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Functional expression of the rat organic anion transporter 1 (rOAT1) in *Saccharomyces cerevisiae*

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Abstract

Organic anion transporter 1 (OAT1) is localized in the basolateral membrane of the proximal tubule in the kidney and plays an essential role in eliminating a wide range of organic anions, preventing their toxic effects on the body. Structural and functional studies of the transporter would be greatly assisted by inexpensive and rapid expression in the yeast *Saccharomyces cerevisiae*. The gene encoding rat OAT1 (rOAT1) contains many yeast non-preferred codons at the N-terminus and so was modified by fusion of the favored codon sequence of a hemagglutinin (HA) epitope preceding the start codon. The modified gene was cloned into several yeast expression plasmids, both integrative and multicopy, with either *ADH*1 promoter or *GAL*1 promoter in order to find a suitable expression system. Compared with the wild type gene, a substantial increase in rOAT1 expression was achieved by modification in the translational initiation region, suggesting that the codon chosen at the N-terminus influenced its expression. The highest inducible expression of rOAT1 was obtained under *GAL*1 promoter in 2 μ plasmid. A large fraction of rOAT1 was glycosylated in yeast, unaffected by growth temperature. The recombinant yeast expressing rOAT1 showed an increase in the uptake of p-aminohippurate (PAH) and this showed a positive correlation with rOAT1 expression level. Location of rOAT1 predominantly in the yeast plasma membrane confirmed correct processing. The importance of glycosylation for rOAT1 targeting was also shown. To our knowledge, this is the first successful functional expression of rOAT1 in the yeast *S. cerevisiae*.

Keywords: Organic anion transporter 1; Membrane protein; *Saccharomyces cerevisiae*; Heterologous gene expression

1. Introduction

Numerous endogenous and exogenous organic anions can have harmful effects on the body. These compounds are either transformed into less active metabolites or are excreted via transport processes by excretory organs such as the kidney, liver and intestine. In the kidney, for example, a multitude of organic anions of varied structure are efficiently eliminated by transport systems in the proximal tubule [1].

rOAT1 is an essential component of the renal secretion system that mediates sodium-independent uptake of anionic compounds across the basolateral membrane into the proximal tubule in exchange for intracellular glutarate. The substrate specificity of rOAT1 is remarkably wide [2] and a variety of drugs have also been demonstrated to be transported by it [3,4]. Efflux of these substances through the luminal membrane is presumed to occur via anion exchangers and/or electrochemical gradient-driven facilitated transporters [1,2,4,5].

So far, structural and functional studies of rOAT1 have been hampered by the lack of a sufficient amounts and heterologous expression in a functional form would be a possible way to solve this problem. Although rOAT1 has been functionally expressed in *Xenopus leavis* oocytes [6,7], manipulation of the test system is time consuming and the protein yield is often poor. The yeast *Saccharomyces cerevisiae* has been successfully used for expression of several mammalian membrane proteins such as cystic fibrosis transmembrane conductance regulator [8], human multidrug resistance [9], vesicular monoamine transporter [10], dopamine receptor [11], and glucose transporter [12] and could thus be considered as a possible vehicle for inexpensive and rapid production of rOAT1.

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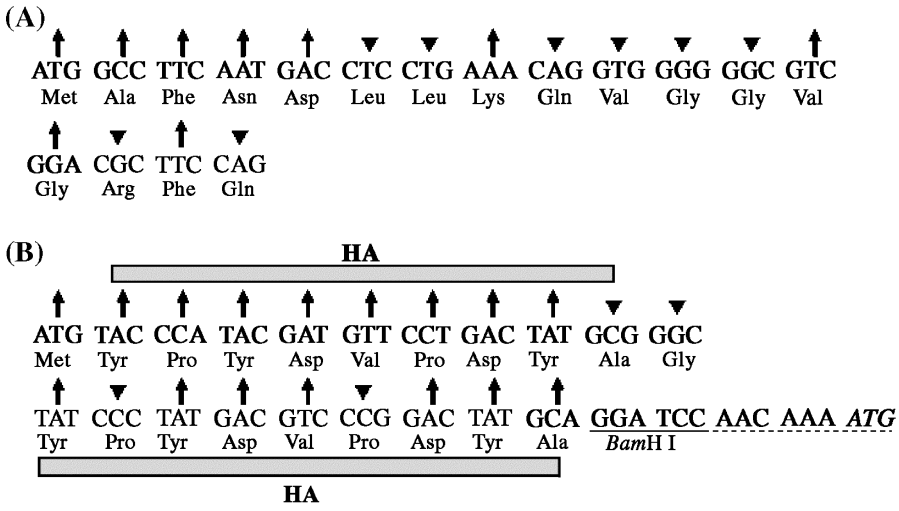

Fig. 1. The first 17 codons encoding the N-terminus of rOAT1 (A). The HA-epitope and the fusion site (B). ↑ represents codon preferred by yeast, ▼ indicate a non-preferred codon, *Bam*HI site is underlined and dot letters show a Kozak consensus sequence upstream of the rOAT1 start codon (italic). Open bars represents boundary of the HA-epitope.

Foreseeable problems with expression of mammalian membrane proteins in *S. cerevisiae* are the strong codon usage bias in yeast and differences in post-translational modification between yeast and mammalian systems, especially for glycosylation. Examination of codon usage at the 5′ end of rOAT1 revealed a trend of yeast non-favored codons. To overcome this problem, we used the strategy of constructing HArOAT1 (Fig. 1) to contain the readily translatable HA (hemagglutinin) epitope fused upstream of the rOAT1 start codon.

To find a suitable expression system for rOAT1, the HA-fusion rOAT1 gene was cloned into different yeast expression plasmids, both integrative and multicopy, with the constitutive *ADH*1 promoter or the *GAL*1 inducible promoter. The effect of growth temperature on glycosylation of yeast-expressed rOAT1 was also studied. In addition, the transport ability and plasma membrane targeting of the protein were examined to determine whether heterologously expressed rOAT1 retained structural and functional characteristics.

2. Materials and methods

2.1. Vector constructions

rOAT1 *cDNA (accession number: AB004559*, GenBank™ nucleotide data bases) was kindly provided by Prof. H. Endou (Kyorin University, Japan) in plasmid pSPORT1. The rOAT1 ORF was previously cloned into pBlueScript SK̄ between the *Bam*HI and *Eco*RI sites to yield pBPII (B. Punlungka unpublished data). The *Bam*HI–*Eco*RI cDNA fragment for rOAT1 was extracted from pBPII and inserted into pFA6a–*TRP*1–*pGAL*1–3HA (obtained from Dr. H. Qui, NIH). The newly constructed plasmid named pFA6a–*TRP*1–*pGAL*1–HArOAT1 harbored the rOAT1 ORF modified at the translation initiation region by fusion with two copies of the HA encoding sequence.

The HA–rOAT1 fusion gene (HArOAT1) was amplified by PCR using a forward primer designed to introduce the sequence TTAGAGCTC*AA*AATG (*Sac*I site underlined, Kozak consensus sequence in italics) to the 5′ end and a reverse primer designed for addition of an *Eco*RI site immediately downstream of the stop codon. The resulting blunt-end PCR fragment was digested with *Eco*RI, to generate a 3′ *Eco*RI cohesive end before it was ligated into pSP72 vector between *Eco*RI and *Eco*RV restriction sites. The correct sequence of the PCR amplified HArOAT1 gene could then be confirmed by DNA sequencing using T7 and SP6 primers. The *Sac*I restriction site of pSP72 located next to the *Eco*RI cloning site could be utilized for subcloning the gene into several expression plasmids. Since the PCR product was rather long, only the modified N- and C-terminal sequences were verified by a single pair of primers. Finally, the middle fragment from the *Bst*EII to *Nhe*I site of the PCR-amplified HArOAT1 was replaced with the original HArOAT1 cDNA from PBPII to yield plasmid pSP72–HArOAT-1.

The parent vector for construction of an integrative HArOAT1 expression plasmid was generated from insertion of an *ADH*1 promoter-multicloning site-*CYC*1 terminator cassette from pTB326 vector (kindly provided by Dr. H. Qui, NIH) into the integrative vector YIPBssHII 211 (obtained from Dr. J. Hauf, Technical University of Darmstadt, Germany). To facilitate subcloning of genes, some restriction sites in YIPBssHII 211 corresponding to the multicloning site of pTB326 firstly had to be removed. YIPBssHII 211 was digested with *Bam*HI and *Eco*RI to cleave out *Sac*I, *Kpn*I and *Sma*I restriction sites, then treated with Mung bean nuclease to destroy *Bam*HI and *Eco*RI restriction sites. The blunt ends of the linearized plasmid were then ligated together before insertion of the *Sp*hI–*Xba*I fragment of the promoter–terminator cassette from pTB326, yielding the integrative yeast expression plasmid YIP211-1a. Then, the *Sac*I fragment of HArOAT1 from pSP72–HArOAT1-1 was ligated into the parent vector YIP211-1a to create the HArOAT1 integrative plasmid pSSI (Fig. 2). By ligation of the same fragment into pTB326, the HArOAT1 multicopy plasmid pSSIV (Fig. 2) was obtained. The multicopy plasmid pSSIII (Fig. 2) for *GAL*1-inducible expression of HArOAT1 was generated by insertion of the gene fragment extract from pSP72–HArOAT1-1 into pYES2 vector (Invitrogen) between *Eco*RI and *Sac*I sites. The wild type rOAT1 gene extracted from pBPII by digestion with *Bam*HI and *Eco*RI was also cloned into pYES2 to yield plasmid pSSII (Fig. 2).

2.2. Strains and media

Escherichia coli strain DH5α (*F*ϕ80*dlac*ZΔ(*lac*ZYA–*arg*F)u169 deoR, *rec*A1, *end* A1, *hsd*R17(r_K-, m_{K+}), *Sup*E44, λ-*thi*-1,*gyr*A96, *rec*1A1) was used for all bacterial work. The *S. cerevisiae* protease deficient strain BJ5462 (*MAT*α, *ura*3–5, *trp*1 *leu*2Δ1, *his*3Δ200, *pep*4::*HIS*3, *prb*1Δ1 1.6R, *can*1, *GAL*) (Yeast Genetic Stock Center, University of California) was used throughout the study. Untransformed yeast cells were grown in SD minimal medium containing 0.67% (w/v) yeast nitrogen base without amino acids (DIFCO Laboratories), 2% (w/v) D-glucose supplemented with an amino acid mixture. The same medium supplemented with an amino acid mixture lacking uracil or lacking tryptophan was used for growing cells transformed with

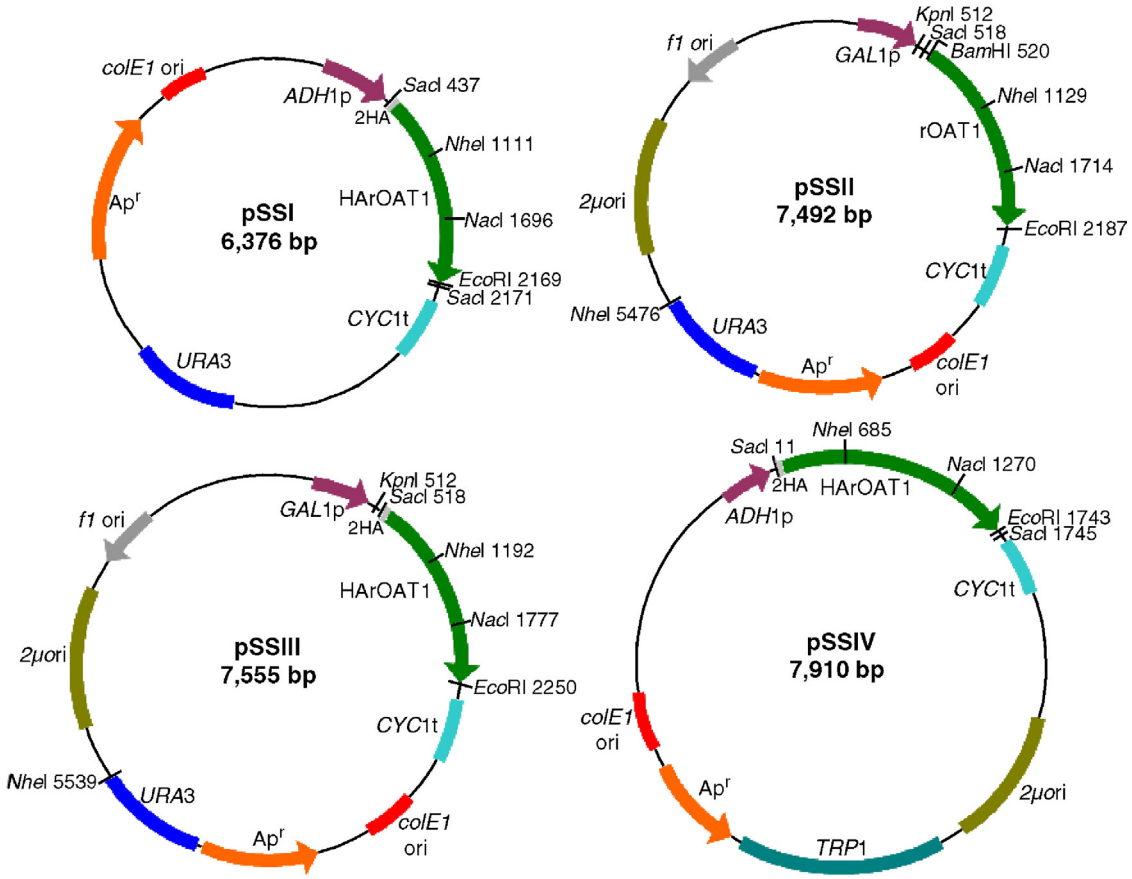

Fig. 2. Physical maps of rOAT1-expressing yeast plasmids. The integrative plasmid pSSI is *URA*3 selectable and encodes the HA-fusion rOAT1 under control of the constitutive *ADH*1 promoter. In the *TRP*1-selectable 2 μ plasmid pSSIV, expression of HArOAT1 is also controlled by *ADH*1 promoter. The galactose-inducible GAL1 promoter was used to control expression of HA-modified rOAT1 and wild type rOAT1 in the multicopy plasmid with *URA*3 selectable marker pSSIII and pSSII, respectively. *CYC*1 transcriptional terminator was used in all plasmids.

*URA*3 plasmid or *TRP*1 plasmid, respectively. Solid media contained an additional 2% (w/v) Bacto-Agar (DIFCO Laboratories).

2.3. Transformation and culture conditions

Yeast cells were transformed by the lithium acetate method described by I to [13] with some modifications [14]. Transformants were identified by auxotrophic selection. All experimental cultures were inoculated at an OD660 of 0.01 from actively growing pre-cultures. Throughout the experiments, cultivation of transformants for gene expression under control of the constitutive promoter *ADH*1 was performed using SD medium containing glucose as the sole carbon source. For inducible expression under control of *GAL*1 promoter, cells were grown in SD medium using 2% raffinose as the carbon source until OD660 reached 0.6–0.8 units (early exponential phase), at which time expression was induced by addition of galactose to a final concentration of 2%. Unless indicated otherwise, the cultures were allowed to grow at 30 °C with constant agitation (200 rpm).

2.4. Isolation of crude yeast membranes and plasma membranes

Yeast cells were grown as above, and crude cellular membranes were prepared by a glass bead lysis protocol [9]. Plasma membranes were purified by the acid precipitation method of Goffeau and Dufour [15]. Protein concentrations were measured by the Bradford method [16].

2.5. Deglycosylation

Endoglycosidase H deglycosylation of proteins was performed as described by Imamura [17] with some modifications. Five μl of 200 mM NaH_2PO_4, pH 5.5 and 0.2% (w/v) SDS were added to 20 μg proteins from yeast plasma membrane extracts. Then 10 mU of endoglycosidase H was added and samples were incubated for 12 h at 37 °C.

2.6. Western blots

Twenty μg protein samples were subjected to 10% SDS-PAGE gel and run at 110 V for about 2 h, followed by transfer onto polyvinylidene difluoride membranes at 0.1 Amp overnight. The membranes were then washed with TBST (20 mM Tris–HCl pH 7.5, 150 mM NaCl and 0.05% (v/v) Tween 20) for 30 min, blocked with 5% skim milk in TBST for 1 h with agitation. Afterward, membranes were incubated with anti-OAT1 rabbit polyclonal antibody (1:5000 dilution) or anti-HA mouse monoclonal antibody (1 μg/ml), for 3 h with gentle agitation. After washing 3 times in TBST (5 min each), membranes were incubated in 1:5000 dilution of anti-rabbit or anti-mouse IgG AP conjugated for 1 h with gentle agitation, washed 3 times in TBST and 2 times in TBS (20 mM Tris–HCl pH 7.5 and 150 mM NaCl) to remove Tween 20. The BCIP/NBT kit (Zymed) was employed for protein detection.

2.7. Anion transport assay

Recombinant cells were cultured for expression of rOAT1 as described above, harvested at mid exponential phase (*GAL*1 inducible expression) or late exponential phase (*ADH*1 constitutive expression), and washed once with Dulbecco's phosphate buffer saline (137 mM NaCl, 3 mM KCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4, 1 mM $CaCl_2$, and 0.5 mM $MgCl_2$) supplemented with 0.15 M D-glucose [9], pH 7.4 that was also used as transport buffer. The cells were then resuspended to 68 $OD_{660} \times 20\ \mu l^{-1}$ for one reaction with the same buffer containing 1 mM glutarate in the presence or absence of 2 mM probenicid

and incubated for 1 h at room temperature. After washing 3 times with the same buffer, uptake experiments were initiated by rapidly mixing 20 μl of cell suspension with 80 μl of transport buffer containing 2 μM [^{14}C]PAH (0.1 mCi/ml). At specific times, reactions were stopped by addition of 1 ml ice-cold transport buffer and the cells were trapped by filtering through Whatman GF/C filters. The filters were washed five times with 1 ml ice-cold transport buffer and trapped radioactivity was quantified by liquid scintillation spectrometry.

3. Results

3.1. rOAT1 is expressed in yeast only when HA is fused preceding its N-terminus

Examination of codon usage at the 5′ end of rOAT1 revealed that the first 17 codons encoding the N-terminus included at least 8 codons seldom used in yeast for highly expressed proteins (Fig. 1). To test whether an intrinsic determinant within the gene impeded rOAT1 expression, 21 codons encoding 2 copies of HA epitope were fused in frame preceding the start codon of rOAT1. This created HArOAT1 that contained only 4 yeast non-favored codons.

Since expression of foreign membrane proteins may interfere with cellular process and may affect cell growth and viability, the HA-modified rOAT1 gene was cloned into different yeast expression plasmids that allowed control of expression levels. As detailed in Materials and methods, four types of plasmids were constructed. All plasmids were introduced by transformation into yeast strain BJ5462. Expression of rOAT1 in the yeast plasma membrane fraction was assessed by Western blot using polyclonal antibody directed against the carboxy terminus of the transporter. No immunoreactive material was detected in plasma membrane prepared from any control strains transformed with parental plasmids YIP211-1a, pTB326 or pYES2 (Fig. 3 lanes 1, 2, 3), or in the strain that carried the wild type rOAT1 expression plasmid pSSII (Fig. 3 lane 4). Addition of HA to the 5′ end changed the expression of rOAT1. The HA-fusion rOAT1 protein encoded by pSSI, pSSIV and pSSIII (Fig. 3 lanes 5, 6, 7) migrated on SDS-PAGE with apparent molecular masses of 60–88 kDa, which was in good agreement with the molecular mass rOAT1 predicted from the amino acid sequence (60 kDa) [2,7,18] and with the molecular mass of glycosylated rOAT1 protein detected in rat kidney with the same antibody (77.8 kDa) [19]. These results indicated the importance of codons at the N-terminus for expression of rOAT1. By N-terminal fusion of short sequence encoding yeast-favored codons, rOAT1 was able to be expressed in yeast. Furthermore, as determined by signal intensity, the highest expression level of HArOAT1 came from *GAL*1 inducible promoter in the high copy plasmid pSSIII; while the constitutive promoter *ADH*1 in the integrative plasmid pSSI showed the least expression level. The same result was also observed when Western blot was performed using mouse monoclonal antibody against HA epitope (data not shown). The small proteins found in the rOAT1-expressed strains might come from protease degradation of rOAT1. This was supported by a large number of predicted proteasomal cleavage sites in rOAT1 protein.

Influenza virus hemagglutinin has a signal peptide that mediates translocation of the protein across the endoplasmic reticulum (ER) membrane. Addition of this transport signal

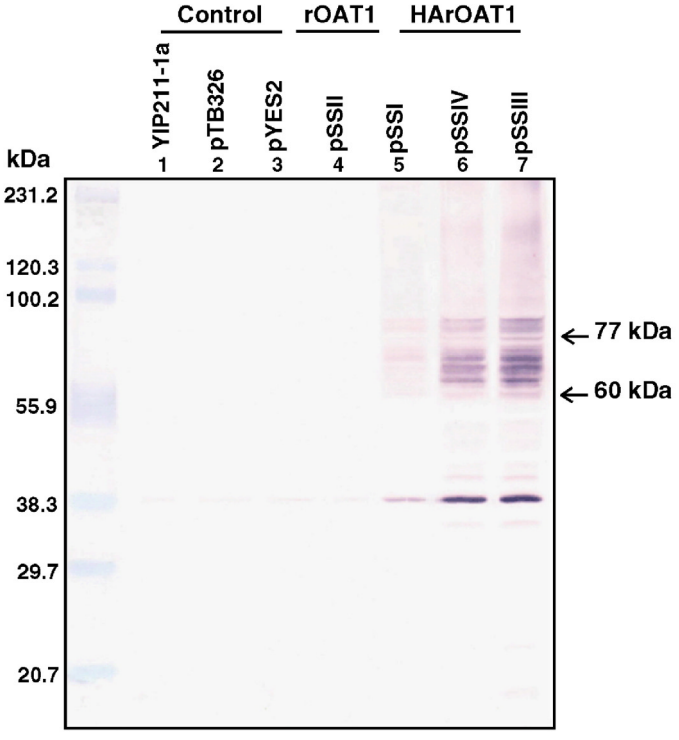

Fig. 3. Expression of the N-terminal modified rOAT1 in yeast. Plasma membranes were prepared from yeast cells with only empty vector YIP211-1a, pTB326 or pYES2; with the wild type rOAT1 plasmid pSSII; and with the HA-fusion rOAT1 (HArOAT1) plasmid pSSI, pSSIV, pSSIII. 20 μg proteins from each sample were subjected to SDS-PAGE and immunoblotted with polyclonal antibody raised against C-terminus of rOAT1.

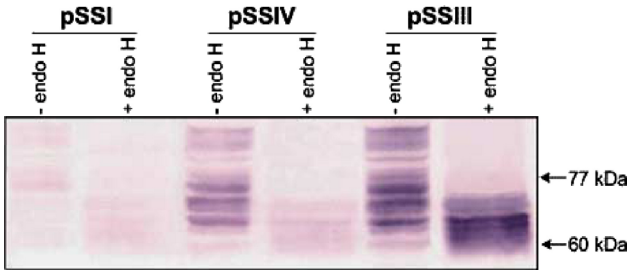

Fig. 4. rOAT1 is glycosylated in yeast. 20 μg endo H-treated and untreated proteins from the cells carrying plasmid pSSI, pSSIV, or pSSIII grown at 30 °C and 16 °C (A) as compared with the 16 °C-growing cells (B), were detected by Western blot using monoclonal antibody against HA epitope.

from hemagglutinin to the N-terminus of yeast protein carboxypeptidase Y (CPY) efficiently directed the protein to the ER in a mammalian expression system [20]. In silico prediction of subcellular localization suggested that 66.7% of the wild type rOAT1 was located in the ER and only 33.3% in the plasma membrane. HA insertion apparently changed the predicted localization of rOAT1. An almost 2-fold increase in plasma membrane targeting (65.2%) was predicted for the HA-fusion protein and only small fractions should have been seen in the ER, Golgi cisternae, vacuolar membranes and nuclear membrane. Immunoblots of total cellular extract with both OAT1 and HA antibody (data not shown) showed the same banding pattern as observed with the plasma membrane. Detection of rOAT1 protein was only in the total cell extract of the HA-rOAT1 recombinant yeast confirmed the role of HA in the enhancement of rOAT1 expression rather than plasma membrane targeting.

3.2. rOAT1 is glycosylated in yeast, unaffected by growth temperature

The native rat OAT1 is glycosylated with up to 17 kDa of carbohydrate [2]. It has been suggested that this carbohydrate is required for sorting of OAT1 to the plasma membrane. Many mammalian membrane proteins expressed in yeast are not glycosylated [9,21–23]. To determine whether the yeast-expressed rOAT1 was glycosylated, plasma membrane isolated from three rOAT1 recombinant strains were treated with enzyme endoglycosidase H, which cleaves the entire high mannose and some hybrid oligosaccharide structure at the glucosamine linkage of N-linked carbohydrates. Immunoblot analysis using the anti-HA monoclonal antibody showed that upon treatment with endoglycosidase H, the electrophoretic mobility of rOAT1 was altered. The apparent molecular masses from over 60 kDa to 88 kDa were reduced mostly to the unglycosylated size of 60 kDa after deglycosylation (Fig. 4). These results indicated that a large fraction of rOAT1 was glycosylated at several levels in yeast.

It has been suggested that an important function of N-linked glycosylation is the quality control for correct protein folding in the endoplasmic reticulum (ER) [24]. Since protein folding and stability often favor low temperatures, glycosylation of rOAT1 in yeast grown at low temperature was investigated. The similar band pattern in immunoblot of the plasma membrane prepared from rOAT1 recombinant strains grown at 30 °C and 16 °C both before and after endoglycosidase H treatment (Fig. 4) indicated the unaltered glycosylation at different temperatures.

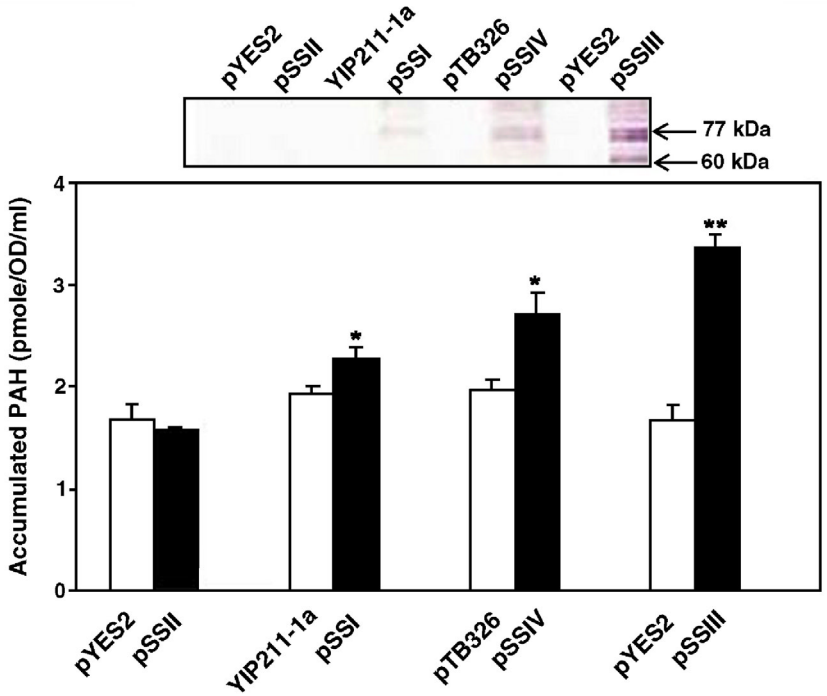

Fig. 5. The rOAT1 expressed in yeast is in functional form [^{14}C] PAH uptake into yeast cells carrying plasmid pSSII and into the rOAT1 expressing cells carrying plasmid pSSI, pSSIV or pSSIII are compared with the uptake in control cells with parent vectors YIP211-1a, pTB326 or pYES2. Each column represents the mean ± S.E. value of accumulated [^{14}C] PAH measured for 10 min in three independent experiments. *P < 0.05; **P < 0.01 indicate significant differences from controls.

3.3. Yeast-expressed rOAT1 is able to transport p-aminohippurate (PAH)

To assess the capability of the yeast-expressed rOAT1 to mediate organic anion exchange, the model substrate PAH was chosen. rOAT1 recombinant strains and control strains carrying only parent vector were cultured for either constitutive or inducible expression of rOAT1. The cells were then loaded with 20 μM [^{14}C] PAH and assayed for anion uptake by measuring the influx of radioactive PAH in exchange for intracellular glutarate [6] (Fig. 5). No apparent difference in the PAH uptake was observed between cells with the wild type rOAT1 plasmid pSSII and those with pYES2 (control cells). By contrast, recombinant yeast carrying the high copy plasmid pSSIV showed very much higher PAH uptake) than control cells carrying parent vector pTB326. Cells carrying pSSIII (highest expression of rOAT1) showed a 2-fold increase in PAH uptake when compared to pYES2-carrying cells. There was a positive correlation between rOAT1 expression level (determined by intensity of 60–88 kDa bands detected in Western blots) and PAH uptake ability in each recombinant strain.

An inhibition study was also performed in order to confirm the specificity of PAH transport by heterologously expressed rOAT1. In the presence of 2 mM probenecid, a classical inhibitor of the uptake system, the amount of PAH taken up by all HArOAT1 recombinant strains was substantially reduced (Table 1). Accumulated PAH in control cells that showed no detectable level of rAOT1 was also reduced in the presence of probenecid. Many intrinsic yeast anion transporters are known to have wide ranges of substrate specificity. The inhibition study suggested that the host yeast *S. cerevisiae* strain BJ5462 had its own transport mechanism for PAH and that probenecid was also able to inhibit that system (Table 1).

3.4. rOAT1 targets plasma membrane

Previous functional assays supported the possibility that rOAT1 is expressed at the cell surface where it mediates PAH entrance into cells. To determine the location of rOAT1 in recombinant yeast strains, Western blot analysis of various cellular fractions was performed (Fig. 6). In all rOAT1 recombinant strains, the greatest amount of rOAT1 protein was detected in the plasma membrane. The same quantity of protein from crude membrane fractions that included both plasma membranes and organelle membranes showed lower levels of rOAT1. A relatively small amount of the transporter was found in the soluble fraction. Interestingly, the presence of only the unglycosylated protein (60 kDa) in the intracellular fluid (i.e., soluble fraction) is in agreement with the suggestion that glycosylation is important for OAT1 targeting to the plasma membrane.

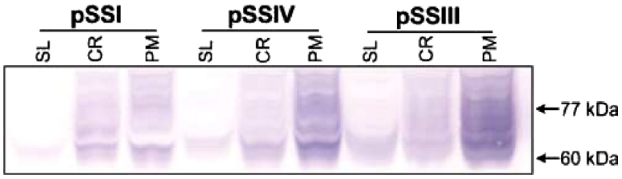

Fig. 6. Localization of rOAT1 in yeast cells. Western blot of plasma membranes (PM), crude cellular membranes (CR), and soluble fractions (SL) prepared from rOAT1 recombinant strains carrying plasmid pSSI, pSSIV and pSSIII, using monoclonal antibody against HA epitope. Each lane contains 20 μg of protein.

4. Discussion

The present study demonstrated that codon usage at the N-terminus of the rat organic anion transporter 1 (rOAT1) is important for the successful expression of rOAT1 in the yeast *S. cerevisiae*. The use of synonymous codons in *S. cerevisiae* is strongly biased [25], mostly due to abundances of different tRNA species [26] and it has been suggested that the efficiency of protein synthesis is increased when required tRNA are not limited. In addition, translation efficiency may also affect the stability of the mRNA transcript. Preferred codons therefore improve both transcriptional and translational processes in protein expression [10]. By synthesizing an ideal gene with yeast preferred codons, successful expression of malarial parasite integral membrane protein Pfcrt in yeast was achieved [27]. Study on the role of biased codon usage in expression of phoshoglycerate kinase (*PGK*1) gene [28] showed that replacement of major codons at the 5′ end of the coding sequence with synonymous minor ones, substantially decreased expression. Instead of substituting minor codons with major codons, we fused an HA sequence consisting of mostly yeast-preferred codons preceding the start codon of rOAT1 in order to simplify initiation of translation. We found that rOAT1 was expressed in yeast with this modification only (Fig. 3). This method has proven useful in a previous study on expression of rat and bovine vesicular monoamine transporters (rVMAT1 and bVMAT2, respectively) in yeast [10]. In addition, we found that the best rOAT1 expression was obtained with a multicopy plasmid in which the expression was controlled by the inducible promoter *GAL*1 (Fig. 3).

The apparent sizes of the yeast-expressed rOAT1 varied from 60 to 88 kDa. Treatment with endoglycosidase H demonstrated that those proteins of over 60 kDa were glycosylated (Fig. 4). Although *S. cerevisiae* is able to glycosylate proteins, it has been shown that oligosaccharide

Table 1
Amount of [^{14}C] PAH accumulated in recombinant yeasts in the presence and absence of 2 mM probenicid

PAH uptake (pmole/OD/ml) Recombinant yeast carrying plasmid	YIP211-1a	pYES2	pTB326	pSSI	pSSII	pSSIII	pSSIV
Without Probinicid	1.932±0.07	1.672±0.15	1.965±0.11	2.260±0.11	1.566±0.02	3.351±0.12	2.693±0.22
With Probinicid	0.145±0.07	0.221±0.19	0.108±0.05	0.182±0.13	0.228±0.12	0.184±0.04	0.324±0.15

Each value represents the mean±S.E from triplicate experiments. The paired results in each column were all significantly different ($P<0.001$) by Student's t test.

structures are significantly different from their authentic versions in mammalian cells [29,30]. Usually, yeast makes extensive mannosylation [31]. Since glycosylated rOAT1 found in the rat kidney is about 77 kDa [17], the larger protein detected in the yeast plasma membrane may have resulted from hypermannosylation.

The effect of cultivation temperature on protein expression and post-translational, modification has been reported for expression of rVMAT1 in yeast [10]. By lowering cultivation temperatures, expression of rVMAT1 was remarkably improved and the unglycosylated protein underwent core glycosylation. In contrast to rVMAT1, glycosylation and expression level of rOAT1 were the same at 30 °C and 16 °C.

Molecular mechanisms that determine membrane localization of proteins in *S. cerevisiae* are not well understood, but the same principles seem to govern membrane trafficking in yeast and higher eukaryotes. An important factor effecting membrane targeting is protein folding. Only proteins that are correctly folded leave the ER and move forward into the Golgi. Misfolded proteins undergo ER-associated degradation and the process involves dislocation of the cargo proteins followed by ubiquitination and proteolytic degradation [32]. Protein modification is associated with folding in the ER and glycosylation may be required for correct folding. Control of proper folding may be a major purpose of modifications. For example, inhibition of glycosylation has been demonstrated to lead to protein misfolding followed by degradation in the ER [32]. However, carbohydrate moieties have also been shown to serve as transport signals for targeting the plasma membrane. Unglycosylated, secretory rat growth hormone fused to the transmembrane and cytosolic domains of vesicular stomatitis virus G protein was blocked in the Golgi complex and unable to reach the plasma membrane. Insertion of N-glycosylation sites into an extracellular domain can enable a protein to target the cell surface [33]. In epithelial Madin-Darby Canine kidney cells, the glycosylated form of rat growth hormone was secreted through the apical membrane, whereas the nonglycosylated native form was randomly secreted both apically and basolaterally [34]. The role of O-glycosylation as a membrane transport signal has also been displayed in the study of the yeast integral membrane protein Fus1p that is involved in cell fusion during yeast mating [35]. Insertion of the Fus1 ectodomain containing several potential O-glycosylation sites between the N-terminal part of invertase and the transmembrane domain of Fus1p altered the localization of the chimeric protein and it accumulated extracellularly. In the mutant yeast pmt4Δ that is unable to glycosylate proteins, the chimeric protein was blocked in the Golgi complex.

The role of N-linked glycosylation in plasma membrane targeting of OAT1 has been clearly demonstrated. Removal of all N-glycosylation sites from mouse and human analogs of OAT1 (mOAT1 and hOAT1, respectively) by replacing asparagine with a glutamine residue resulted in failure to target to the plasma membrane and accumulation intracellular compartments [36]. This was in good agreement with previous results of the same group showing that treatment of mOAT1-expressing COS-7 cells with tunicamycin to inhibit N-linked glycosylation resulted in failure of protein translocation to the plasma membrane [37]. Examination of rOAT1 localization in recombinant yeasts strongly supports these results. Glycosylated OAT1 was found exclusively in the yeast plasma membrane while the unglycosylated protein was found in the intracellular fluid (Fig. 6).

The possible cellular mechanisms underlying the function of carbohydrate moieties that determine apical transport of glycoproteins in epithelial cells have been proposed. One model suggests that hypothetical lectins bind to the glycans and bring the glycoproteins to transport carriers in the trans-Golgi network [34]. Another model suggests that glycans change the biophysical properties of proteins to facilitate the presentation of sorting signals to their receptors [35,38].

The ability of hetorologously expressed rOAT1 to transport PAH indicated that the structure and function of the transporter was at least partially preserved in *S. cerevisiae* (Fig. 5). PAH transport by proximal tubules in the rat kidney has been examined by various methods, including the stopped flow capillary perfusion method. PAH was transported into proximal tubular cells with a K_m of 0.08 ± 0.01 mM and Vmax of 1.1 ± 0.1 pmol $\times s^{-1} \times cm^{-1}$ [39]. Comparison of the transport ability of yeast-expressed rOAT1 with the native protein in the rat kidney requires purification and reconstitution of the protein into synthetic vesicles for further study of transport kinetics. In addition, it has been reported that transport function of mOAT1 and hOAT1 was almost completely lost when the putative glycosylation site Asp-39 was disrupted. However, it was found that this putative site was used for glycosylation only in hOAT1 and not in the mouse homolog. In addition, disruption of other putative glycosylation sites in both proteins had no effect on the transport activity of either mOAT1 or hOAT1 [36]. The transport function of heterologously expressed rOAT1 in this study was assessed using a whole cell transport assay, in which only the function of the plasma membrane inserted rOAT1 could be examined.

In summary, we reported the first successful expression of functional rOAT1 protein in yeast. This will lead the way to further studies on molecular structure and transport mechanisms and perhaps eventually to the development of in vitro systems for studying drug kinetics or for screening drug nephrotoxicity.

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