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Evaldas Čiplys

ANALYSIS OF MATURATION OF MEASLES VIRUS HEMAGLUTININ IN YEAST *S. CEREVISIAE* AND *P. PASTORIS* SECRETORY PATHWAY AND HUMANIZATION OF YEAST CELLS

Summary of doctoral dissertation
Physical sciences, biochemistry (04 P)

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Evaldas Čiplys

TYMŲ VIRUSO HEMAGLIUTININO BRENDIMO PROCESŲ MIELIŲ *S. CEREVISIAE* IR *P. PASTORIS* LĄSTELIŲ SEKRECINIAME KELYJE TYRIMAS IR MIELIŲ HUMANIZAVIMAS

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Introduction

The importance of therapeutic proteins has grown rapidly since the emergence of the biotechnology industry more than 30 years ago. One of the main challenges for production of biopharmaceuticals is discovery of novel, safe and cheap expression systems, suitable for synthesis of glycoproteins, and improvement of current ones. Protein-based drugs constitute about a quarter of new approvals with a majority (about 70 %) being glycoproteins (Sethuraman and Stadheim, 2006). The rate of approval of new biopharmaceuticals has slowed over the past four years. Only 25 new biological entities came onto the US or EU market in 2006-2010 period. From those only 4 were produced in yeast *S. cerevisiae* (4) and *P. pastoris* (1). Most glycoproteins, mainly antibodies (13 from 25), used for medicine development, were synthesized in mammalian cell cultures (Walsh, 2010). However, volumetric productivity, product heterogenecity, media cost, retroviral contamination, and the time required to generate stable cell lines are generally viewed as drawbacks of mammalian cell culture (Choi et al., 2003; Sethuraman and Stadheim, 2006). Intensive search for alternative expression systems still comes with no suitable result. Yeast, like plant or insect expression systems for several reasons are linked for this purpose. Yeast are generally regarded as safe organism with extremely well studied and understood genetics, biochemistry and physiology. Still only handful of proteins synthesized in yeast (insulin, hirudin, somatotropin, surface proteins of hepatitis B and polyomavirus) were used to develop medicine and vaccines (Walsh, 2010). Thus, improvement and adaptation of yeast expression system for production of complex human and virus proteins and glycoproteins are highly pursuable.

Most glycoproteins, significant for medicine, must obtain their natural conformation to be active and functional. In yeast cells synthesis of secreted recombinant glycoproteins is blocked or impaired in different stages of protein secretory pathway: translocation into and exit from the endoplasmic reticulum (ER), entrance into the Golgi apparatus (GA) or hyper mannosylation (Schroder, 2007; Čiplys et al., 2011a). Yeast protein secretory and post-translational modification pathways must be modified and adapted for efficient mammalian recombinant glycoproteins synthesis. Successful example in this area is humanization of yeast *P. pastoris* N-glycosylation pathway which

replicate the most essential glycosylation pathways found in mammals and allows production of glycoproteins in yeast with glycosylation of mammalian manner (Hamilton and Gerngross, 2007). Still, number of glycoproteins produced in yeast, suitable for pharmaceutical industry, is very low. Mostly, in yeast cells mammalian glycoproteins are improperly folded and modified, while essential reasons, explaining drawbacks of yeast to produce such kind of proteins, are unexplained (Sakamoto et al., 1999). Early stages of yeast protein secretory pathway – translocation and maturation into the ER – have never been comprehensively modified to adapt synthesis of human cell glycoproteins. Also experiments, enabling yeast cells to produce active complex glycoproteins have never been carried out.

According to the problems mentioned above, **objects of this study** were formulated as follows:

1. Characterize maturation of measles virus hemagglutinin (MeH) in yeast protein secretory pathway in molecular level and reveal the reasons for unsuccessful expression of this glycoprotein in yeast cells.
2. Generate a stable yeast strains with integrated genes of protein secretory pathway of human cells and to examine influence of coded human proteins on MeH maturation.

According to these objects, **aims of this study** were:

1. Synthesis and characterization of measles virus glycoprotein in yeast *S. cerevisiae* and *P. pastoris*.
2. Proteomic analysis of yeast cells, synthesizing MeH and identification of yeast proteins responsible for stress, caused by MeH synthesis, response.
3. Data analysis and definition of reasons for unsuccessful expression of MeH in the yeast cells.
4. Cloning of protein secretory pathway genes of human cells into the yeast *S. cerevisiae* vectors and expression in yeast cells.
5. Co-expression of human secretory pathway proteins together with MeH and evaluation of their influence on MeH maturation.
6. Identification of combinations of human secretory pathway proteins that guarantee proper folding and maturation of measles virus glycoprotein in yeast cells.

7. Construction of yeast strains, capable to synthesize complex mammalian glycoproteins suitable for medicine, with integrated human protein secretory pathway genes.

Scientific novelty. Properties of recombinant measles virus hemagglutinin, as also other virus glycoproteins, synthesized in yeast have never been described. Also, reasons for inability of yeast cell to produce such kind of glycoproteins have not been identified. Proteomic analysis of yeast cells, expressing MeH protein, allows detailed study of processes happening during synthesis of recombinant protein and ability to describe important issues. This work is part of a new trend in biotechnology – synthetic biology, which aims to establish new valuable features to commonly used microorganisms, which naturally they do not have. Yeast are widely used in this area and first results not only are published in most prestigious journals, but also saves lives (Ro et al., 2006). In this study yeast are "taught" to produce virus glycoproteins by introducing human secretory pathway proteins. This is innovative work, because we try to create totally new yeast cell with features that are important for biotechnology and biomedicine.

Practical value. This work is important both practically and academically. Identification of factors limiting successful production of virus glycoproteins in yeast cells demonstrate difference between yeast and human cell that occurred during evolution. Bottlenecks for effective production of biologically active MeH were revealed. Introduction of human ER chaperones and evaluation of their influence to MeH maturation determined differences between properties of human and yeast ER chaperones. Basis for generation of yeast cell capable to produce virus surface glycoproteins were set.

Materials and methods

Reagents, enzymes and kits

All reagents used in this work were the highest quality available. All enzymes, their reaction buffers and kits were provided by UAB „Fermentas, Lithuania“(Lithuania) and used according to the manufacturers‘ recommendations.

Oligonucleotides

Oligonucleotide primers for PCR were purchased from Metabion GmbH.

Bacterial, yeast strains and plasmids

Bacterial recombinants were screened in *Escherichia coli* DH5α (F^- *gyrA96* (Nal^r) *recA1 relA1 endA1 thi-1 hsdR17* ($r_k^- m_k^+$) *glnV44 deoR* Δ*(lacZYA-argF)U169* [φ80dΔ*(lacZ)M15*]) cells. Yeast strains *P. pastoris* GS115 (*his4*) was obtained from Invitrogen (JAV). *S. cerevisiae* strains AH 22 (*MATa leu2 his3*) was used for expression. Plasmids pPIC3.5K and pPIC9K (Invitrogen, USA) were used as *P. pastoris* and plasmid pFGG3 (Slibinskas et al., 2004) and pFDC (Čiplys et al., 2011b) as *S. cerevisiae* expression vectors.

Bacterial, yeast media, transformation and growing conditions

E.coli strains were grown in LB medium at 37ºC. Transformations of *E.coli* were carried out by the $CaCl_2$-heat shock method (Sambrook and Russell, 2001). *S .cerevisiae* strains were grown at 30ºC in YEPD medium, transformed with plasmid DNA by LiAc/ssDNA/PEG method (Guthrie and Fink, 1991). For selection and growing of *S. cerevisiae* transformants YEPD medium was supplemented with 5 mM formaldehyde, for induction of recombinant gene expression YEPG medium (yeast extract 1%, peptone 2%, and galactose 3%) was used. Transformation of *P. pastoris* GS115 and SMD1168 strains was performed by electroporation (Cregg et al., 2000). Determination of a copy number was carried out exactly as recommended in *Pichia* protocols (Romanos et al., 1998).

DNA preparation and manipulation

PCR amplification, DNA hydrolysis with restriction endonucleases, filling-in recessed 3'-termini of double-stranded DNA, dephosphorylation of DNA 5'-termini and DNA ligation was performed using UAB "Fermentas" (Lithuania) enzymes and kits, according to manufacturers' recommendations.

SDS-PAGE and Western blotting

SDS-PAGE and Western blotting were performed according to standard protocols (Ausubel, 1999; Sambrook and Russell, 2001).

Preparation of crude yeast lysate

Cell pellets of 10–20 mg were collected into a 1.5-mL microcentrifuge tube by centrifugation, then washed with distilled water and resuspended in 10 volumes (v/w) of breaking buffer (50mM sodium phosphate, pH 7.2, 5mM EDTA and 1mM phenylmethylsulfonyl fluoride). An equal volume of glass beads was added and cells were lysed by vortexing. Then, an equal volume of 2xSDS-PAGE sample buffer (125mM Tris-HCl, pH 6.8, 20% glycerol, 8% SDS, 150mM dithiothreitol, 0.01% bromphenol blue) was added directly to the same tube, mixed and immediately boiled at 100 1C for 10 min. Four microliters of prepared whole cell lysate were loaded onto an SDS-polyacrylamide gel (up to 20 mg protein in each lane).

Fractionation of yeast lysates

Crude cell extracts were prepared using glass beads as described above. Crude lysates were cleared by centrifuging at 800 g for 5 min at 4 1C to remove undisrupted cells. Clarified lysates (Fraction 1) were further fractionated by centrifugation at 16 000 g for 45 min at 4^0C. After the first centrifugation, the supernatant (Fraction 2) was removed, the pellets (Fraction 3) were resuspended in breaking buffer with 1% Triton X-100 for 1h on ice and the centrifugation stage was repeated. The supernatant containing proteins soluble in nonionic detergents was collected (Fraction 4), whereas insoluble pellets represented Fraction 5.

Two dimensional (2D) protein electrophoresis

Cells were lysed by mechanical disruption using glass beads in denaturing IEF buffer (7 M urea, 2 M thiourea, 2% CHAPS detergent, 0,5% ampholytes, 0,002% Bromphenol Blue; 75 mM DTT), cell debris were removed by centrifugation at 16000 × g for 15 min. at 16°C. Supernatants (whole cell lysates) were applied onto 7 cm length IPG strips. Invitrogen (USA) ZOOM IPGRunner system was used for IEF according to manufacturer's recommendations. Soluble fractions, achieved during fractionation of yeast lysates under native conditions, were added to IEF buffer directly or (in the case of high salt concentration) treated with 2-D Clean-Up Kit (Amersham Biosciences, USA) prior to use. After IEF the strips were incubated in equilibration buffer (50 mM Tris-HCl pH 8.8, 2% SDS, 6 M urea, 30% glycerol, 0,002% bromphenol blue) containing, in course, reducing (75 mM DTT) and alkylating (125 mM 2-iodoacetamyde) agents (treated for 15 min. by both). Equilibrated strips were applied onto SDS-polyacrylamide gels and SDS-PAGE was run for the second dimension. Proteins in 2D gels were visualized with Coomassie brilliant blue R-250.

Analysis of 2D gels

2D gel images were analyzed using the ImageMaster 2D Platinum 7.0 software (GE Healthcare, USA). Detected protein spots were quantified by the relative volumes (%Vol), indicating percentage of volumes of a separate spots among volume of all protein spots in a gel.

Results

1. Expression of measles virus hemagglutinin in yeasts *S. cerevisiae* and *P. pastoris*

The expression of hemagglutinin from the measles virus (MeH) in yeast cells has not been examined previously. Schwartz strain (accession no. AF266291) MeH gene, containing C-terminal 6His-tag was used in the expression experiments. MeH gene was inserted into the yeast *S. cerevisiae* expression vector pFGG3 (Fig.1A) under the control of galactose-inducible promoter of yeast *GAL7* gene and the resulting plasmid pFGG3-MeH was used for expressing MeH in *S. cerevisiae* cells. For intracellular expression of MeH in yeast *P. pastoris* the same His-tagged MeH gene was cloned into *P. pastoris* vector pPIC3.5K (Invitrogen, USA) under control of AOX1 promoter. For secreted expression the 5' part of MeH gene encoding N-terminal transmembrane (TM) anchor domain was removed and the rest of the gene was in frame fused with 269 bp fragment encoding the α-factor signal sequence by cloning into *P. pastoris* vector pPIC9K (Invitrogen, USA) under the same AOX1 promoter (Fig. 1B). Resulting plasmids pPIC3.5K-MeH and pPIC9K-MeHΔTM were linearized and electroporated into *P. pastoris* strain GS115 with subsequent selection of multicopy transformants, resistant to various concentrations of antibiotic G418.

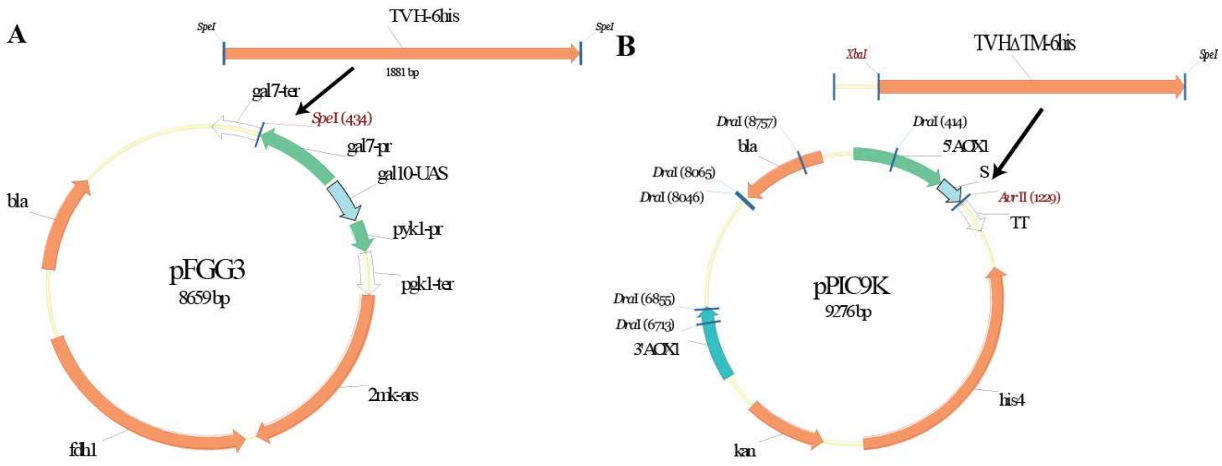

Fig. 1. Construction scheme of *S. cerevisiae* (A) and *P. pastoris* (B) MeH expression vectors. 2 mk-as – 1.74 kb fragment of yeast 2 μm plasmid; fdh1 – *FDH1* gene of *Candida maltosa*, conferring resistance to formaldehyde; gal7-ter – *S. cerevisiae GAL7* gene transcription terminator; gal7-pr – *S. cerevisiae GAL7* gene promoter; gal10-UAS – *GAL10* gene UAS sequence; pyk1-pr – *S. cerevisiae PYK1* gene promoter; pgk1-ter – of *S. cerevisiae PGK1* gene transcription terminator; bla – gene conferring resistance of *E. coli* to ampicillin; kan – gene conferring resistance of *P. pastoris* to antibiotic G418; his4 – *HIS4* gene of *P. pastoris*; 5'AOX1 – *P. pastoris AOX1* gene promoter; 3'AOX1 – *P. pastoris* sequence downstream *AOX1* gene promoter; S – α-factor from *S. cerevisiae*.

SDS-PAGE of yeast lysates (a), obtained from *S. cerevisiae* transformants containing the plasmid pFGG3-MeH and corresponding Western blots (b) with anti-tetraHis specific monoclonal antibodies (Qiagen, Germany) are presented in Fig. 2. SDS-PAGE and Western blot revealed that MeH protein in PAA gels formed two bands, the main band of 65 kDa and minor band of 75 kDa (Fig. 2b, lanes 1, 4 and 6). Probably, the latter stood for the glycosylated form of MeH protein. To determine whether the 75-kDa species exhibited itself as the glycosylated form of the 65-kDa species, the crude lysates were treated with N-glycosidase F (New England BioLabs, USA) before Western blotting. After N-glycosidase F treatment the upper band (75 kDa) disappeared in comparison with the control (lacking enzyme) (Fig. 2b, lanes 1 and 2). This suggests that a small amount of MeH protein represented in the upper band was transported to the endoplasmic reticulum (ER) and N-glycosylated. Centrifugation and extraction of yeast lysates revealed that both glycosylated and unglycosylated forms of MeH synthesized in yeast were insoluble as demonstrated in Fig. 2, lanes 3–6. Virtually all MeH was found in insoluble fractions of lysates (Fig. 2, lanes 4 and 6) and only the traces of MeH was found in Fraction 4 (Fig. 2, lane 5) after extraction with solution containing nonionic detergent Triton-X100. It is worth to note that the MeH glycoprotein isolated from native measles viruses is fully soluble in solutions containing 1% Triton X-100 (Kidokoro et al., 2002).

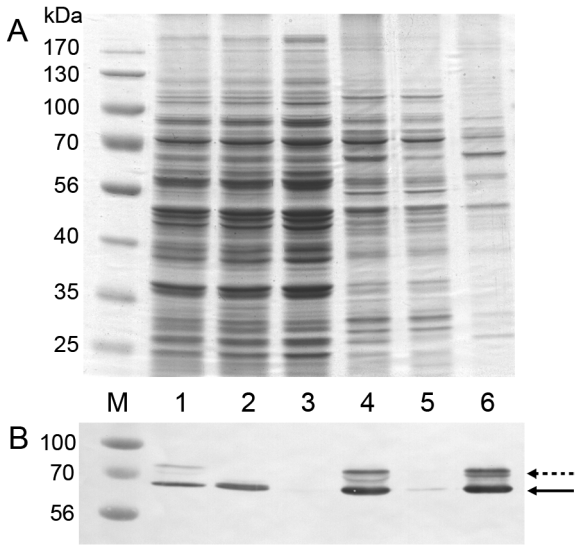

Fig. 2. Analysis of recombinant MeH protein in yeast. (a) SDS-PAGE of crude yeast lysates. (b) Western blot using monoclonal anti-tetraHis antibodies (Qiagen, UK). M – molecular mass standards (Fermentas, Lithuania, SM0671). All samples were obtained from AH22 strain transformed with pFGG3-MeH. Lane 1, yeast crude lysate (Fraction 1); lane 2, yeast crude lysate (Fraction 1) treated with N-glycosidase F; lane 3, supernatant, obtained after centrifugation of yeast lysate (Fraction 2); lane 4, pellets obtained after centrifugation of yeast lysate (Fraction 3), represent proteins insoluble in the disruption buffer; lane 5, proteins extracted with disruption buffer containing 1% Triton X-100, represent proteins soluble in the 1% Triton X-100 (Fraction 4); lane 6, pellets obtained after extraction with 1% Triton X-100, represent proteins insoluble in 1% Triton X-100 (Fraction 5). Solid arrow indicates unglycosylated MeH protein precursors, whereas dashed arrow points to glycosylated MeH forms.

A comprehensive study was performed on MeH expression in *P. pastoris* system that gives much more options for such experiments than *S. cerevisiae*. The expression analysis of pPIC3.5K-MeH transformants with increasing expression level of MeH, conferring different resistance to G418, revealed the following results. The expression of MeH in transformants with one copy of integrated MeH gene is too low to be detected by Western blot. A range of MeH expression level is detected in multicopy pPIC3.5K-MeH transformants, resistant to various G418 concentrations (Fig. 3). When MeH is expressed at the low level, all recombinant product is translocated into ER and glycosylated (Fig. 3B, lane 3). Increasing the expression level, the protein begins to accumulate in the cytosol in the form of unglycosylated precursors. Further increasing gene dosage results in the accumulation of large amounts of unglycosylated MeH precursors (Figure 3B, lanes 1-2), similar to that observed for overexpressed MeH in *S. cerevisiae*. Unfortunately, lowering the expression level has no effect on properties of recombinant MeH protein. All synthesized protein was detected in the insoluble fractions

in transformants with both high and low level expression of MeH (not shown). The only effect of lower expression level of MeH was a less pronounced stress response (described below) (Figure 3A, lane 3 in comparison to lanes 1-2).

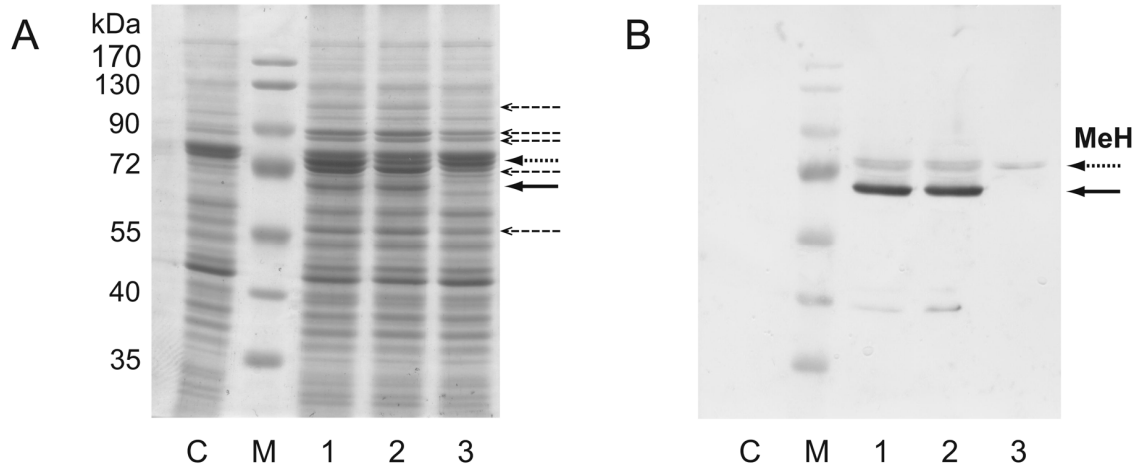

Fig. 3. Analysis of MeH expression in *P. pastoris* transformants. (A) SDS-PAGE of whole cell lysates from *P. pastoris* transformants carrying empty vector or multicopy MeH expression cassettes. (B) Western blotting of the same samples using anti-tetraHis antibody (Qiagen, UK). M – molecular mass standards (Fermentas, Lithuania, SM0671), C – Control (pPIC3.5K MutS), 1,2,3 – multicopy pPIC3.5K-MeH transformants for intracellular expression of MeH, resistant to various concentrations of antibiotic G418. Solid arrows indicate unglycosylated MeH polypeptide precursor, dotted arrows point to glycosylated MeH form and dashed arrows show cellular proteins, upregulated in response to MeH expression.

Expression of chimeric α-MeHΔTM in pPIC9K-MeHΔTM *P. pastoris* transformants, showed that, majority of recombinant α-MeHΔTM protein was translocated into ER and glycosylated (Fig. 4). However, this chimeric protein aggregated in the ER lumen and was not secreted in the culture media. Thus, we did not achieve active recombinant protein by this manipulation. The gene dosage effect was similar as in the case of native MeH sequence variant. When the expression level exceeded the limit of successful translocation, the unglycosylated α-MeHΔTM precursors began to accumulate in the cytoplasm (Fig. 4, lanes 1 and 3, compared to lane 2). Similarly, as in the case of native sequence MeH variant expression in both yeast genera, recombinant α-MeHΔTM protein was insoluble and was not recognized by measles positive human sera. Therefore, the standard approaches to achieve successful expression of viral protein did not help in this case.

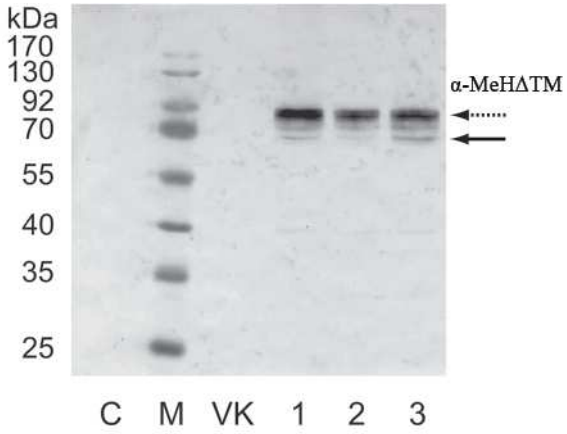

Fig. 4. Analysis of the expression of α-MeHΔTM chimeric protein in *P. pastoris.* Whole cell lysates of methanol induced *P. pastoris* cells expressing a-MeH chimeric protein containing *S. cerevisiae* α-factor signal sequence instead of native TM anchor domain were resolved by SDS-PAGE, blotted onto nitrocellulose membrane and analysed by Western blotting using anti-tetraHis antibody (Qiagen, UK). M – molecular mass standards (Fermentas, Lithuania, SM0671), C – control(pPIC9K), VK – one-copy pPIC9K-MeHΔTM transformant, 1,2,3 – multicopy pPIC9K-MeHΔTM transformants. Solid arrow indicates unglycosylated MeH polypeptides, dotted arrow – glycosylated MeH forms.

2. Analysis of yeast cells, producing MeH protein

Effect of MeH synthesis on yeast cells and reasons for improper maturation were examined in this study. First of all, it was noticed that expression of and MeH inhibited yeast growth. Yeast harboring empty vectors without viral gene doubled every 3 h in galactose media (YEPG), whereas the doubling time slowed to about 7 h for yeast expressing MeH. Also, about 1.5-fold increase in protein accumulation was observed in yeast cell producing MeH comparing to control cells. In comparison, synthesis of measles virus nucleocapsid protein had no inhibitory effect on the yeast growth, despite the high-level of recombinant nucleoprotein expression (Slibinskas et al., 2004). Thus, the synthesis of measles virus surface glycoprotein specifically disorder homeostasis of yeast cells.

Analysis of whole cell lysates showed that synthesis of viral surface proteins causes overexpression of some cellular proteins (Fig. 3, dashed arrows). Two-dimensional (2D) gel electrophoresis and matrix assisted laser desorption/ionization-mass spectrum (MALDI-MS) fingerprinting were used to separate and analyse the proteins (Fig. 5, table 1).

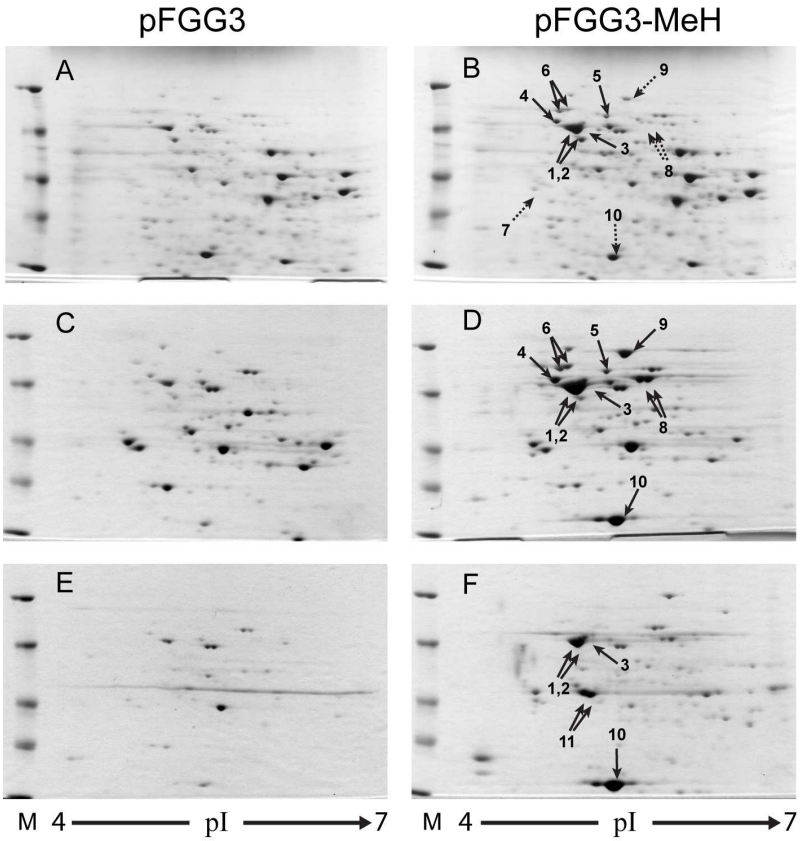

Fig. 5. Analysis of specific stress response in yeast cells, induced by recombinant MeH. (A-F) 2D gel electrophoresis of yeast proteins. Samples were taken from yeast cells of *S. cerevisiae* AH22 strain, expressing MeH (B, D, F) and from control AH22 cells, transformed with empty vector pFGG3 (A, C, E). At the top (A, B) whole cell lysates, in the middle (C, D) fractions of proteins, soluble at high salt concentration, and in the bottom panel (E, F) proteins, insoluble under native conditions are shown. Solid arrows in B indicate proteins, identified by MS directly from whole cell lysates, whereas dotted arrows point to the proteins, identified from soluble and insoluble fractions. Numbers of identified protein spots correspond to those, given in Table 1. Protein molecular mass markers (120, 66, 45, 35, and 25 kDa) were run simultaneously in the left lane (M) of each 2D gel.

Table 1. Identification of yeast proteins involved in recombinant MeH expression.

Spot No.	Name[a]	Fold Change[b]	Function, Process[c]	Localization	Fraction overexpressed[d]
1,2	Ssa1/2	**2.4±0.2**	Chaperone, Stress response	Cytoplasm, cell wall	All fractions except insoluble under denaturing conditions
3	Ssa4		Chaperone, Stress response	Cytoplasm, nucleus	All fractions except insoluble under denaturing conditions
4	Kar2	**3.8±0.4**	Chaperone, UPR	Endoplasmic reticulum	Soluble at high salt concentration
5	Sse1	**2.3±0.2**	Co-chaperone, Stress response	Cytoplasm	Soluble at high salt concentration

6	Hsc82	**2.1±0.3**	Chaperone, Stress response	Cytoplasm, mitochondrion	Soluble at high salt concentration
7	Sgt2	**1.6±0.2**	Co-chaperone, Response to heat	Cytoplasm	Soluble in non-ionic detergent
8	Sti1	**1.6±0.3**	Co-chaperone, Stress response	Cytoplasm	Soluble at high salt concentration
9	Hsp104	**2.6±0.3**	Chaperone, Stress response	Cytoplasm, nucleus	Soluble at high salt concentration
10	Hsp26	**0.7±0.1**	Chaperone, Stress response	Cytoplasm, nucleus	Insoluble under native conditions
11	Hsp42	ND[f]	Chaperone, Stress response	Cytoplasm, cytoskeleton	Insoluble under native conditions
12	Tef2	1.0±NA[f]	Elongation factor, Translation	Cytoskeleton, Ribosome	Insoluble under denaturing conditions

[a]Accepted name from the *Saccharomyces* genome database (SGD).
[b]Fold change represents ratio between the relative protein amounts (%Vol) in whole cell lysates of the MeH expressing and control cells, respectively, ±SD (calculations are given in Table S1).
[c]Molecular function, biological process and localization are noted according to UniProtKB and SGD.
[d]Lysates were fractionated into 5 different fractions based on protein solubility under various conditions as described in Experimental Procedures. Fractions with the strongest overexpression of identified yeast proteins in MeH variants compared to control samples are indicated.
[f]ND – not determined; NA – not assayed.

As indicated in Table 1, most of the identified proteins, overexpressed in response to synthesis of MeH, are chaperones and co-chaperones involved in cellular stress responses. All of them, except the ER-resident chaperone Kar2p/BiP, are localized in the cytoplasm. Ssa1/2p, Ssa4p and Sse1p represent cytosolic Hsp70 family, Hsc82p is a member of Hsp90 family and Hsp104p belongs to Hsp110 family, respectively. Sti1p and Sgt2p are co-chaperones, which interact with identified Hsp70, Hsp90 and Hsp110 chaperones and thereby coordinate and regulate the activities of the large Hsps in chaperone complexes (Angeletti et al., 2002; Wegele et al., 2003). Proteomic analysis of *S. cerevisiae* cells expressing MeH revealed a specific stress response that, according to the list of induced proteins, is similar to recently reported cytosolic unfolded protein response (UPR-Cyto) (Metzger and Michaelis, 2009).

Fractionation of cell lysates revealed that Hsp70 chaperones Ssa1/2p and Ssa4p were similarly overexpressed in all soluble fractions, whereas other identified proteins

were found in separate fractions (Table 1), showing different degrees of interactions with MeH. Treatment with Triton X-100 fully solubilized only co-chaperone Sgt2p (not shown), indicating hydrophobic interaction with protein aggregates, whereas large Hsps were solubilized from MeH at high salt concentration (Fig. 5D), suggesting ionic interactions. Analysis of protein fractions insoluble under native conditions by 2D gel electrophoresis showed three major cellular components, exhibiting the strongest interaction with MeH: small heat shock proteins 26 and 42 (Hsp26 and Hsp42), and Hsp70 chaperones (Fig. 5F, spots 10, 11 and 1-3 accordingly). Small heat shock proteins (sHsps) also appeared to be involved in cellular stress response; however these proteins displayed different interactions MeH. The vast majority of overexpressed large Hsps were solubilized under native conditions, whereas the majority of sHsps could only be removed from insoluble viral protein aggregates under denaturating conditions. This demonstrates the formation of irreversible protein aggregates containing sHsps, which can not be recovered in vivo and under native conditions in vitro. Moreover, in contrast to large Hsps, expression of Hsp26 was not upregulated, whereas Hsp42 expression was too low to be detected in whole cell lysates on 2D gels. These results demonstrate a specific cellular stress with different action and regulation of sHsps versus large Hsps in response to synthesis of MeH. Moreover, analysis of yeast proteins unsoluble under denaturating conditions (8M urea), revealed that, virtually all yeast proteins were solubilized by this treatment in control samples, while the vast majority of viral protein remained insoluble (Fig. 6). SDS-PAGE analysis of the insoluble fractions revealed that major cellular protein associated associated with MeH is eukaryotic Translation Elongation Factor 1A (eEF1A or Tef1/2; Fig. 6 short arrow; Table 1, protein number 12). eEF1A was removed from recombinant protein aggregates only by including a reducing agent (at least 60-100 mM 2-mercaptoethanol) which also solubilized MeH. It suggests that eEF1A is involved in formation of complex with MeH multimers.

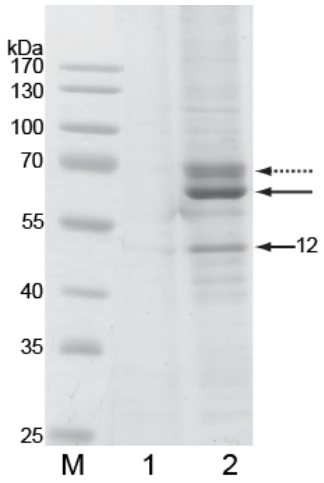

Fig. 6. Analysis of recombinant MeH protein fractions insoluble under denaturating conditions. Proteins of *S. cerevisiae* strain AH22 transformed with empty vector (1) and with pFGG3-MeH plasmid insoluble in 8M urea solution. Solid arrow indicates unglycosylated MeH polypeptides, dotted arrow – glycosylated MeH forms, short solid arrow – yeast eEF1A protein. M – molecular mass standards (Fermentas, Lithuania,

These results demonstrate that viral surface proteins in yeast cytoplasm form separate aggregates. The core of these aggregates consists of MeH disulfide-linked multimers involving eEF1A and is closely associated with small Hsps that can be removed only under denaturing conditions. Complexes of large Hsps seem to be bound to aggregate core peripherally as they can be easily removed at high salt concentrations.

Similar data, describing changes in yeast cell while expressing MeH glycoprotein, was obtained analyzing yeast *P. pastoris* using the same methods as described here (unpublished data, Čiplys, 2007, graduation dissertation). It is obvious that irreversible changes in yeast cells caused by synthesis of MeH are common to both yeast species.

Taken together, this data suggest that MeH synthesis in both yeast genera is inefficient mostly due to bottlenecks in translocation of viral protein precursors and maturation in the ER (Čiplys et al., 2011a).

3. Humanization of yeast *S. cerevisiae* cotranslational protein translocation apparatus

In eukaryotic cells SRP and Sec61 protein complexes interact and ensure cotranslational translocation of proteins through the ER membrane. In mammalian cells MeH is translocated to the ER using this translocation pathway, thus, differently from yeast cells, MeH polypeptide is not exposed in the cytoplasm. In yeast posttranslational translocation pathway is dominant, therefore humanization of first steps of secretory pathway in yeast seems necessary. Moreover, new data suggest that interaction of SRP and Sec61 complexes with translocated polypeptide facilitates its proper folding (Skach, 2007; Zimmermann et al., 2011).

Cotranslational protein translocation into proteoliposomes can be reconstituted from purified proteins of SRP, Sec61 complexes and TRAM1 (Gorlich and Rapoport, 1993). Accordingly, cDNA of all genes coding these human proteins were cloned in this work (Table 2).

Table 2. Human genes cloned for humanization of yeast cotranslational translocation pathway.

Gene	Accession no. in GenBank database	Human protein	Accession no. in UniProt database
SRP72	AF077019	SRP72	O76094
SRP68	AK074698	SRP68	Q9UHB9
SRP54	U51920	SRP54	P61011
SRP19	BC010947	SRP19	P09132
SRP14	X73459	SRP14	P37108
SRP9	U20998	SRP9	P49458
SRPR	X06272	SRα	P08240
SRPRB	AF141882	SRβ	Q9Y5M8
SEC61A1	AF077032	Sec61α1	P61619
SEC61B	CR456883	Sec61β	P60468
SEC61G	CR456979	Sec61γ	P60059
TRAM1	X63679	TRAM1	Q15629

First of all, human SEC61A1, SEC61B, SEC61G and TRAM1 genes were individually cloned into the yeast constitutive expression cassettes of *PGK1* gene and resulting expression cassettes were integrated in to the genomic IGS1 locus of ribosomal DNA (rDNA) array of yeast *S. cerevisiae* strain AH22, using specially constructed plasmid (Fig. 7). This plasmid allows selection of integrants using yeast *URA3* gene as a marker. *URA3* gene is flanked with repeated sequences, which makes this marker reusable using 5-fluororotic acid.

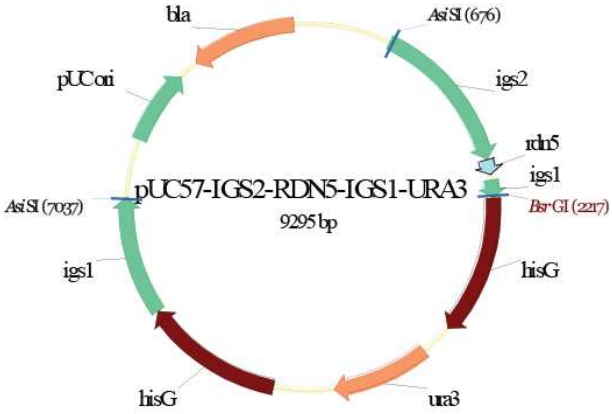

Fig. 7. Scheme of plasmid for integration of target gene into the IGS1 locus of *S. cerevisiae.* bla – gene conferring resistance of *E. coli* to ampicillin; igs1 ir igs2 – intergenci spacers of *S. cerevisiae* ribosomal DNA array; rdn5 – 5S ribosomal RNA gene; ura3 – *URA3* gene of *S. cerevisiae*; hisG – repeated sequences, designed for pop out of *URA3* gene during homologous recombination.

In the *S. cerevisiae*, the rDNA array comprises of between 150 and 200 tandem repeats, which each consists of four ribosomal RNA genes (26S, 18S, 5.8S and 5S) as well as two internal transcribed spacers (ITS1 and ITS2), two external transcribed spacers (ETS1 and ETS2), and a large intergenic spacer (IGS) (James et al., 2009). Thus, after transformation of yeast cells, subsequent selection of strain with best expression of integrated human genes is necessary, because different number of cassettes is integrated. *S. cerevisiae* strain with optimal number of integrated human SEC61A, SEC61B and SEC61G (Fig. 8 lane 5) was selected as it is shown in Fig. 8. Antibodies against human TRAM1 protein were commercially unavailable, therefore there is no direct evidence that this protein is produced in yeast and only assumptions can be made from indirect results.

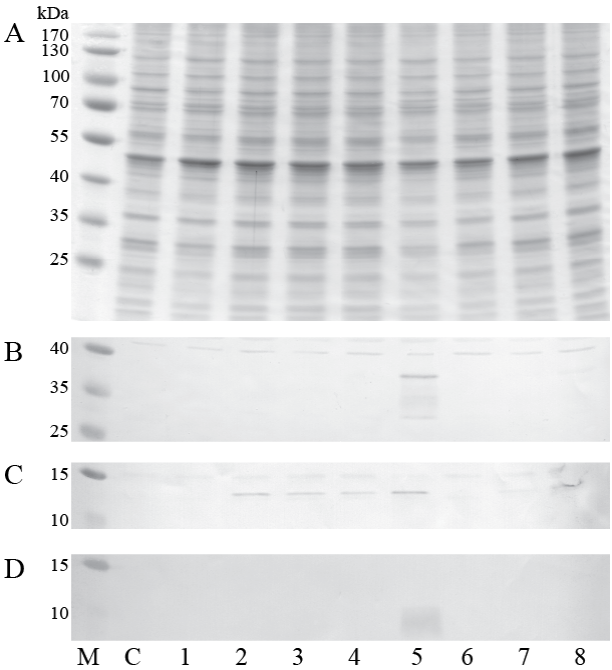

Fig. 8. Selection of humanized yeast *S. cerevisiae* strain, with optimal number of integrated human SEC61A1, SEC61B, SEC61G and TRAM1 genes. SDS-PAGE (A) and Western blot using specific antibodies against human Sec61α1 (B) Sec61β (C) and Sec61γ (D) proteins. 1-8 – samples of membrane protein fraction from different yeast strains. M – molecular mass standards (Fermentas, Lithuania, SM0671).

Selected *S. cerevisiae* strain AH22_IGS1-Sec61c-TRAM1 was further humanized by replacing yeast Sec61 complex protein coding genes *SSH1* and *SBH1* with human homologues SEC61B and SEC61G. Sss1p is the essential yeast protein, thus replacement with human Sec61γ protein with no effect on yeast physiology, shows that human Sec61γ protein fully substitutes functions of yeast Sss1p protein. On the other hand, yeast Sec61p protein, which is also essential protein of yeast Sec61 complex, is not functionally replaced by human homologue Sec61α1, resulting in no viable yeast strains in the same experiments. After these manipulation humanized yeast strain AH22_ SBH1::SEC61B_SSS1::SEC61G_IGS1-Sec61c-TRAM1 was generated. This and AH22_IGS1-Sec61c-TRAM1 strains were transformed with pFGG3-MeH plasmid and influence of human Sec61 complex proteins on MeH translocation in yeast cells determined (Fig. 9).

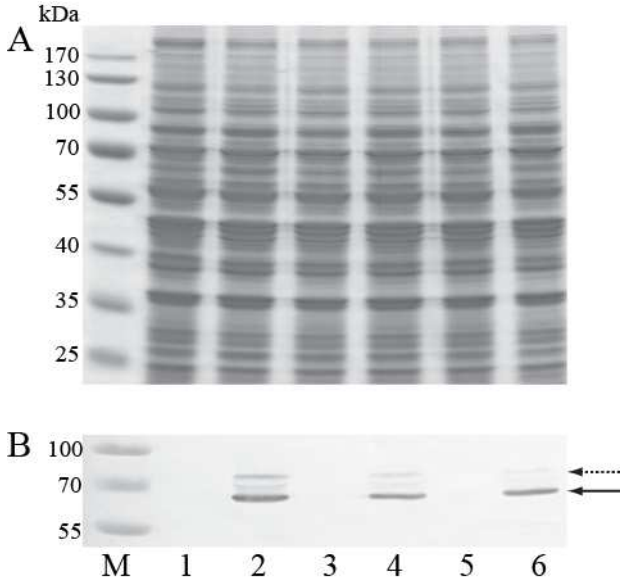

Fig. 9. Analysis of MeH protein produced in humanized yeast strains. SDS-PAGE (A) and Western blot using anti-tetraHis antibody (Qiagen, UK) of crude yeast lysates of yeast *S. cerevisiae* strains AH22 (lanes 1 and 2), AH22_IGS1-Sec61c-TRAM1 (3 and 4) and AH22_SBH1::SEC61B_ SSS1::SEC61G_IGS1-Sec61c-TRAM1 (5 and 6) tranformed with pFGG3 (1, 3 and 5) and pFGG3-MeH (2, 4 and 6) plasmids. Solid arrow indicates unglycosylated MeH polypeptides, dotted arrow – glycosylated MeH forms M – molecular mass standards (Fermentas, Lithuania, SM0671).

Translocation rate of MeH was determined using GE Healthcare ImageQuant TL software. Results show, that in AH22 strain about 20% (Fig. 9, lane 2), in AH22_IGS1-Sec61c-TRAM1 about 10% (Fig. 9, lane 4) and in AH22_SBH1::SEC61B_ SSS1::SEC61G_IGS1-Sec61c-TRAM1 (Fig. 9, lane 6) strain about 4% of total amount

of MeH were translocated into the ER. Interestingly, translocation of recombinant MeH protein in yeast cell decreases as Sec61 complex is more humanized. Moreover, humanization of yeast Sec61 complex had no effect on yeast physiology or homeostasis, also protein pattern in SDS-PAGE gel (Fig. 9A) seems to be unaffected. Thus it seems, that human Sec61 complex is fully functional in yeast cell and biogenesis of yeast secreted proteins is unaltered, but it is unable to translocate MeH precursors into the ER. It can be explained by the fact, that for proper cotranslational translocation correct interaction between SRP and Sec61 complexes is necessary (Helmers, 2003). As posttranslational, SRP independent, translocation pathway is dominant in yeast cells, improper interaction between yeast SRP and human Sec61 complex proteins might disorder translocation of MeH protein in yeast cells. Thus, in order, to reconstitute fluent mammalian like translocation nature of MeH in yeast cells, human SRP complex must be transferred into the yeast cells.

For this purpose, genes coding human SRP proteins were inserted into the yeast constitutive expression cassettes of *PGK1* gene and integrated into the genomic IGS1 locus of *S. cerevisiae* strains AH22, AH22_IGS1-Sec61c-TRAM1 and AH22_SBH1::SEC61B_SSS1::SEC61G_IGS1-Sec61c-TRAM1, as described above. Resulting humanized *S. cerevisiae* strains AH22_IGS1-SRPc, AH22_IGS1-Sec61c-TRAM1-SRPc and AH22_*SBH1*::SEC61B_*SSS1*::SEC61G_IGS1-Sec61c-TRAM1-SRPc, with optimal number of integrated human genes, were selected from pool of generated integrants by Western blot against human SRβ protein (Fig. 10). Further synthesis of all proteins in selected strains was confirmed by Western blot using specific antibodies against all other human SRP proteins (Fig. 11). (Western blot against human SRP54, even using two different antibodies, was very dim, not shown in Fig. 11).

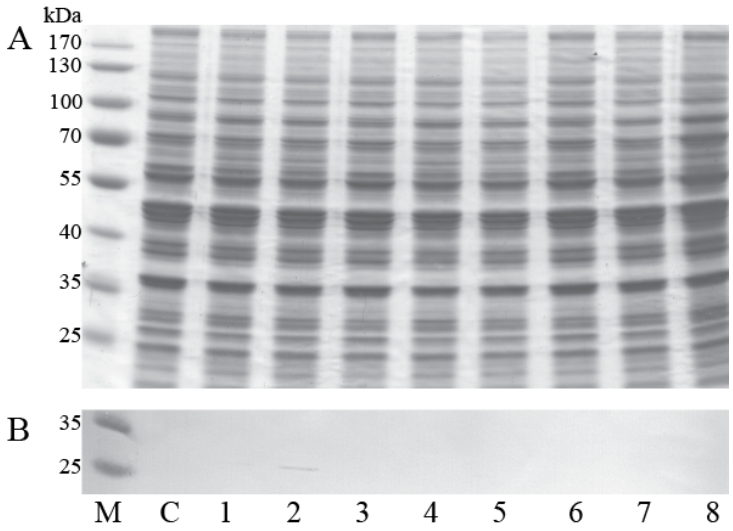

Fig. 9. Selection of humanized yeast *S. cerevisiae* AH22_IGS1-SRPc strain, with optimal number of integrated human SRP genes. SDS-PAGE (A) and Western blot using specific antibodies against human SRβ (B) protein. 1-8 – samples of crude yeast lystaes from different yeast strains. M – molecular mass standards (Fermentas, Lithuania, SM0671). Optimal *S. cerevisiae* strains AH22_IGS1-Sec61c-TRAM1-SRPc and AH22_*SBH1*::SEC61B_*SSS1*::SEC61G_IGS1-Sec61c-TRAM1-SRPc were selected accordingly.

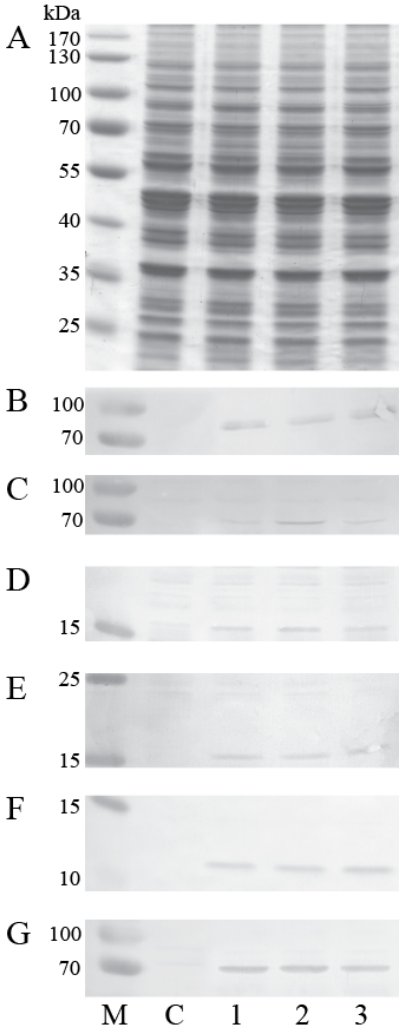

Fig. 11. Analysis of selected yeast strains with integrated human SRP genes. SDS-PAGE (A) and Western blot against human SRP72 (B), SRP68 (C), SRP19 (D), SRP14 (E), SRP9 (F) and SRα (G) proteins of crude yeast lysates of selected *S. cerevisiae* strains AH22_IGS1-SRPc (1), AH22_IGS1-Sec61c-TRAM1-SRPc (2) and AH22_*SBH1*::SEC61B_*SSS1*::SEC61G_IGS1-Sec61c-TRAM1-SRPc (3) C – samples from crude yeast lysate of AH22 strain. M – molecular mass standards (Fermentas, Lithuania, SM0671).

Further, plasmid for human 7SL RNA, main compound of SRP complex, synthesis in yeast was constructed. Human 7SL RNA gene was commercially synthesized with promoter and terminator of RNA polymerase of bacteriophage T7 on 5' and 3' end of the gene accordingly. T7 RNA polymerase gene (T7RNApol) was cloned into vector pFDC (Fig. 13) under constitutive promoter of yeast *PGK1* gene, subsequently MeH coding gene was cloned under control of galactose inducible promoter of yeast *GAL7* gene and 7SL RNA gene cloned just after terminator of *PGK1* gene. Resulting plasmid pFDC-TVH-T7RNRpol-7SL was used to transform humanized *S. cerevisiae* strains AH22_IGS1-SRPc, AH22_IGS1-Sec61c-TRAM1-SRPc and AH22_ *SBH1*::SEC61B_*SSS1*::SEC61G_IGS1-Sec61c-TRAM1-SRPc. Synthesis of T7 RNA polymerase was confirmed by SDS-PAGE of crude yeast lysates, while synthesis of human 7SL RNA was confirmed by reverse transcriptase-PCR analysis (not shown). After confirming that all components of human SRP complex are produced in yeast cell, synthesis of MeH was induced and effect of human SRP complex on MeH translocation into the ER in yeast cell determined (Fig. 12).

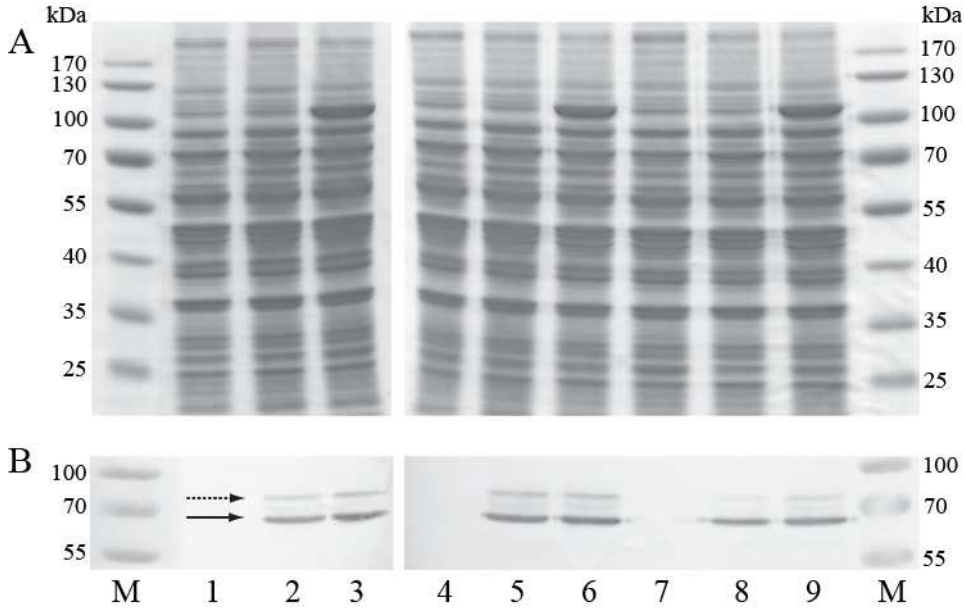

Fig. 12. Analysis of recombinant MeH, synthesized in humanized yeast strains. SDS-PAGE (A) and Western blot using anti-tetraHis antibody (Qiagen, UK) of crude yeast lysates of yeast *S. cerevisiae* strains AH22_IGS1-SRPc (lanes 1, 2 and 3), AH22_IGS1-Sec61c-TRAM1-SRPc (4, 5 and 6) ir AH22_*SBH1*::SEC61B_*SSS1*::SEC61G_IGS1-Sec61c-TRAM1-SRPc (7, 8 and 9) transformed with pFDC (1, 4 and 7), pFDC-MeH (2, 5 and 8) and pFDC-TVH-T7RNRPol-7SL (3, 6 and 9) plasmids. Solid arrow indicates unglycosylated MeH polypeptides, dotted arrow – glycosylated MeH forms. M – molecular mass standards (Fermentas, Lithuania, SM0671).

Interestingly, results show, that full human SRP complex can partially regenerate translocation of MeH into the ER in the *S. cerevisiae* strain AH22_*SBH1*::SEC61B_*SSS1*::SEC61G_IGS1-Sec61c-TRAM1-SRPc, where, as in the strain AH22_*SBH1*::SEC61B_*SSS1*::SEC61G_IGS1-Sec61c-TRAM1, translocation of MeH is impaired due to humanization of yeast Sec61 complex (Fig. 12, lanes 8 and 9; Fig. 9, lane 6). Analysis with GE Healthcare ImageQuant TL software shows that translocation rate of MeH from 4% of total amount of MeH in the *S. cerevisiae* strain AH22_*SBH1*::SEC61B_*SSS1*::SEC61G_IGS1-Sec61c-TRAM1-SRPc (Fig. 12, lane 8) increases to 12% of total MeH amount with human 7SL RNA present (Fig. 12, lane 9). Such results evidence that human SRP complex is functional in yeast cell and recognize MeH precursors. Though, when functional yeast SRP and Sec61 complexes are present, human SRP and Sec61 complexes do not increase translocation rate of recombinant MeH glycoprotein (Fig. 12, lanes 2, 3, 5 and 6). Therefore, expression of human Sec61, SRP, TRAM1 proteins and 7SL RNA must be optimized and humanization of yeast SRP complex carried out. Also additional components of human cotranslational translocation pathway (such as TRAP complex) might be necessary.

4. Coexpression human ER chaperones with MeH in yeast *S. cerevisiae*

As mentioned above, about 20% of MeH protein is translocated into the ER, but still is aggregated and have no biological activity. Lack of necessary chaperones might be bottleneck for production of active MeH glycoprotein in yeast. In this study five main human chaperones were cloned (Table 3) and their influence on maturation of MeH in yeast cells assessed (Čiplys et al., 2011b).

Table 3. Human ER chaperones cloned in this study.

Gene	Accession no. in GenBank database	Human protein	Accession no. in UniProt database
HSPA5	AF216292	BiP/GRP78	P11021
CANX	L18887	Calnexin	P27824
CALR	M84739	Calreticulin	P27797
P4HB	X05130	PDI	P07237
PDIA3	U42068	ERp57	P30101

A set of expression vectors, pFDC-MeH-BiP, pFDC-MeH-CNX, pFDC-MeH-CRT, pFDC-MeH-ER57 and pFDC-MeH-PDI, containing expression cassettes for MeH under the control of the galactose inducible GAL7 promoter, and selected human chaperone genes HSPA5, CANX, CALR, PDIA3 and PH4B under the control of the constitutive promoter PGK1, were constructed on the basis of vectors pFDC (Fig. 13). Strong constitutive yeast PGK1 promoter was used for the expression of human chaperone genes in yeast in order to make an environment constantly enriched with individual human chaperones during the induction of MeH synthesis.

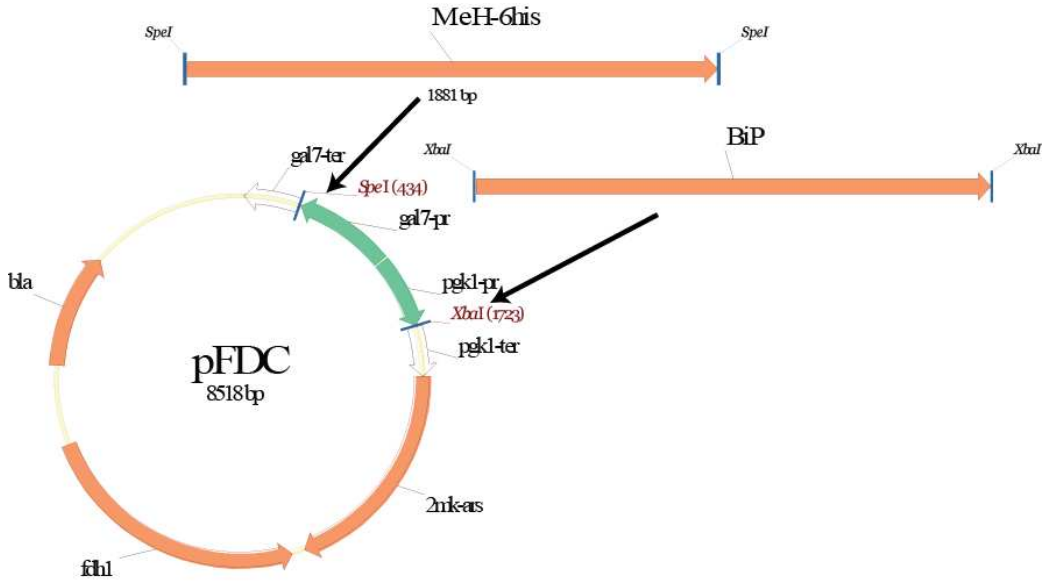

Fig. 13. Construction scheme of *S. cerevisiae* vector for coexpression MeH and human BiP chaperone. 2 mk-as – 1.74 kb fragment of yeast 2 μm plasmid; fdh1 – *FDH1* gene of *Candida maltosa*, conferring resistance to formaldehyde; gal7-ter – *S. cerevisiae GAL7* gene transcription terminator; gal7-pr – *S. cerevisiae GAL7* gene promoter; pgk1-pr – *S. cerevisiae PGK1* gene promoter pgk1-ter – *S. cerevisiae PGK1* gene transcription terminator; bla – gene conferring resistance of *E. coli* to ampicillin. All other human chaperones were cloned accordingly.

Specific antibodies against each human chaperone were used for Western blotting. The expression of selected human chaperone genes in yeast is presented in Fig. 14. All five analyzed chaperones formed specific bands in Western blots, which correspond to the predicted molecular masses of the analyzed proteins (Fig. 14, lanes T and S). It is worth noting that all five proteins were totally soluble in nonionic detergent and were found in the supernatant fraction (Fig. 14, lanes S).

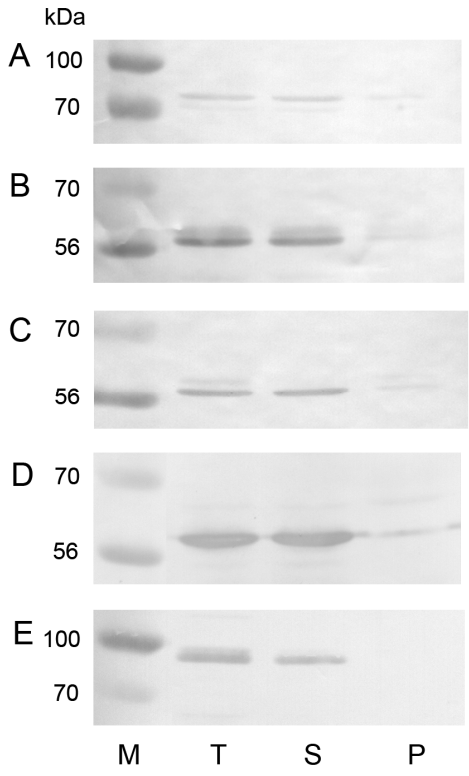

Fig. 14. Analysis of recombinant human chaperones. Synthesis of human chaperones BiP (a), calreticulin (b), ERp57 (c), PDI (d) and calnexin (e) in yeast *S. cerevisiae*. Western blots with corresponding monoclonal or polyclonal antibodies are shown. Lysates were obtained from yeast transformants containing recombinant plasmids pFDC-MeH-BiP (a), pFDC-MeH-CRT (b), pFDC-MeH-ER57 (c), pFDC-MeH-PDI (d) and pFDC-MeH-CNX (e). Lane T, pellets of total membrane fraction, obtained after centrifugation of yeast lysates (Fraction 3); lane S, extract of the pellets with disruption buffer containing Triton X-100, represent proteins soluble in the solution with 1% Triton X-100 (Fraction 4); lane P, pellets obtained after extraction with 1% Triton X-100, represent proteins insoluble in 1% Triton X-100 (Fraction 5).

Further, synthesis of MeH was induced and influence of human chaperons on properties of recombinant protein determined. First of all, no significant changes in the expression level and in translocation rate of MeH in comparison with corresponding controls without chaperones were observed (not shown). After that, influence of human ER chaperones on the solubility of MeH was assessed. As mentioned above, the MeH glycoprotein isolated from native measles viruses is soluble in solutions containing 1% Triton X-100, while the same protein synthesized in yeast forms insoluble multimeric aggregates. Analysis showed, that coexpression of the human calnexin gene resulted in an increased solubility of the glycosylated form of MeH (Fig. 15).

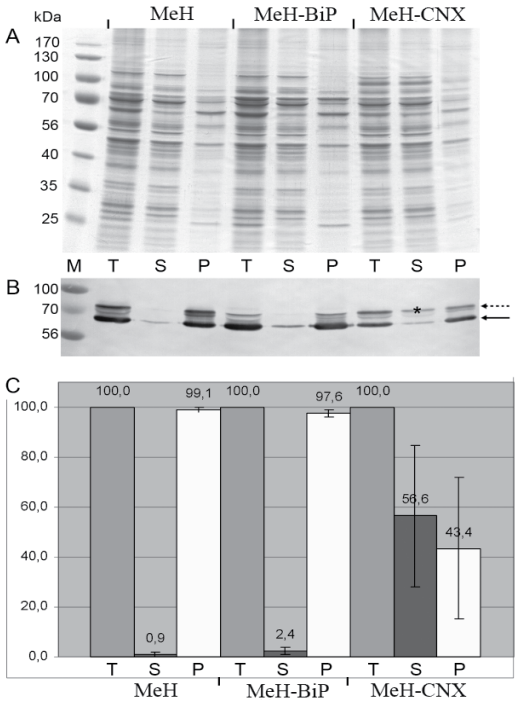

Fig. 14. Effect of coexpression of human chaperones on the solubility of recombinant MeH protein. (a) SDS-PAGE of membrane protein fractions. (b) Western blot of the same samples using monoclonal anti-tetraHis antibody (Qiagen, UK). (c) Quantitative values for solubility of glycosylated MeH protein form. M – molecular mass standards (Fermentas, Lithuania, SM0671). MeH indicate samples obtained from pFDC-MeH transformants expressing MeH alone, MeH-BiP indicate fractions from yeast cells transformed with pFDC-MeH-BiP coexpressing MeH and human BiP, whereas MeH-CNX indicate samples obtained from pFDC-MeH-CNX transformant coexpressing MeH with human calnexin. T – total membrane protein fraction (Fraction 3); S – soluble membrane protein fraction extracted with nonionic detergent (Fraction 4); P – pellets containing proteins, insoluble in Triton X-100 (Fraction 5). Solid arrow in (b) indicates unglycosylated MeH protein precursors, whereas dashed arrow point to glycosylated MeH form. Additional band of soluble glycosylated MeH protein, detected only in pFDC-MeH-CNX transformant, is indicated by asterisk. Values in (c) represent the percentage of glycosylated MeH protein amounts (unglycosylated MeH protein form was not calculated) in membrane fractions (total membrane protein fraction T is taken as 100% and further divides into soluble S and pellet P fractions, according to MeH solubility in Triton X-100). Quantitative analysis was performed by Western blots using the same dilution and loading the same volume of Fractions 3, 4 and 5 on each lane in the same gel. Values above the columns are the average calculated from at least three independent experiments and the bars represent SD.

Quantitative analysis, using GE Healthcare ImageQuant TL software, of solubility of glycosylated MeH form showed that approximately half of the product (56.6±28.3%; average and SD are calculated from five independent experiments) is soluble in nonionic detergent (Fig. 14C, MeH-CNX, column S). We noticed that the amount of soluble MeH glycoprotein can be increased up to 90% using higher dilution of Fraction 4 with buffer containing Triton X-100. Coexpression of other human chaperones encoded by human HSPA5, CALR, PDIA3 and PH4B genes failed to change the solubility of MeH in

comparison with corresponding controls. Coexpression of human HSPA5 gene, encoding GRP78/BiP protein, is shown in Fig. 14 (MeH-BiP) and accounted for 2.4±2.1% of soluble MeH glycoprotein, i.e. the difference from the expression of MeH alone was not statistically significant. Coexpression of other human chaperones revealed similar outcome as in the case of human BiP (data not shown).

The results obtained using human calnexin raised the question concerning the impact of yeast calnexin on the solubility of MeH. The yeast S. cerevisiae *CNE1* gene, encoding calnexin, was overexpressed together with MeH in the same manner as human chaperones. For the confirmation of Cne1p overexpression, lysates of recombinant yeast with *CNE1* gene in a high-copy-number plasmid and lysates of yeast without *CNE1* gene in a high-copy state were compared. A more intense band corresponding to the molecular mass of yeast calnexin was visible after SDS-PAGE of lysates obtained from yeast with overexpressed *CNE1*. The identity of this band was confirmed by two-dimensional electrophoresis and MS analysis (not shown). Still, overexpression of yeast *CNE1* in the same vector system as the human calnexin gene has no significant effect on the solubility of MeH (not shown).

Reasons for inability of recombinant human BiP, calreticulin, ERp57 and PDI chaperones to influence maturation of MeH in yeast cells were also assessed. Sequence and structure analysis of human chaperones, used in this study, revealed, that differently from calnexin, which is membrane bound protein, all other human chaperones are retained in the ER lumen in human cells thanks to KDEL amino acid sequence in the C terminus of protein, which is recognized by KDELR protein and serves as retention signal. In yeast cells such retention signal is comprised from HDEL amino acids and is recognized by Erd2p protein. Ability of yeast Erd2p protein to recognize and retain in the ER proteins containing KDEL motive of amino acids is unknown. Moreover, Erd2p and HDEL retention system is known to be leaky in yeast (Dean and Pelham, 1990). Thus, culture medium after incubation of yeast cells transformed with pFDC-TVH-BiP, pFDC-TVH-CNX, pFDC-TVH-CRT, pFDC-TVH-PDI ir pFDC-TVH-ERp57 was analyzed by SDS-PAGE (Fig. 15).

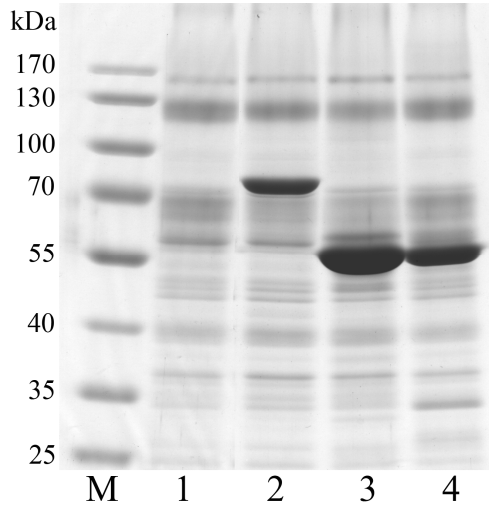

Fig. 15. Analysis of culture media of yeast cells producing human chaperones. SDS-PAGE of 50 times concentrated culture media of yeast *S. cerevisiae* AH22 strain transformed with pFDC (1), pFDC-TVH-BiP (2), pFDC-TVH-CRT (3) ir pFDC-TVH-ERp57 (4) plasmids. M – molecular mass standards (Fermentas, Lithuania, SM0671).

Results show, that recombinant human BiP, calreticulin and ERp57 chaperones are not retained in the ER, but instead are secreted in to the culture medium, as calnexin, as expected, and PDI are not. To solve this problem C terminus of human HSPA5, CALR and PH4B genes were fused with coding sequences of transmembrane domains of yeast Cne1p and Ost1p proteins. Also, to retain flexibility, stem comprising of repeated serine and glycine amino acids was inserted between human chaperone and transmembrane domain. Such chimeric human chaperones were retained in the yeast ER, but as in their native sequence had no effect on MeH maturation (not shown).

5. Construction of yeast *S. cerevisiae* strains with integrated genes of human chaperones

As described earlier, human calnexin was the only human chaperone that had positive effect on maturation on MeH protein in yeast cell. Thus, firstly, yeast *PGK1* gene expression cassette with human calnexin coding gene was inserted in to the yeast *S. cerevisiae* genome replacing yeast *URA3* gene. One, two and three copies of CANX yeast expression cassettes were integrated. Three copies of human CANX gene, integrated in the genome and regulated by yeast *PGK1* gene promoter, were almost enough to produce the same amount of calnexin protein as using multi-copy plasmid pFDC-MeH-CNX (not shown). Resulting AH22-CNX strain, carrying three copies of human calnexin gene, was used for following experiments.

Further, genes, coding native and chimeric sequences of human BiP, calreticulin, ERp57 and PDI chaperones were cloned into the yeast expression cassettes of *PGK1* or

TEF2 genes and integrated into the genomic IGS of yeast *S. cerevisiae* strain AH22-CNX, and selection of optimal strains was carried out, as described above (not shown). Native and chimeric sequences of human HSPA5, CALR, PDIA3 and PH4B genes were integrated individually and in different combinations, resulting in every possible combination of five human chaperones. All these strains were transformed with pFGG3-MeH plasmid and analysis of influence of different combinations of human chaperones on MeH maturation in yeast cell was made. Shortly, maturation of MeH in the yeast ER was not improved further by any combination of human BiP, calreticulin, ERp57 or PDI with human clanexin chaperone (not shown).

Discussion

Overexpression of MeH in *S. cerevisiae*, as well as in *P. pastoris*, results in the accumulation of unglycosylated protein precursors within the yeast cytoplasm. Unglycosylated MeH molecules are not detected in the preparations of this protein after synthesis in mammalian expression system (Kidokoro et al., 2002), however the expression of the MeH protein in insect cells by baculovirus system resulted in a large amount (approximately half of the total product corresponding to 65 kDa species) of unglycosylated MeH protein molecules (Vialard et al., 1990). The recombinant MeH protein, produced in transgenic carrot plants also had lower molecular weight than the viral protein, suggesting a different glycosylation pattern, but this was not further explored (Marquet-Blouin et al., 2003). It appears that maturation of MeH protein is increasingly impaired switching from mammalian cell culture to less complex expression systems, and in lower eukaryotes (e.g. yeast) we eventually observe a majority of MeH molecules in immature form of non-glycosylated precursors. However, we found that minor amounts of MeH protein are yet glycosylated in yeast (Figure 2D). It indicates that small amounts of viral protein precursors are successfully translocated into ER lumen and glycosylated, but this process is rather ineffective in yeast compared to higher eukaryotes.

Aggregation of unglycosylated viral protein precursors with cytoplasmic yeast proteins demonstrates that the vast majority of recombinant product is localized in the cytoplasm of yeast cells. Proteomic analysis of *S. cerevisiae* cells expressing MeH revealed a specific stress response that, according to the list of induced proteins, is similar to recently reported cytosolic unfolded protein response (UPR-Cyto) (Geiler-Samerotte et al., 2011). A key feature of this response is the formation of extremely large aggregates involving macromolecular structures of eEF1A. The eukaryotic translation elongation factor 1A (eEF1A, encoded by two identical yeast genes TEF1 and TEF2, also known as Tef2p) is localized in cell cytoplasm and has several important functions including delivery of aminoacyl-tRNA to the elongating ribosome (Grosshans et al., 2000), cytoskeleton organization via actin filament-binding and -bundling activities (Gross and Kinzy, 2005), quality surveillance of newly synthesized proteins (Hotokezaka et al., 2002) and ubiquitin-dependent degradation (Chuang et al., 2005). It

was shown that mammalian homolog of yeast eEF1A protein has chaperone-like activity and, unlike other ribosome-associated factors, interacts with unfolded polypeptide chains after their release from the ribosome (Gross and Kinzy, 2005). This data as well as reports that eEF1A promotes degradation of cotranslationally damaged proteins imply that eEF1A play a role in quality surveillance of newly synthesized proteins. Results of this study suggest that eEF1A may participate in both protein quality control and cellular stress response in yeast. The function of eEF1A in stress response in yeast cells has not been described before. Interestingly, eEF1A appears to be involved in heat shock response in mammalian cells where both heat shock RNA-1 (HSR1) and eEF1A are required for activation of the heat-shock transcription factor 1 (HSF1) (Shamovsky et al., 2006). It seems likely that eEF1A interactions with MeH precursors may result in activation of stress response in yeast utilizing a similar mechanism as in mammalian cells.

Native viral surface protein precursors are transported through ER membrane cotranslationally and depend on SRP, whereas significant posttranslational insertion into ER membrane does not occur (Wilson et al., 1987). The use of either SRP-dependent cotranslational or SRP-independent posttranslational translocation pathway in yeast is determined by signal sequence hydrophobicity (Ng et al., 1996). Hydrophobicity plots show that signal sequences of SRP-independent substrates have peaks that do not exceed +2.0 U (as defined by Kyte and Doolittle, 1982), whereas those that use SRP have peaks approaching +3.0 U. As viral surface proteins possess highly hydrophobic signal sequences, e.g. hydrophobicity of MeH TM domains peaks over 3.0U, they should use SRP-dependent translocation pathway in yeast cells. It is consistent with the results showing that the replacement of MeH native TM domain by *S. cerevisiae* α-factor signal sequence greatly enhances the amount of translocated and glycosylated viral protein (Fig. 4). It is well established that prepro-α-factor is translocated posttranslationally by SRP-independent route (Ng et al., 1996), thus increased translocation rate may be simply explained by higher protein load in *S. cerevisiae* posttranslational versus SRP-dependent translocation pathway. Taken together, results suggest that MeH synthesis is inefficient mostly due to bottleneck in translocation of viral protein precursors and induces specific cytoplasmic stress leading to accumulation of insoluble protein aggregates in yeast cells.

Also results of this study suggest that, maturation of MeH is impaired due to the lack of folding assistants in the ER of yeast cell. Human RNA viruses have relatively small genomes, during evolution they created multifunctional proteins consisting of domains with individual activities. Maturation of such complex proteins in the human cell requires all repertoire of ER chaperones and folding enzymes (Braakman and van Anken, 2000). Variety of human ER folding assistant is far greater than in yeast cell. For comparison, human PDI family consists of 19 proteins (Appenzeller-Herzog and Ellgaard, 2008), whereas in yeast there are only 5 such proteins (Norgaard et al., 2001). Moreover, the human proteome set contains 5.8 times as many protein architectures as yeast. This difference is most prominent in the recent evolution of novel extracellular and transmembrane architectures in the human lineage (Lander et al., 2001). All these facts show, that yeast protein secretion pathway is primitive comparing with human cells and is not equipped to deal with complex virus glycoproteins. It is consistent with the results showing that the replacement of MeH native TM domain by *S. cerevisiae* α-factor signal sequence greatly enhances the amount of translocated and glycosylated viral protein, but still no biologically active protein is produced.

These problems regarding improper synthesis and maturation of MeH in yeast cells were attempted to solve by introducing human genes coding proteins of human cell protein secretion pathway. As mentioned above efficient mammalian like cotranslational translocation pathway for MeH using human SRP, Sec61 complexes and TRAM1 protein was not established in the yeast cell during this study. Data show, that human Sec61 and SRP complexes are functional and specifically recognize MeH in yeast cell. Thus, it seems that yeast *S. cerevisiae* is suitable organism for generation of mammalian like cotranslational translocation pathway. Optimization of expression of human genes and transfer of additional human accessories of secretion pathway should help achieve this goal. Further experiments are performed.

Coexpression of MeH protein in the yeast cells constantly overexpressing human calnexin enhanced the quantity of the soluble glycosylated form of MeH significantly. Almost all glycosylated MeH is solubilized by human calnexin. Such impact on the solubility of the MeH glycosylated form was characteristic only for human calnexin. Coexpression of other ER chaperones, such as BiP, calreticulin, ERp57 or PDI did not change the solubility of MeH. In mammalian cells, calnexin and calreticulin form a part

of the quality-control system for glycoproteins in the ER. They bind to terminal glucose residues on N-linked oligosaccharides and retain misfolded glycoproteins in the ER (Williams, 2006). However, there is increasing evidence that calnexin can perform chaperone functions by associating directly with the polypeptide backbone, in addition to binding to carbohydrate moieties. In addition, calnexin possesses binding sites for ATP, Ca^{2+}, non-native polypeptides and ERp57. Thus, it was demonstrated that mammalian calnexin can associate with a protein substrate that completely lacks N-linked oligosaccharides *in vivo* (Leach and Williams, 2004). Measles virus, evolving in human cells, successfully adopted the calnexin system for the maturation of virus glycoproteins (Pieren et al., 2005). The impact of human calnexin on the solubility of MeH was characteristic only for human calnexin. Overexpression of yeast *CNE1* gene, encoding the functional and the structural analog of human calnexin (24% amino acid identity), had no impact on the solubility of MeH. It suggests that human calnexin and yeast *S. cerevisiae* ortholog proteins are not functionally equivalent where glycoproteins of mammalian virus are concerned.

Measles virus hemaglutinin during maturation in human cells interact with calreticulin, BiP and calnexin (Bolt, 2001). Thus it is interesting why human BiP and calreticulin as also PDI and ERp57 had no effect on properties of MeH. As it is shown in figure 15 human chaperones of native sequence are secreted out of the yeast cell. Immobilization of human proteins using yeast transmembrane domains also gave no positive result. Recent model of retention of proteins in the ER, suggest that ER resident proteins interact with KDELR (Erd2p in yeast) in Golgi apparatus and only then, together with KDLER protein are transferred back to the ER (Capitani and Sallese, 2009). It seems that chaperones function in very dynamic environment. Thus, inadequate retention signal or immobilization can make them ineffective. Different approach must be taken to solve this problem.

Taken together all the data of presented in this study, allows to state that humanization of yeast protein secretory pathway adapting for production of complex virus proteins seems possible. Yeast *S. cerevisiae* is perfect host for such manipulations and large amount of data gathered during this study enables precise projection of further necessary experiments. Thus, results, presented here could be considered the first

successful step in the construction of yeast specialized for the production of virus glycoproteins.

Conclusions

1. Wild yeast *S. cerevisiae* and *P. pastoris* strains are not suitable for synthesis of measles virus hemagglutinin (MeH). This glycoprotein aggregates and remains insoluble and biologically inactive in yeast cells.
2. Synthesis of MeH causes cytoplasmic unfolded protein stress in yeast cells. Response to this stress involves overexpression of large Hsp proteins and formation of irreversible aggregates in yeast cell cytoplasm comprised from MeH precursors, eEF1A, small and large Hsp proteins.
3. Synthesis of MeH in yeast cell is inefficient due to bottlenecks in translocation of viral protein precursors and maturation in the ER.
4. Fluent mammalian like translocation nature of MeH in yeast cells was not reconstituted in this study following transfer of human SRP, Sec61 and TRAM1 proteins. Results suggest that human Sec61 and SRP complexes are functional in yeast cell.
5. Human calnexin rescues glycosylated MeH from insolubility in yeast cells. Soluble MeH is not biologically active.
6. Human BiP, calreticulin, ERp57 and PDI chaperones have no influence on maturation of MeH in yeast cells. Possible identified reason – incorrect cellular localization of these human proteins.
7. Effective system for synthesis and purification of human BiP, calreticulin and ERp57 proteins was established in *S. cerevisiae.*

Publications

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Čiplys E, Sasnauskas K, Slibinskas R. Overexpression of human calnexin in yeast improves measles surface glycoprotein solubility. FEMS Yeast Res. 2011 Sep;11(6):514-23.

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Slibinskas R, Sasnauskas K, **Čiplys E**. Coexpression of human calnexin rescues measles virus hemagglutinin glycoprotein from insolubility in the yeast expression system. 9th International Calreticulin Workshop. 29-31 August 2011, Statens Serum Institut (SSI), Copenhagen, Denmark.

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TYMŲ VIRUSO HEMAGLIUTININO BRENDIMO PROCESŲ MIELIŲ S. CEREVISIAE IR P. PASTORIS LĄSTELIŲ SEKRECINIAME KELYJE TYRIMAS IR MIELIŲ HUMANIZAVIMAS

REZIUMĖ

Vienas pagrindinių biomedicininės paskirties baltymų gamybos iššūkių yra pigių ir saugių raiškos sistemų, tinkamų glikobaltymų sintezei, paieška bei esamų sistemų tobulinimas. Vaistai, sukurti baltymų pagrindu, sudaro apie ketvirtadalį naujai patvirtinamų vaistų rinkos, o apie 70% jų sudaryti iš glikobaltymų (Sethuraman ir Stadheim, 2006). Dabar glikobaltymų sintezei naudojamos žinduolių ląstelių kultūros turi keletą trūkumų. Jose gaunamų rekombinantinių baltymų kaina yra didelė, ribotas tūrinis našumas, ląstelės lėtai dauginasi ir auga, būna užkrėstos retrovirusais, gaunamas heterogeniškas produktas ir užima daug laiko sukurti stabilią ląstelių liniją (Choi ir kt., 2003; Sethuraman ir Stadheim, 2006). Itin intensyviai vykstantis tinkamų raiškos sistemų kūrimas kol kas nedavė norimų rezultatų. Mielių, kaip ir augalų bei vabzdžių, raiškos sistemos, dėl keleto priežasčių yra įvardijamos kaip vienos pagrindinių kandidatų užimti šią vietą. Visų pirma, mielės yra pripažintos kaip saugus organzimas, jų genetika, biochemija ir fiziologija yra gerai ištirta.

Dauguma medicinai reikšmingų rekombinantinių glikobaltymų turi įgyti natūralią formą, kad būtų veiklūs ir galėtų atlikti savo funkcijas. Mielėse sekretuojami rekombinantiniai glikobaltymai susiduria su jų sintezę stabdančiomis ar ribojančiomis problemomis. Norint pašalinti rekombinantinių glikoproteinų sintezę mielių ląstelių sekrecinio kelio etapuose ribojančius veiksnius, reikia modifikuoti mielių baltymų sintezės ir po-transliacinių modifikacijų kelius pritaikant žmogaus glikobaltymų sintezei.

Pirmoji šio darbo dalis skirta apibūdinti žmogaus tymų viruso glikobaltymo brendimo procesus mielių sekreciniame kelyje, nustatant neefektyvios šių baltymų raiškos mielėse priežastis. Darbo eigoje pirmą kartą buvo aprašytos tymų viruso hemagliutinino (TVH) sintezės galimybės mielėse *Saccharomyces cerevisiae* ir *Pichia pastoris* ir parodyta, kuriuose mielių baltymų sekrecinio kelio etapuose yra sutrikęs rekombinantinio baltymo brendimas (Čiplys ir kt., 2011a). Klasikinės mielių rūšys ir

standartiniai rekombinantinių baltymų raiškos ir gryninimo protokolai nėra tinkami diagnostikai ir vakcinų kūrimui reikalingo TVH baltymo gavimui.

Proteominė *S. cerevisiae* ląstelių, sintetinančių TVH baltymą, analizė leido nustatyti specifinį mielių atsaką į stresą, kurį sukelia TVH baltymo agregatai. Nustačius mielių baltymus, kurių raiška ląstelėje padidėja dėl TVH baltymo sintezės, parodyta, TVH sintezė mielėse sukelia neseniai literatūroje aprašytą citoplazminį nesusivyniojusių baltymų atsaką (UPR-cyto) (Metzger ir Michaelis, 2009). Pagrindinis šiame darbe aprašyto atsako į stresą požymis yra ypatingai didelių baltymų agregatų, kurių šerdį sudaro TVH ir mielių eEF1A baltymai, susidarymas.

Rezultatai leidžia aiškiai teigti, kad TVH sintezė mielėse yra sutrikusi ankstyvosiose baltymo brendimo stadijose. Mielių ko-transliacinio baltymų perkėlimo į ET ir ET baltymų sulankstymo mechanizmai nėra pritaikyti sudėtingų virusinių baltymų brendimui.

Antrojoje šio darbo dalyje aprašyti bandymai išspręsti mielių sekrecinio kelio trūkumus į mielių ląsteles perkeliant žmogaus sekrecinio kelio komponentus. Pirmiesiems mielių sekrecinio kelio humanizavimo etapams buvo pasirinkti žmogaus baltymai atsižvelgiant į nustatytas neefektyvaus TVH baltymo brendimo mielių ląstelėse priežastis. Žmogaus tipo ko-transliacinį baltymų pernešimą į ET mielių ląstelėse bandyta atkurti perkeliant žmogaus SRP, Sec61 kompleksų ir TRAM1 baltymus, o siekiant sukurti tinkamas TVH baltymo brendimui sąlygas, mielių ląstelių ET buvo sintetinami pagrindiniai žmogaus ląstelių ET šaperonai – BiP, kalretikulinas, kalneksinas, PDI ir ERp57.

Darbo metu mielių ląstelėse nepavyko sukurti efektyvaus žinduolių tipo ko-transliacinio tipo visą sintetinamą TVH baltymą į ET perkeliančio mechanizmo. Žmogaus SRP ir Sec61 bei TRAM1 baltymų kompleksas į ET perkelia mažiau TVH baltymo nei mielių homologai. Vis tik, rezultatai rodo, kad žmogaus baltymai mielių ląstelėje yra lokalizuoti tinkamuose kompartmentuose ir panašu, kad formuoja veiklius kompleksus, o mielių ląstelė yra tinkama perkelti visus žmogaus sekrecinio kelio komponentus. Taip pat eksperimento eigoje surinkta daugybė naudingos informacijos padėsiančios optimizuoti turimus humanizuotus mielių kamienus, todėl mielių sekrecinio kelio pirmųjų etapų humanizavimas, pritaikant virusinių glikobaltymų sintezei, atrodo yra įmanomas.

Vykdant žmogaus šaperonų ir TVH baltymo sintezės mielių ląstelėse eksperimentus, nustatyta, kad vienintelis žmogaus kalneksino baltymas turėjo teigiamos įtakos TVH baltymo brendimui. Optimizavus sąlygas, virš 90% glikozilinto TVH baltymo, sintetinamo kartu su žmogaus kalneksinu, buvo apsaugota nuo agregacijos mielių ET. Kiti žmogaus ET šaperonai, sintetinami tiek natyvios sekos, tiek įmobilizuoti ET membranoje sujungiant su mielių baltymų transmembraniniais domenais bei visose įmanomose kombinacijose, jokios įtakos TVH baltymui neturėjo. Darbo metu nustatyta, kad galima to priežastis – nevykstantis šių žmogaus šaperonų išlaikymas mielių ET ertmėje.

Mielių kalneksinas, net ir stipriai padidinus jo raišką, TVH baltymo brendimui įtakos neturėjo. Tai parodo skirtumus, atsiradusius evoliucijos eigoje tarp žmogaus ir mielių ląstelių sekrecinio kelio komponentų. Vis tik, nors žmogaus kalneksinas ir apsaugojo beveik visą glikozilintą TVH baltymą nuo agregacijos ET, nepadarė jo biologiškai aktyviu.

Viską apibendrinant, šio darbo metu buvo nustatytos neefektyvios TVH sintezės mielėse priežastys bei atlikti pirmieji žingsniai jas sprendžiant. Nors šiame darbe nepavyko sukurti mielių kamienų, sintetinančių aktyvų TVH baltymą, mielių sekrecinio kelio humanizavimas, pritaikant virusinių baltymų sintezei, atrodo įmanomas. Darbo metu sukonstruota naujų vektorių, skirtų genų integracijai į genomą bei rekombinantinių baltymų raiškai, surinkta daugybė informacijos ir numatyti tolimesni konkretūs šito darbo etapai.

Sintetinant žmogaus BiP, kalretikulino ir ERp57 šaperonus mielėse *S. cerevisiae* nustatyta, kad jie yra sekretuojami į auginimo terpę. Šių šaperonų sekrecija yra labai efektyvi, todėl terpėje aptinkami dideli kiekiai rekombinantinių baltymų. Nors tai ir nebuvo šio darbo tikslas, pastebėtas fenomenas leido sukurti efektyvią rekombinantinių šaperonų raiškos sistemą su itin paprastu baltymų gryninimu.