to efficiently adopt their native conformation after translocation to the endoplasmic reticulum (ER) lumen [1–3] where misfolded protein molecules are recognized and eliminated by the, so-called, ER quality control machinery ERQC (for review, see [4]). Alternatively, some recombinant or aberrant proteins entering yeast secretory pathway can be sorted from the *trans*-Golgi compartments to the vacuole for degradation by the vacuolar protease complex [1,5]. Vacuolar proteases can be also involved in the undesired cleavage of target proteins during secretion [6].

Many vacuolar proteins such as soluble vacuolar hydrolases, e.g. carboxypeptidase Y (CPY), travel through the early stages of the secretory pathway to the late compartment of the Golgi apparatus, where

* Corresponding author. Tel.: +7 095 414 6738; fax: +7 095 414 6733/6699.
E-mail address: aga@cardio.ru (M. Agaphonov).
[1] Present address: PharmedArtis GmbH, Forckenbeckstr. 6, 52074 Aachen, Germany.

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they are sorted away from secretory proteins destined for delivery to the cell surface (for review see [7]). Many genes involved in protein trafficking from the Golgi apparatus to the vacuole were identified in screens for mutants defective in CPY enzymatic activity (*pep* mutants) [8] or for mutants that secrete CPY due to inability to properly sort it to the vacuole (*vps*) [9,10]. Vps10p receptor has been shown to be implicated in the recognition and delivery of some vacuolar proteins from the late Golgi to the prevacuolar compartment [11–13]. This receptor also can target misfolded or aberrant proteins, which exit the ER, to the vacuole for degradation [5]. Here, we show that a lesion in the machinery mediating protein traffic between the Golgi apparatus and vacuole causes an increase in the efficiency of proteolytic processing of a recombinant secretory protein in the yeast *Hansenula polymorpha*.

The methylotrophic yeast *H. polymorpha* is currently in use as an efficient host for recombinant protein production and as a model organism for studies of peroxisome metabolism, nitrate and nitrite utilization, regulation of gene expression [14], protein secretion and glycosylation [15–17]. These studies have been greatly facilitated recently due to sequencing of more than 90% of the *H. polymorpha* genome [18]. A high frequency of non-homologous integration of transforming DNA in *H. polymorpha*, the trait that complicates targeted inactivation of chromosomal genes, allowed development of an efficient, so-called RALF, approach for the functional analysis of its genome [19]. This approach includes obtaining mutations via transformation with a linear DNA fragment, which randomly integrates within the genome, and subsequent identification of mutant loci by the isolation of the transforming DNA fragment with the adjoining chromosomal sequences.

At present, several independently isolated *H. polymorpha* strains, differing from each other by some genetic and physiological properties, are in use in biotechnology and in fundamental studies [14]. Combination of advantages provided by different strains can be exploited to facilitate molecular genetic manipulations with *H. polymorpha*. For example, the strain CBS4732 can mate and sporulate and was shown to be suitable for obtaining mutations via the RALF approach [19]. However, low frequency of homologous integration of transforming DNA hampers the targeted disruption of chromosomal genes in this strain. In this respect another *H. polymorpha* strain, DL-1, has advantages over CBS4732 because of higher frequency of homologous recombination (our unpublished data). In this study, we used both *H. polymorpha* strains: mutations were induced via the RALF approach in a strain derived from CBS4732, and the mutant phenotypes in some cases were then confirmed in corresponding gene disruptants obtained in a derivative of DL-1.

2. Materials and methods

2.1. Strains

Standard nomenclature was used for designation of yeast genes. When necessary, *H. polymorpha* genes were marked with "*Hp*". Strains M14 (*leu2 ade2 mox::GOX [MOX][PDI1:uPA, GAP1:MF1α-KDEL]*) and u23m25 (*leu2 ade2 ura3::ADE2 mox::GOX [MOX][PDI:uPA, GAP1:MF1α-KDEL]*) possessed uPA expression cassette (*PDI1:uPA*) consisting of the DNA fragment encoding this protein fused to a 5′-end portion of the *PDI1* gene (the promoter region and a portion of the ORF coding for the secretion signal). Although only secretion of uPA has been analyzed in this work, these strains were designed for the selection of mutations with other manifestations (to be published elsewhere) and, in this connection, they possessed expression cassettes of two more reporter proteins, *Aspergillus niger* glucose oxidase (*GOX*) and *Saccharomyces cerevisiae*-derived MFα1 – provided with the ER retention signal KDEL (*MFα1-KDEL*). Square brackets in the genotypes of the M14 and u23m25 strains designate genes which were carried by the exogenous DNA fragments co-integrated into an unidentified chromosomal locus (see below).

The strain M14 was obtained from the 1B (*ade2 leu2*) strain [20], which is a derivative of *H. polymorpha* CBS4732 (ATCC 34438). The *MOX* gene in the 1B strain was replaced with the *GOX* expression cassette [21] via the approach described earlier [22]. Then, the *Xba*I and *Stu*I-digested pNR45 plasmid, which contained the expression cassettes of uPA and the modified *S. cerevisiae*-derived MFα1, was introduced into the resulting *MOX*-negative strain by co-transformation with a DNA fragment possessing the *MOX* gene as selection marker. One of the methanol-utilizing (Mut^+) transformants, which produced uPA, was designated M14. Strain u23m25 was obtained from the M14 strain by disruption of the *URA3* gene with the *ADE2* selection marker. The strain DLQ (*leu2 mox::uPA-*Q^{302}) expressing a mutant uPA that lacks the *N*-glycosylation site (uPA-Q^{302}), which derived from *H. polymorpha* DL1 (ATCC 26012), has been described earlier [23]. The strain DLQ-VL with disrupted *VPS10* (*leu2 mox:: uPA-*Q^{302}*vps10::LEU2*) was obtained via transformation of strain DLQ with the pKAD15 plasmid digested with *Hpa*I and *Sal*I [24].

The *S. cerevisiae* strain BY4741 (*MATa his3-Δ1 leu2-Δ0 met15-Δ0 ura3-Δ0*) [25] and its derivatives disrupted for the *VPS8*, *VPS10*, and *VPS17* genes were obtained from the Euroscarf collection. To study uPA secretion, these strains were transformed with the *Eco*RV-digested pNR9 plasmid [23].

2.2. Generation of mutants via the RALF approach and their further analysis

To obtain mutants by the random integration of linear DNA fragments, the u23m25 and m14 strains were transformed with the *Eco*RV-digested pCAD24 plasmid, or *Eco*RI-digested pKNR51 and pKNR56 plasmids (Fig. 1). Transformants were replica-plated onto plates with fibrin-containing medium, where secretion of uPA was detected by haloes of fibrin lysis. Selected transformants were subjected to identification of the integration locus of transforming plasmid as described previously [19]: their chromosomal DNA was digested with *Hin*cII and self-ligated. As the transforming DNA fragments did not possess *Hin*cII recognition sites, the resulting plasmids contained genomic DNA fragments flanking the integration sites. The ligated DNA was used for *E. coli* transformation. Sequences of genomic DNA inserted into the plasmids, which were recovered by the *E. coli* transformation, were compared with the genome databases. Access to the *H. polymorpha* genome database was kindly provided by Rhein Biotech GmbH.

2.3. Plasmids construction

The *S. cerevisiae* uPA expression vector pNR9 was described elsewhere [23]. Plasmid pNR45 consists of the 5.4-kb *Age*I-*Kpn*I fragment of the pNR9 plasmid, 0.46-kb *Age*I-*Eco*RI fragment of the pGAG418-43 plasmid [26], an *Eco*RI-*Sac*I fragment of the PCR product encoding the *S. cerevisiae* MFα1 precursor extended with the C-terminal KDEL sequence, and a 0.5-kb *Sac*I-*Hin*cII fragment of plasmid p120CMC [24] harbouring the 5′-portion of the *HpPDI1* gene. To allow ligation of the *Hin*cII and *Kpn*I-generated DNA ends, the 3′-overhang of the *Kpn*I end was removed by the Klenow enzyme. Plasmid pCAD24 was constructed by insertion of the 0.6-kb *Xho*I-*Eco*RV fragment of plasmid pTZMOX [22] into the *Sal*I and *Eco*RV sites of the pCHLX [27]. Plasmid pKNR51 was obtained by insertion of the 1.2-kb *Xho*II-*Xho*II fragment of plasmid p18B1 [28] and the 0.6-kb *Bam*HI-*Xho*I fragment of plasmid pCAD24 between the *Sal*I and *Bgl*II sites of vector pUK21 [29]. The plasmid pKNR56 was obtained by deletion of the 117 bp *Kpn*I-*Eco*RI fragment in the plasmid pKNR51.

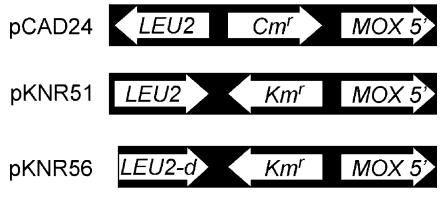

Fig. 1. Schemes of linearized plasmids pCAD24, pKNR51, and pKNR56. *LEU2*, *H. polymorpha LEU2* gene; *LEU2-d*, *H. polymorpha LEU2* gene with the truncated promoter region; Cm^r, chloramphenicol resistance marker; Km^r, kanamycin resistance marker; *MOX*-5′,5′-end portion of the *MOX* gene containing the promoter region and first seven codons of its ORF.

2.4. Analyses of uPA expression

A qualitative test for the ability of yeast transformants to secrete uPA was performed by examination of their capability to create haloes during growth on a fibrin-containing medium as described previously [22]. Amounts of uPA secreted by DLQ and DLQ-VL strains were compared by probing appropriately diluted culture supernatants with the anti-uPA antibody specific to the uPA protease domain. The amounts of uPA in culture medium were normalized to the levels of total cellular protein. Production of uPA in *H. polymorpha* was induced by cultivation in liquid medium under conditions either inhibiting or stimulating its proteolytic processing. Prior to the induction, cells were grown overnight in YPD medium containing 0.1 mM NaCl. To stimulate uPA proteolysis, the overnight cultures were 6-fold diluted in induction medium A (1% yeast extract, 3% peptone, 0.1 mM NaCl, 2% methanol) and incubated for ~70 h at 37 °C in polypropylene culture tubes. To inhibit uPA proteolysis, the overnight cultures were 7.5-fold diluted by induction medium B (1% yeast extract, 3% peptone, 25 mM $NH_4H_2PO_4$, 25 mM $(NH_4)_2HPO_4$, 0.1 mM NaCl, 2% methanol) and incubated at 37 °C for ~70 h in glass culture tubes. To induce uPA expression in *S. cerevisiae* yeast transformants were grown at 30 °C for ~30 h in medium containing 1% yeast extract, 2% peptone, 25 mM $NH_4H_2PO_4$, 25 mM $(NH_4)_2HPO_4$, 0.1 mM NaCl, and 2% galactose. Proteins from the culture supernatants were 60-fold concentrated by precipitation with trichloroacetic acid, treated with Endoglycosidase H, and analyzed by Western blotting. Amounts of samples loaded on the gel were normalized to the total cellular protein.

2.5. Antibody and Western blotting

The immunoblots were developed using chemiluminescent detection (Amersham Pharmacia Biotech, Sunnyvale, CA). Antisera against carboxypeptidase Y (CPY) were isolated from mice immunized with a polypeptide representing the ^{6}His-tagged mature part of the *H. polymorpha* DL-1 CPY. The respective gene sequence was expressed in *E. coli* from the pCNR2 expression vector and the protein purified using the "TALON" resin (Clontech Laboratories, Palo Alto, CA) according to the manufacturer's instructions. The anti-uPA antibody specific to the uPA protease domain (IMTEK, Moscow, Russia) has been described elsewhere [30].

3. Results

3.1. Mutants with increased activity of extracellular uPA arise due to defects of vacuolar protein sorting

The u23m25 strain was mutagenized by transformation with the linearized plasmids pCAD24, pKNR51, and pKNR56 (see Section 2 and Fig. 1). The transforming DNA fragments were flanked by a selection marker at one side, and by the promoter and short 5′-end portion of the *MOX* gene ORF at the other side. The plasmid pKNR56 was equipped with the *LEU2* selection marker, which lacks most of its promoter region (*LEU2-d*). This non-expressed *LEU2* gene can acquire a promoter sequence via random integration of the pKNR56 DNA fragment into the promoter region of a chromosomal gene. In this case, the *MOX* promoter at the opposite flank of the transforming DNA fragment should be directed to the ORF of the same chromosomal gene, affecting its expression. For the detection of extracellular uPA the obtained transformants were replica-plated onto fibrin-containing medium, and clones forming haloes of increased size were selected. In total, 196 transformants with haloes of increased size have been identified. Out of this collection, twenty three transformants forming the largest haloes were tested for the ability to secrete vacuolar protein CPY, which served as indication of a defect in vacuolar protein sorting. Increased amount of CPY was detected in culture medium of eight integrants (data not shown), and mutant loci were identified in six of them. In all cases, mutants arose due to defects of genes responsible for protein traffic between the Golgi apparatus and the vacuole. The identified genes were homologues of the *S. cerevisiae VPS8*, *VPS17*, *PEP3*, *VPS35*, and *VPS10* genes. *VPS10* was disrupted in two independently obtained integrants (Table 1).

It is notable that among the transformants with identified mutant loci, strain u170 was the only one obtained by transformation with the pKNR56 plasmid, which harbors the *LEU2-d* selection marker (Table 1). As predicted, the transforming DNA fragment integrated between the promoter region and ORF of the *PEP3* gene in an arrangement where the *LEU2-d* is fused to the *PEP3* promoter, while the promoter and portion of ORF of the *MOX* gene (Fig. 1) is directed to the *PEP3* ORF. Since the *PEP3* ORF remained intact, one could assume that cultivation of cells on glucose- or methanol-containing medium and the resulting repression or induction of the *MOX* promoter may influence the manifestation of the mutant phenotype. However, this integrant formed haloes of increased size on fibrin plates independently of the carbon source used. Apparently, the lack of its own promoter prevented expression of the *PEP3* ORF on glucose-containing medium, while induction of the *MOX* promoter did not promote *PEP3* expression due to the presence of the *MOX* ORF segment, whose ATG start codon was not in frame with the *PEP3* ORF (data not shown).

Table 1
Mutant loci identified in transformants with increased activity of extracellular uPA

Transformant	Strain used for obtaining mutants	Plasmid used for obtaining mutants	*S. cerevisiae* homologue of the inactivated gene
u15	M14	pCAD24	*VPS10*
u25	M14	pCAD24	*VPS10*
u96	u23m25	pKNR51	*VPS17*
u118	u23m25	pCAD24	*VPS8*
u170	u23m25	pKNR56	*PEP3*
v17	M14	pCAD24	*VPS35*

3.2. Disruption of the VPS10 gene affects proteolytic processing of uPA

The screen for mutants with increased activity of extracellular uPA allowed us to identify genes involved in the control of sorting of vacuolar proteins. In yeast, recombinant proteins can be sorted by means of the Vps10p receptor to vacuole for degradation [5,1]. One can suggest that the lack of the Vps10p receptor decreases the rate of uPA degradation, which, in turn, results in increased amount of its secreted form. uPA productivity of the strain used for mutant generation was very low, since expression of the corresponding gene was governed by the relatively weak *PDI1* promoter. On one hand, this facilitated selection of the mutants by the increased halo size, but complicated their further analysis on the other hand, since the amount of secreted uPA was insufficient for detection by Western blotting even after 100-fold concentration (data not shown). To study whether human uPA produced in *H. polymorpha* can be sorted to the vacuole for degradation, we inactivated the *VPS10* gene in the DLQ strain expressing uPA-Q^{302} under the control of the *MOX* promoter. The obtained *vps10-Δ* strain formed haloes of increased size during growth on fibrin-containing medium (Fig. 2(a)).

Previously we have observed that the uPA secreted by *H. polymorpha* cells undergoes proteolysis resulting in a 30-kDa polypeptide chain. Such proteolysis most likely occurs at the activation site of the uPA zymogene [23]. According to our unpublished observations, proteolysis of uPA in *H. polymorpha* can be inhibited or stimulated by variation of cultivation conditions (see Section 2). uPA in culture medium of the isogenic *vps10-Δ* and *VPS10* strains, grown in conditions stimulating uPA proteolysis, migrated in SDS-PAGE as a protein of 30 kDa. The titer of secreted protein was not increased in the mutant strain (Fig. 3(a)). When grown under inhibitory conditions for uPA proteolysis, the enzyme secreted by the wild-type strain migrated as the full-length 48-kDa protein, while that secreted by

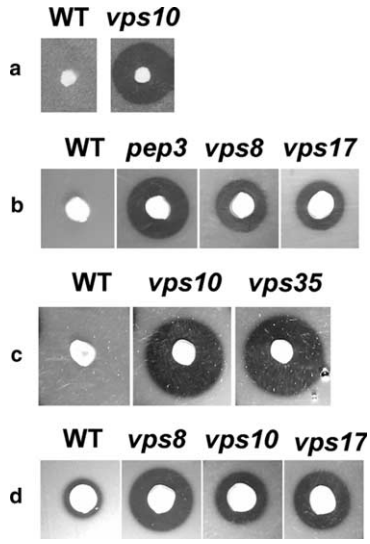

Fig. 2. Haloes on fibrin-containing medium formed by yeast transformants expressing uPA. (a) *H. polymorpha* DL-1 derivatives DLQ (WT) and DLQ-VL (*vps10-Δ*); (b) *H. polymorpha* CBS4732 derivatives u23m25 (WT), u170 (*pep3*), u118 (*vps8*), and u96 (*vps17*), (Table 1); (c) *H. polymorpha* CBS4732 derivatives M14 (WT), u25 (*vps10*), and v17 (*vps35*) (Table 1); (d) *S. cerevisiae* strain BY4741 (WT) and its *vps8-Δ*, *vps10-Δ*, and *vps17-Δ* derivatives bearing the uPA expression vector pNR9.

the mutant in comparable amounts migrated as a 30-kDa polypeptide (Fig. 3(b)). Remarkably, uPA obtained from the culture medium of the *vps10-Δ* caused lysis of fibrin gel when present in much lower concentration than the uPA from culture medium of the strain with wild-type *VPS10* (Fig. 4). These data indicate that the proteolytic cleavage of uPA in the *vps10-Δ* mutant leads to its activation.

3.3. Deletions of VPS genes influence uPA secretion in S. cerevisiae

We transformed *S. cerevisiae* strain BY4741 and its derivatives defective in the *VPS8*, *VPS10*, or *VPS17* gene with the pNR9 plasmid bearing uPA expression cassette under control of the *GAL-CYC* promoter. Prior to the transformation, the plasmid was cleaved inside of

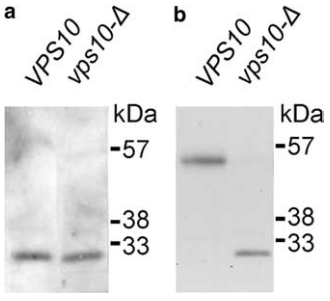

Fig. 3. Western blot analysis of uPA in culture supernatants of strains DLQ (*VPS10*) and DLQ-VL (*vps10-Δ*) grown in conditions stimulating (a) or inhibiting (b) proteolytic processing of uPA (for details, see Section 2).

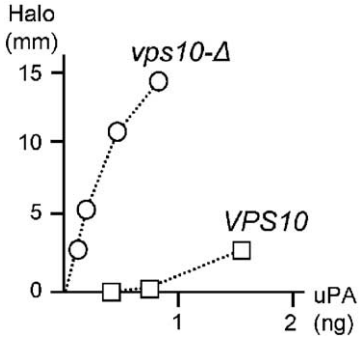

Fig. 4. Dependence of ability of secreted uPA to lyse fibrin on amount of the enzyme loaded. Serially diluted culture supernatants of the strains DLQ (*VPS10*) and DLQ-VL (*vps10-Δ*), grown in conditions inhibiting proteolytic processing of uPA (for details, see Section 2), were loaded on fibrin gel and incubated at 37 °C for 18 h. Amounts of uPA in culture supernatants were determined as described in Section 2.

the *URA3* marker to direct integration of the plasmid into the corresponding locus in a single copy. Transformants of the mutant strains formed larger haloes on fibrin-containing medium than the wild-type strain (Fig. 2(d)), though the effect was not as pronounced as in *H. polymorpha* (Fig. 2(a)–(c)). Western-blot analysis of uPA secreted by transformants grown in liquid medium revealed that uPA from culture supernatants of transformants of the wild-type strain migrates in SDS-PAGE as a 50-kDa protein, while amounts of uPA in culture broth of the mutant transformants were not sufficient for the detection by immunoblotting even after 50–60-fold concentration (data not shown).

4. Discussion

In this work, we have performed screening for *H. polymorpha* mutants with increased productivity of human uPA. This protein is synthesized as 48-kDa zymogen, which is converted into active enzyme by proteolytic cleavage of the K^{158}–I^{159} peptide bond producing a 30-kDa polypeptide chain (for review, see [31]). This enzyme induces lysis of fibrin clots and its secretion can be detected by transparent haloes around yeast colonies grown on plates with fibrin-containing medium. By the RALF technique we have obtained mutants forming large haloes on fibrin plates and demonstrating a vacuolar protein sorting defect. Mutant loci have been identified in six such strains. The mutations damaged genes involved in protein traffic between the Golgi apparatus and the vacuole. To study the impact of the vacuolar sorting defect on uPA, one of the identified genes, *VPS10*, was disrupted in the strain expressing an unglycosylated uPA variant, uPA-Q^{302}. Like the randomly selected mutations, *VPS10* disruption led to increased halo-forming ability, although the amount of uPA secreted into the liquid medium was not elevated. At the

same time, under certain conditions the wild-type strain secreted mostly unprocessed 48-kDa uPA, while uPA secreted by the *vps10-Δ* mutant was proteolytically cleaved (it migrated as a 30-kDa protein) and caused fibrin lysis at a much lower concentration than uPA secreted by the wild-type strain. This allowed us to conclude that inactivation of the *VPS10* gene led to a bulk cleavage of uPA at the activation site. Thus, the larger haloes on the fibrin-containing medium formed by the *vps10-Δ* strain resulted from the activation of uPA, but not from an increased rate of its secretion.

Although the uPA proteolysis was found to depend on vacuolar protein sorting, vacuolar proteases are unlikely responsible for it since: (i) deletion of *PEP4*, which encodes the key vacuolar protease (for review see [32]), does not influence the pattern of uPA proteolysis (data not shown) and (ii) this cleavage can be induced by certain growth conditions without disruption of vacuolar protein sorting. Moreover, inactivation of the gene for the medial Golgi Ca^{2+} pump Pmr1p in *S. cerevisiae* causes a vacuolar protein sorting defect [33], though it appears not to stimulate proteolysis of uPA [34]. According to our unpublished data, the same is true for the *H. polymorpha pmr1* mutation. One can suggest that a lesion in the protein traffic between the Golgi apparatus and vacuole makes uPA more exposed to some protease(s), present in late compartments of the secretory pathway. If uPA and this unknown protease pass through the same secretory compartment(s), the increased proteolysis can be due to increased expression of this protease, or lower dynamics of the secretory compartment(s), where uPA undergoes proteolysis, causing prolonged exposition of uPA to the cleavage. Alternatively, the defects of vacuolar protein sorting can somehow direct uPA flow to a certain protease-containing compartment, which is normally bypassed.

Disruptions of the *VPS8*, *VPS10*, and *VPS17* genes in *S. cerevisiae* also led to increased halo-forming ability on fibrin plates, though the effect was less pronounced than in *H. polymorpha*. We suggest that, like in *H. polymorpha*, this was also due to proteolytic activation of the uPA zymogen, since disruptions of the mentioned genes, despite the increase in the halo-forming ability, noticeably decreased the amount of uPA in the culture medium (we were unable to detect it by Western blotting), while uPA secreted by the wild-type cells migrated in SDS-PAGE as a 48-kDa protein. The lower effect of the *S. cerevisiae vps* mutations on the halo-forming ability compared to that in *H. polymorpha* was probably due to the substantially decreased uPA secretion.

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