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The oligosaccharyltransferase complex from pig liver: cDNA cloning, expression and functional characterisation

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Oligosaccharyltransferase (OST) is an oligomeric protein complex which catalyses the transfer en bloc of Glc_3-Man_9-$GlcNAc_2$ from Dol-PP to specific asparagine residues in the nascent polypeptide chain. In order to study the function of the pig enzyme subunits, we have cloned OST48, ribophorin I and ribophorin II and characterized these proteins after *in vitro* translation as well as after expression in COS-1 cells. The individual full-length cDNAs contained open reading frames (ORFs) encoding polypeptides with calculated molecular masses of ~48.9 kDa (OST48), ~68.7 kDa (ribophorin I) and ~69.3 kDa (ribophorin II), respectively. A Kyte and Doolittle hydrophobicity analysis revealed that OST48, ribophorin I and ribophorin II possess a type I membrane topology with the bulk of their polypeptide chains directed towards the ER-lumen. In contrast to OST48, ribophorin I and II contain, respectively, three or two potential N-glycosylation sites of the Asn-Xaa-Thr/Ser type; only one is found to function as the acceptor site in each protein.

Transfection of COS-1 cells with vector constructs encoding either OST48, ribophorin I, or a ribophorin I variant tagged with a myc-peptide sequence, resulted in the over-expression of polypeptides whose molecular masses were similar to those calculated from the respective cDNA ORFs. None of these three polypeptides, or ribophorin II, were found to display OST activity when over-expressed alone. By contrast, a modest but reproducible ~25% increase of activity was observed when OST48 together with ribophorin I, or OST48 and myc-tagged ribophorin I, were co-expressed, indicating that these two subunits are probably responsible for the catalytic activity in the hetero-oligomeric OST complex. The only modest over-expression of transferase activity suggests that either the dimeric enzyme complex is catalytically unstable, or that the OST48 and ribophorin I polypeptides are unable to fold properly when other subunit components of the hetero-oligomeric OST complex are lacking. OST48 as well as ribophorin I are expressed in COS-1 cells as ER-resident proteins. Whereas OST48 carries a double-lysine motif in the −3/−5 position of its cytosolic C-terminal domain, ribophorin I does not contain recognizable ER-retention information. Replacing the lysine residue in the −3 position by leucine resulted in plasma membrane expression of the OST48-Leu polypeptide, indicating that this sequence motif may be able to influence OST48 localisation. No cell surface staining was observed when OST48-Leu was co-expressed with ribophorin I. This suggests that localisation of OST48 in the ER is mediated by interaction with ribophorin I rather than by the double-lysine motif.

Keywords: **oligosaccharyltransferase from pig liver and pig muscle, OST48, ribophorin I and II, cDNA cloning, structural properties, subcellular localisation, COS-1 cell expression**

Introduction

Asparagine-linked glycosylation is a highly conserved protein modification occuring in all eukaryotic cells. Synthesis of the N-glycosidic linkage is catalysed by oligosaccharyltransferase (OST) which transfers $GlcNAc_2$-Man_9-Glc_3 en bloc from Dol-PP to specific asparagine residues in the nascent polypeptide chain [1]. It is well established that asparagine residues acting as acceptor sites in OST catalysed glycosylation, must be located within a triplet sequence of the Asn-Xaa-Thr/Ser/Cys type, with Xaa being any amino acid except proline [2,3]. Model studies on the functional role of this consensus sequence have shown that the hydroxy amino acid not only takes part in substrate binding but also in OST catalysis. This probably involves hydrogen-bond formation with the asparagine side chain, as a result of which the β-acid amide reactitivity is assumed to be enhanced [4,5].

OST has been purified from different mammalian tissues and species including dog pancreas [6], hen oviduct and liver [7,8], human liver [9] and pig liver [10]. Except for the pig liver enzyme which contained an additional ~40 kDa protein, OST

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activity was found to be associated with a hetero-oligomeric protein complex consisting of three subunits with molecular masses of ~48 kDa, ~66 kDa and ~63 kDa, the latter two proteins being identical with ribophorin I and ribophorin II, respectively. More recently, a ~12 kDa polypeptide (DAD1) has been identified as a putative subunit of the OST complex by crosslinking and immunological studies [11]. The specific function of the individual subunits remains unknown at present. There is evidence, however, suggesting that either OST48, or ribophorin I, or even both subunits may be responsible for catalytic activity: (i) the tetrameric OST complex purified from pig liver and separated by ConA-Sepharose chromatography, gives rise to a catalytically active enzyme containing only these two subunits [10]; (ii) both OST48 and ribophorin I were double-labelled specifically after incubation of the pig liver enzyme in the simultaneous presence of Dol-PP-[^{14}C]oligosaccharides and an inhibitor hexapeptide in which the hydroxy amino acid within the Asn-Xaa-Thr/Ser consensus sequence was replaced by epoxyethylglycine [12]; (iii) finally, a photoactivatable peptide derivative with p-benzoylphenylalanine in the Xaa position of the consensus sequence reacted with the Ostl p subunit of the yeast OST complex which is assumed to be equivalent with mammalian ribophorin I. This indicates that the probe must have been bound to, or at least in close contact, with this subunit [13].

The subunits of the OST complex which include OST48, ribophorin I and II, have been cloned from different mammalian species but not from pig tissue [14–17]. Extending our studies of the pig liver enzyme, we describe here the cDNA cloning of pig OST48, ribophorin I and II, as well as the structural and functional characterisation of the subunit polypeptides encoded for by the respective cDNA ORFs. Single subunits when over-expressed in COS-1 cells were without catalytic activity. Co-expression of OST48 and ribophorin I, on the other hand, resulted in a modest but reproducible (~1.25-fold) increase in OST activity, supporting the assumption that OST48 and ribophorin I are the catalytic species. The modest increase in OST activity indicates that additional factors and/or subunit(s) may be required for the expression of a stable and fully active OST enzyme during biosynthesis.

Materials and methods

Materials

Materials and chemicals were obtained from the following sources: mixed-primed pig liver cDNA library pL1011a in λgt10, *E. coli* C600 host strain (Clontech); oligo(dT)-primed-porcine muscle cDNA library in Uni-ZAP XR, XL1-blue MRF host strain (Stratagene); anti-c-myc mouse monoclonal antibody, pUC BM20 vector, oligo(dT)cellulose (Roche); mammalian expression vector pcDNA3 (Invitrogen); synthetic oligonucleotides, Taq polymerase, Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (Life Technologies); restriction endonucleases, DNA modifying enzymes (MBI Fermentas); Prime-a-Gene-labelling system, TNT-T7Quick coupled transcription/translation system (Promega); ^{T7}Sequencing kit, DEAE dextran, L-[^{35}S]methionine (specific activity > 1000 Ci/mmol); Hybond N membranes, ProteinA-sepharose (Amersham/Pharmacia); COS-1 cells (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH); goat anti-rabbit IgG alkaline phosphatase conjugate, goat anti-mouse IgG alkaline phosphatase conjugate (Sigma); nitrocellulose membranes (Schleicher & Schuell); fluoresceine (DTAF)-conjugated AffiniPure goat anti-rabbit IgG, rhodamine (TRITC)-conjugated AffiniPure goat anti-mouse IgG (Dianova); [α-^{32}P]dATP (specific activity 3000 Ci/mmol), [^{35}S]dATP-α-S (specific activity > 1000 Ci/mmol), [γ-^{32}P]-ATP (specific activity > 3000 Ci/mmol) (Hartmann Analytic); X-OMAT AR films (Kodak); bacterial expression vector pQE30, Ni-NTA-agarose (Qiagen). All other compounds used were of analytical grade purity.

Pig liver first-strand cDNA synthesis and preparation of specific screening probes

Total mRNA was isolated from pig liver tissue using caesium chloride density centrifugation, followed by separation of the poly(A)$^+$ mRNA fraction on an oligo(dT)-cellulose column. First-strand cDNA which was used as the template for the synthesis of screening probes, was prepared by incubating the poly(A)$^+$ mRNA fraction with reverse transcriptase in the presence of oligo(dT) and "random" hexanucleotide primers. A 338 bp screening probe specific for OST48 was amplified by PCR using sense-primer A derived from the N-terminal sequence of pig liver OST48 and anti-sense primer B derived from the coding sequence of dog OST48 (see Table 1 for sense and anti-sense primers) [17]. The ribophorin I-specific screening probe (1295 bp) was synthesized by PCR using C as the sense

Table 1. Primers used for PCR and vector construction

A	AT(A/T/C) GA(T/C) CA(C/T) CA(T/C) AA(T/C) TA(T/C) GA(T/C)
B	(A/G)TC (A/G)TA (A/G)TT (A/G)TG (A/G)TG (A/G)TC (A/G/T)AT IAC
C	ATG GAG GCG CCA GCC GCC GGC TTG TTT CTG
D	GTA CAG GGC ATC CAG GAT GTG GTC GAT CTT G
E	ATG GCI CCI CCI GGI TCI (A/T)(G/C)I ACI GTI TT(T/C) (C/T)TI (C/T)TI GCI (C/T)TI ACI AT(C/T/A) AT(C/T/A) GC
F	CTA TTT AGG TGA CAC TAT AGA AGG T
G	CCG GAA TTC GGA CCA CCT CGG CCG TCA CCT
H	CCG GAA TTA CCA TGG AGG CGC CCG CAG T
I	ATA GCG GCC GCC TAG AGG TCC TCC TCG GAG ATG AGC TTC TGC TCC AGT GCA TCC AAG ATG TGG TCG ATC TGG ATA GCG GCC GCC TAG AGG TCC TCC TCG GAG ATG AGC TTC TGC TCC AGT GCA TCC AAG ATG TGG TCG ATC TGG

and D as the anti-sense primer, both derived from human ribophorin I [14]. The PCR products were purified by agarose gel electrophoresis and characterized by sequencing using the dideoxynucleotide chain termination method. Ribophorin II-specific λ-clones were isolated by library screening with the degenerate 53 bp oligonucleotide E encoding a peptide sequence located at the N-terminus of human ribophorin II [14].

Library screening

The PCR products encoding for OST48 and ribophorin I were radio-labelled with [α-^{32}P]dATP by primer extension, whereas the ribophorin II-specific 53 bp-oligonucleotide was radio-labelled with [γ-^{32}P]ATP and a kinase reaction. The radio-labelled probes were then used to screen a mixed-primed cDNA library in λgt10 from pig liver (pL1011a, Clontech) and/or an oligo(dT)-primed pig muscle cDNA library constructed in Uni-ZAP XR (Stratagene), applying standard hybridisation methods. OST48-specific cDNA inserts in the λ-clones were excised with EcoRI and subcloned into pUC BM20 for sequencing. In case of ribophorin I and II, the cDNAs were excised *in vivo* as pSK(-)Bluescript-plasmids and sequenced directly.

Construction of recombinant expression vectors and COS-1 cell transfection

The OST48-full length cDNA was reconstructed from fragment inserts excised from two independent λ-clones by EcoRI cleavage, taking advantage of an internal EagI-restriction site. The cDNA construct was then subcloned into the EcoRI site of the mammalian expression vector pcDNA3 to give the recombinant vector pcOST48. In case of ribophorin II, the full-length-cDNA was excised from pSK(-) Bluescript-plasmid by EcoRI/NotI restriction, followed by subcloning of the insert into pcDNA3 (pcRII). The full length-ribophorin I cDNA was reconstructed as follows: a cDNA fragment covering the 5′-region of the ribophorin I ORF was amplified by PCR using pig muscle Uni ZAP XR cDNA library template clones, the cloning vector-derived oligonucleotide F as sense and the ribophorin I specific oligonucleotide G as the anti-sense primer. The 291 bp PCR product containing a 184 bp sequence specific for ribophorin I, was 5′-ligated to the 1906 bp cDNA fragment obtained by EagI cleavage from a ribophorin I-specific Uni ZAP XR clone, taking advantage of the internal EagI restriction site. The cloning vector-specific sequence at the 5′-end of the cDNA construct was removed using EcoRI, followed by subcloning of the resulting 2090 bp cDNA fragment into the EcoRI/NotI site of pcDNA3 (pcRI). The cDNA encoding for the ribophorin I polypeptide tagged at its C-terminus with a myc-peptide sequence, was synthesized by PCR using ribophorin I-specific sense H and anti-sense I primers, the latter containing the myc-specific nucleotide sequence. The resulting PCR fragment was then subcloned into the EcoRI/NotI site of pcDNA3 (pcRI-myc). Expression vectors containing the cDNA for both OST48 and ribophorin I (pcOST48-RI) or OST48 and the myc-tagged ribophorin I variant (pcOST48-RI-myc), were prepared by cleaving pcOST48 with PvuI and NruI, followed by ligation of ribophorin I- and ribophorin I-myc-specific cDNA fragments which were obtained by PvuI and BstZ17I restriction cleavage from pcRI and pcRI-myc, respectively.

COS-1 cells were propagated in DMEM containing 10% fetal calf serum, 100 U/ml penicillin and 100 µg/ml streptomycin, and then subcultured 24 h prior to transfection. At $\sim$60% confluency the cells were transfected with 10 µg vector plasmid-DNA using the DEAE-dextran/chloroquine method [18]. 48 h after transfection cells were washed twice with 50 mM Tris/HCl, pH 7.2, harvested by scraping and solubilized in lysis buffer containing 50 mM Tris/HCl, pH 7.2, 0.8% Triton X-100, 1 M sucrose, 0.5 M NaAc, 10 mM $MnCl_2$ and 0.2% phosphatidylcholine.

Determination of oligosaccharyltransferase activity

OST activity in COS-1 cells transfected with pcDNA3 (control) or the various vector plasmids was measured by adding 40 µl aliquots of detergent extracts to 60 µl of lysis buffer (see above) containing 2000 cpm Dol-PP-[^{14}C]$GlcNAc_2$ and 1 mM acceptor tripeptide (N-benzoyl-Asn-Gly-Thr-$NHCH_3$). Reactions were run at 25°C and terminated by the addition of 500 µl of methanol. The methanol phase was separated, 750 µl chloroform and 150 µl water added, and the phases then separated by centrifugation. [^{14}C]Labelled glycopeptides partition into the upper aqueous phase and these were directly quantitated by scintillation counting [4].

In vitro transcription and translation

In vitro translation of OST48, ribophorin I and ribophorin II polypeptides were carried out by incubating aliquots of the TNT-T7 coupled transcription/translation system (Promega) in the presence of vector plasmids and [^{35}S]methionine as detailed in the supplier's manual. Translated polypeptides were then analysed by SDS/PAGE and autoradiography.

Preparation and purification of a polyclonal antibody against OST48

A 690 bp cDNA fragment encoding a 230 amino acid sequence of OST48 was subcloned in the correct ORF into the expression vector pQE30 in order to introduce a His_6-tag at the N-terminus. *E. coli* cells (ER2267) transformed with the resulting vector plasmid, were induced by addition of IPTG (1 mM). After 4 h, cells were harvested by centrifugation (4000 × g for 20 min) and suspended in a lysis buffer containing 8 M urea, 100 mM NaH_2PO_4 and 10 mM Tris/HCl HCl buffer, pH 8.0. After centrifugation, the supernatant was removed, supplemented with Ni-NTA-agarose and the suspension stirred for 3 h. The Ni-NTA-agarose was then separated by decantation and washed several times with lysis buffer, pH 8.0 and 6.8, respectively. The His_6-tagged OST48 polypeptide

was then eluted from the resin with 100 mM NaH_2PO_4/10 mM Tris/HCl, pH 4.5, containing 8 M urea. Aliquots of the eluate containing ~100 μg of the His_6-tagged OST48 protein, were emulsified with an equal volume of complete Freund's adjuvant and the solution injected subcutaneously into a rabbit. Immunization was repeated with ~100 μg protein in incomplete Freund's adjuvant after 3 and 6 weeks. The blood taken 3 weeks after the last injection was allowed to clot at room temperature and centrifuged at low speed to yield the serum fraction. The serum was passed over a ProteinA-Sepharose column and the bound IgG fraction eluted with 100 mM glycine, pH 3.0, followed by neutralisation of the acidic eluate with 1 M Tris/HCl, pH 8.0. The anti-OST48-antibody was then separated from the IgG fraction by affinity chromatography on a column (2 ml) containing the antigen covalently bound to Sepharose 4B [19]. The affinity eluate containing the monospecific anti-OST48-antibody was dialysed against PBS before storage at −20°C.

General methods

SDS-PAGE and Western- and immunoblotting as well as library screening, PCR amplification and site directed mutagenesis were carried out as described [19–22]. OST48 was purified by preparative SDS-PAGE and the N-terminal amino acid sequence determined by Edman degradation [23]. Immunofluorescence microscopy was carried out as detailed by Bieberich and Bause using DTAF-conjugated goat anti-rabbit IgG and TRITC-conjugated anti-mouse IgG for differential staining [18]. Nucleotide sequencing was performed using the Pharmacia ^{T7}Sequencing kit and [^{35}S]dATP-α-S as the tracer [24]. Hydrophobicity profiles were calculated using the Kyte and Doolittle method with a window size of seven residues [25]. Homology searches were carried out using the GenBank database, BLASTN and BLASTP programs. All other methods were carried out as described by Ausubel *et al.* [26] and Sambrook *et al.* [27]. The cDNA sequences of pig OST48, ribophorin I and ribophorin II were submitted to the EMBL Data Bank (accession numbers AJ293581, AJ293582 and AJ293583).

Results and discussion

cDNA cloning of OST48, ribophorin I and ribophorin II

OST48

Multiple screening of the mixed-primed cDNA library from pig liver with an OST48-specific 338 bp oligonucleotide yielded two λ-clones, allowing reconstruction of a full-length cDNA of 1618 base pairs (Figure 1A). The 1618 bp construct contained a single 1320 bp ORF corresponding to $A^{19}TG$ and TGA^{1339}, encoding a 439 amino acid polypeptide with a molecular mass of ~48.9 kDa. The authenticity of the cDNA construct was verified by recovery of the N-terminal peptide sequence of OST48 from pig liver, as well as by the observed sequence similarity at nucleotide and amino acid level with the human and dog OST48 equivalent. The 279 bp 3′-untranslated region in the 1618 bp construct terminated in a 18 bp polyA-stretch. This was preceeded by a typical AATAAA polyadenylation signal located 23 bp upstream. Analysis of the encoded OST48 polypeptide sequence using the Kyte and Doolittle method [25], revealed two stretches of hydrophobic amino acids, one being located at the N-terminus and the other close to the C-terminus (Figure 2A). The hydrophobic sequence at the N-terminus probably functions as a cleavable signal sequence. This view is supported by the observation that the N-terminal amino acid of the purified OST48 polypeptide aligns with Ser-26 in the 1320 bp ORF (Figure 1A). Cleavage of the OST48 precursor protein at Ala25-Ser26 is consistent with the specificity of signal peptidases, known to be greater for small amino acids at the −3/−1 position of the cleavage site [28]. The hydrophobic peptide sequence between Tyr-411 and Leu-430, on the other hand, displays typical features of a membrane domain, indicating that OST48 is a type I transmembrane protein, with the bulk of its N-terminal domain directed towards the lumen and a short cytosolic sequence consisting of 9 amino acids. The cytosolic peptide sequence is extremely polar containing three acidic and three basic amino acids including a double-lysine motif at the −3/−5 position, assumed to act as ER-retrieval signal [29,30]. In contrast to ribophorin I and II (see below), OST48 lacks any Asn-Xaa-Thr/Ser consensus sequence, excluding its N-glycosylation.

Ribophorin I

Screening of an oligo-dT-primed pig muscle cDNA library subcloned in UniZAP XR, using a 1295 bp cDNA probe specific for ribophorin I resulted in the isolation of a clone with a 2068 bp insert containing most of the ribophorin I ORF, as well as the complete 3′-UTR including a 20 bp polyA-stretch. The short sequence, which was lacking from the 5′-coding region, was obtained by PCR amplification using cDNA library clones as the template, a ribophorin I-specific oligonucleotide derived from the 5′-region of the 2068 bp cDNA insert as the antisense and an UniZAP XR cloning vector specific sequence as the sense primer. Under these conditions a 291 bp PCR product was amplified that contained a 107 bp sequence at its 5′end originating from the cloning vector followed downstream by 184 bp specific for ribophorin I. With this additional information a 2090 bp full-length cDNA could be reconstructed containing a 1827 bp ORF framed by a 17 bp long 5′- and by a 244 bp 3′-untranslated region. The 20 bp polyA-stretch at the 3′-end was preceded by a ATTAAA polyadenylation signal located 28 bp upstream (Figure 1B).

The 1827 bp ORF encodes 608 amino acids corresponding to a ~68.7 kDa polypeptide. The sequence of the ~68.7 kDa protein contains three potential N-glycosylation sites located at Asn-191, Asn-221 and Asn-300, respectively. Only one of these sites (Asn-300) can function as N-glycosyl acceptor site

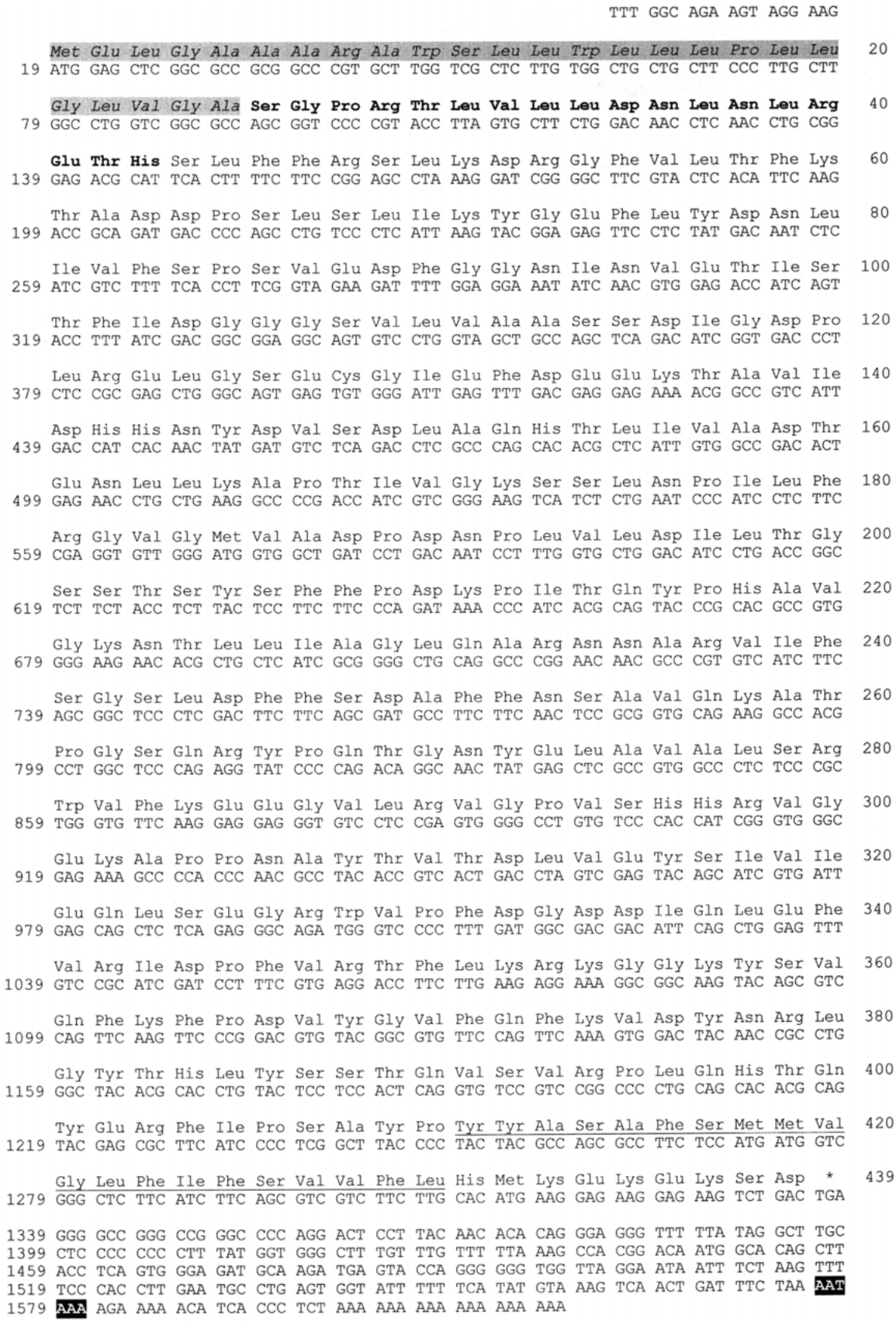

Figure 1. cDNA and derived amino acid sequence of pig OST48, ribophorin I and ribophorin II. Numbers on the left designate the nucleotide sequence of reconstructed full-length cDNAs, numbers on the right amino acids with #1 corresponding to the initiator methionine. The peptide sequence derived from pig liver OST48 (A) is shown in heavy type. Cleavable signal sequences are given in grey. Hydrophobic domains are underlined. Potential glycosylation sites are shown in boxes; polyadenylation signals are given as white on black. (**A**) OST48; (**B**) ribophorin I; (**C**) ribophorin II.

B.

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     1                                                                                                                    CG CCG GAG GTC TGG GCC

     Met Glu Ala Pro Ala Val Cys Leu Leu Pro Leu Leu Leu Leu Leu Trp Ala Trp Ala Pro Ala Pro Gly Arg Ala Ser Pro Glu Ala Leu Pro Leu Val Asn Glu  35
  18 ATG GAG GCG CCC GCA GTC TGC CTG CTA CCG CTG CTG CTG TTG CTC TGG GCC TGG GCC CCG GCG CCG GGC CGC GCC TCC CCC GAG GCG CTG CCG CTG GTC AAC GAG

     Asp Val Lys Arg Thr Val Asp Leu Ser Ser His Leu Ala Lys Val Thr Ala Glu Val Val Leu Ala His Ala Gly Ser Ser Ser Ser Pro Arg Ala Ala Ser Phe  70
 123 GAC GTG AAG CGC ACG GTG GAT CTG AGC AGC CAC CTG GCT AAG GTG ACG GCC GAG GTG GTC CTG GCG CAC GCG GGC AGC AGC TCC TCG CCC CGC GCC GCC TCC TTC

     Leu Leu Ala Leu Glu Pro Glu Leu Glu Ala Arg Leu Ala His Leu Gly Val Gln Val Lys Gly Glu Asp Glu Glu Glu Asn Asn Leu Glu Val Arg Glu Thr Lys 105
 228 CTA CTA GCC CTG GAG CCC GAG CTG GAG GCC CGC TTG GCG CAC CTC GGC GTG CAG GTG AAG GGA GAG GAT GAG GAA GAG AAC AAT CTG GAA GTG AGA GAA ACC AAA

     Ile Lys Gly Lys Arg Gly Arg Phe Phe Thr Val Thr Leu Pro Val Ala Leu Asp Pro Gly Ala Lys Ile Ser Val Thr Val Glu Thr Val Tyr Thr His Val Leu 140
 333 ATT AAG GGT AAA CGC GGG AGA TTC TTC ACA GTC ACA CTT CCA GTT GCT CTT GAT CCT GGG GCC AAG ATT TCA GTC ACT GTG GAA ACC GTT TAC ACC CAC GTG CTT

     Gln Pro Tyr Pro Thr Gln Ile Thr Gln Ser Glu Lys Gln Phe Val Val Phe Glu Gly Asn His Tyr Phe Tyr Ser Pro Tyr Pro Thr Lys Ser Gln Ser Met Arg 175
 438 CAG CCA TAT CCA ACA CAG ATC ACC CAA TCA GAG AAA CAG TTT GTG GTT TTC GAG GGG AAC CAT TAT TTC TAT TCT CCC TAC CCA ACC AAG TCA CAA AGC ATG CGT

     Val Lys Leu Ala Ser Arg Asn Val Glu Ser Tyr Thr Lys Leu Gly [Asn Pro Thr] Arg Ser Glu Asp Leu Leu Asp Tyr Gly Pro Phe Arg Asp Val Pro Pro Tyr 210
 543 GTG AAG CTC GCC TCC CGA AAC GTG GAA AGC TAC ACC AAG CTG GGG AAC CCC ACG CGC TCT GAG GAC CTC CTG GAT TAC GGG CCT TTC CGA GAT GTC CCT CCC TAT

     Ser Gln Asp Thr Phe Lys Val His Ser Glu [Asn Asn Ser] Pro Phe Leu Thr Ile Thr Ser Met Thr Arg Val Ile Glu Val Ser His Trp Gly Asn Ile Ala Val 245
 648 AGT CAG GAT ACC TTT AAA GTA CAT TCC GAG AAC AAC AGC CCC TTC CTG ACC ATC ACC AGC ATG ACA CGA GTC ATT GAA GTC TCC CAC TGG GGT AAT ATT GCT GTG

     Glu Glu Asn Val Asp Leu Lys His Thr Gly Ala Val Leu Lys Gly Pro Phe Ser Arg Tyr Asp Tyr Gln Arg Gln Pro Asp Ser Gly Ile Ser Ser Ile Arg Ser 280
 753 GAA GAA AAT GTG GAC CTG AAG CAC ACG GGG GCT GTG CTG AAG GGG CCG TTT TCC CGC TAT GAC TAT CAG AGA CAG CCA GAC AGT GGG ATC TCC TCC ATC CGT TCT

     Phe Lys Thr Ile Leu Pro Ala Ala Ala Gln Asp Val Tyr Tyr Arg Asp Glu Ile Gly [Asn Val Ser] Thr Ser His Leu Leu Ile Leu Asp Asp Ser Val Glu Met 315
 858 TTT AAG ACC ATC CTT CCT GCT GCT GCC CAG GAT GTT TAT TAC CGG GAT GAG ATT GGC AAT GTT TCC ACC AGC CAC CTT CTT ATT TTG GAC GAT TCT GTA GAG ATG

     Glu Ile Arg Pro Arg Phe Pro Leu Phe Gly Gly Trp Lys Thr His Tyr Ile Val Gly Tyr Asn Leu Pro Ser Tyr Glu Tyr Leu Tyr Asn Leu Gly Asp Gln Tyr 350
 963 GAA ATC CGG CCT CGC TTC CCT CTT TTT GGC GGG TGG AAG ACC CAC TAC ATC GTT GGC TAC AAC CTC CCA AGC TAC GAG TAC CTC TAT AAT TTG GGA GAC CAG TAT

     Ala Leu Lys Met Arg Leu Val Asp His Val Phe Asp Glu Gln Val Ile Asp Ser Leu Thr Val Lys Ile Ile Leu Pro Glu Gly Ala Lys Asn Ile Gln Val Asp 385
1068 GCA CTG AAG ATG AGG CTT GTG GAC CAT GTG TTT GAT GAG CAA GTG ATA GAC TCT CTG ACT GTG AAG ATC ATC TTG CCT GAA GGG GCC AAG AAC ATC CAG GTG GAC

     Ser Pro Tyr Glu Ile Ser Arg Ala Pro Asp Glu Leu His Tyr Thr Tyr Leu Asp Thr Phe Gly Arg Pro Val Ile Val Ala His Lys Lys Asn Leu Val Glu Gln 420
1173 AGT CCC TAC GAG ATC AGC CGA GCC CCT GAC GAG CTG CAC TAC ACC TAC CTG GAC ACG TTT GGC CGC CCC GTG ATC GTG GCC CAC AAG AAG AAC CTG GTG GAA CAG

     His Ile Gln Asp Ile Val Val His Tyr Thr Phe Asn Lys Val Leu Met Leu Gln Glu Pro Leu Leu Val Val Ala Ala Phe Tyr Ile Leu Phe Phe Thr Val Ile 455
1278 CAT ATT CAG GAC ATT GTG GTG CAC TAC ACA TTC AAC AAG GTG CTC ATG CTG CAG GAG CCC CTG CTG GTG GTG GCC GCC TTT TAC ATC CTG TTC TTC ACC GTC ATT

     Val Tyr Val Arg Leu Asp Phe Ser Ile Thr Lys Asp Pro Ala Ala Glu Ala Arg Met Lys Val Ala Cys Ile Thr Glu Gln Val Leu Thr Leu Val Asn Lys Arg 490
1383 GTC TAC GTC AGG CTG GAC TTC TCC ATC ACA AAG GAT CCG GCT GCA GAG GCC AGG ATG AAG GTG GCC TGC ATC ACA GAG CAG GTC TTG ACC CTG GTG AAC AAG AGA

     Ile Gly Leu Tyr Arg His Phe Asp Glu Thr Ile Asn Arg Tyr Lys Gln Ser Arg Asp Val Ser Thr Leu Asn Ser Gly Lys Lys Ser Leu Glu Leu Glu His Lys 525
1488 ATA GGT CTC TAC CGC CAC TTT GAT GAG ACG ATC AAT AGA TAC AAA CAG TCC CGG GAT GTG TCC ACC CTC AAC AGC GGC AAG AAG AGC CTG GAG TTG GAG CAC AAG

     Ala Leu Thr Ser Glu Val Ala Leu Leu Gln Ser Arg Leu Lys Thr Glu Gly Ser Asp Leu Cys Asp Lys Val Ser Glu Met Gln Lys Leu Asp Ala Gln Val Lys 560
1593 GCC TTG ACC AGT GAG GTG GCA CTG CTA CAG TCC AGG CTG AAG ACA GAA GGC TCT GAC CTC TGT GAC AAA GTA AGC GAA ATG CAG AAA CTG GAC GCA CAG GTC AAG

     Glu Leu Val Leu Lys Ser Ala Val Glu Ala Glu Arg Leu Val Ala Gly Lys Leu Lys Lys Asp Thr Tyr Ile Glu Asn Glu Lys Leu Ile Ser Gly Lys Arg Gln 595
1698 GAG CTG GTA CTG AAG TCA GCG GTG GAG GCC GAG CGG CTG GTG GCT GGC AAG CTC AAG AAA GAC ACA TAC ATT GAG AAT GAG AAG CTT ATC TCG GGA AAG CGC CAG

     Glu Leu Val Thr Lys Ile Asp His Ile Leu Asp Ala Leu  *                                                                                  608
1803 GAG CTG GTC ACC AAG ATC GAC CAC ATC TTG GAT GCA CTG TAG CCA TGT ACT TAC TTT AAG AAG GCA GAA TGG GGG TGG TGA CCA CCC AGG CCA GCA AAT GCT GTC

1908 TTC TGT GTC CTG CAG AAG ACC TTC AAG GAA GAG AGA AGC CAG GCC CTG CCT CAG GCA ACA CAA GAA GCT TAG TTG TTG TCC CCA GAG TTT TCT TTT TTC TTT TTT
2013 TTC CAA AAA ACT ACC ACC ACT TAA ATC CCA TTA AAA ACA AAA TAT TTT TGT GTT TTG AAA AAA AAA AAA AAA AAA AA
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Figure 1. Continued

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C.  1                                                                                                                        CG GCT CGG GCC CTG GTG GAC CTG CTC GGA GGA

     Met Ala Pro Pro Gly Ser Arg Thr Val Leu Leu Leu Ala Leu Thr Ile Ile Ala Arg Thr Gln Ala Leu Lys Pro Thr His Tyr Leu Thr Lys His Asp Val Glu  35
  33 ATG GCG CCG CCG GGT TCA AGA ACT GTC TTG CTG TTG GCC CTG ACA ATC ATA GCC AGA ACC CAG GCT CTG AAG CCC ACC CAC TAC CTC ACC AAG CAT GAC GTG GAG

     Arg Leu Lys Ala Ser Leu Asp Arg Pro Phe Thr Ser Leu Glu Ser Ala Phe Tyr Ser Ile Val Gly Leu Ser Ser Leu Gly Ala Gln Val Pro Asp Glu Lys Lys  70
 138 AGA CTG AAA GCC TCA CTG GAC CGC CCT TTC ACA AGT TTG GAA TCT GCT TTC TAC TCC ATT GTG GGA CTC AGC AGC CTT GGT GCC CAG GTG CCA GAT GAA AAG AAA

     Ala Cys Thr Phe Ile Lys Ser Asn Leu Asp Pro Ser Asn Val Asp Ser Leu Phe Tyr Pro Pro Gln Ser Ser Gln Ala Leu Ser Gly Cys Glu Ile Ser Ile Ser 105
 243 GCT TGC ACC TTC ATT AAG TCA AAC CTT GAT CCC AGC AAC GTG GAT TCC CTT TTC TAC CCT CCC CAG TCC AGC CAG GCC CTC TCA GGG TGT GAG ATC TCT ATT TCA

    [Asn Glu Thr] Lys Asp Leu Leu Leu Ala Ala Val Ser Glu Asp Ser Ser Val Thr Gln Ile Tyr His Ala Val Ala Ala Leu Ser Gly Phe Gly Leu Pro Leu Ala 140
 348 AAT GAG ACC AAA GAC CTG CTT CTG GCA GCT GTC AGT GAA GAC TCC TCT GTG ACC CAG ATC TAC CAT GCA GTT GCA GCT CTC AGT GGC TTT GGC CTT CCC TTG GCT

     Ser Gln Glu Ala Leu Gly Ala Leu Thr Ala Arg Leu Ser Lys Glu Glu Thr Val Leu Ala Thr Val Gln Ala Leu Gln Thr Ala Ser Tyr Leu Ser Gln Gln Ala 175
 453 TCC CAA GAA GCG CTC GGT GCT CTC ACT GCT CGC CTC AGC AAG GAA GAG ACC GTG CTA GCA ACG GTC CAG GCT CTG CAG ACA GCA TCC TAC CTG TCC CAG CAG GCT

     Asp Leu Arg Ser Ile Val Glu Glu Ile Glu Asp Leu Val Ala Arg Leu Asp Glu Leu Gly Gly Val Tyr Leu Gln Phe Glu Glu Gly Leu Leu Glu Thr Thr Ala 210
 558 GAC CTG AGA AGC ATC GTG GAG GAG ATT GAG GAC CTT GTC GCT CGC CTG GAT GAA CTG GGA GGT GTG TAT CTC CAG TTT GAA GAA GGA CTG CTG GAA ACA ACA GCG

     Leu Phe Val Ala Ala Thr Tyr Lys Leu Met Asp His Glu Gly Thr Glu Pro Ser Ile Lys Glu Asp Gln Val Ile Gln Leu Met Asn Thr Ile Phe Ser Lys Lys 245
 663 TTA TTT GTA GCT GCC ACC TAC AAG CTC ATG GAC CAC GAG GGG ACT GAG CCG TCC ATT AAG GAG GAT CAG GTC ATC CAG CTA ATG AAC ACC ATC TTT AGC AAG AAG

     Asn Phe Glu Ser Leu Pro Glu Ala Phe Ser Val Ala Ser Ala Ala Ala Ala Leu Ser Gln Asn Arg Tyr His Val Pro Val Val Val Val Pro Glu Gly Ser Pro 280
 768 AAC TTT GAG TCA CTC CCA GAA GCC TTC AGT GTG GCC TCT GCT GCC GCT GCA CTC TCC CAG AAT CGC TAC CAT GTG CCT GTT GTG GTT GTG CCT GAA GGC TCT CCT

     Ser Asp Thr Gln Glu Gln Ala Phe Leu Arg Leu Gln Val Thr Asn Val Leu Ser Gln Pro Leu Thr Gln Ala Thr Val Lys Leu Glu His Ala Lys Ser Val Ala 315
 873 TCC GAC ACT CAG GAA CAG GCT TTC CTG CGG TTG CAG GTC ACC AAC GTT CTG TCC CAG CCT CTG ACT CAG GCC ACT GTT AAA CTA GAA CAT GCT AAG TCT GTT GCT

     Ser Arg Ala Thr Val Leu Gln Lys Thr Ser Phe Thr Pro Val Gly Asp Val Phe Glu Leu Asn Phe Val Asn Val Lys Phe Ser Ser Gly Tyr Tyr Asp Phe Ser 350
 978 TCC AGA GCC ACG GTC CTC CAG AAG ACG TCT TTC ACC CCT GTG GGA GAT GTT TTT GAG CTA AAC TTC GTG AAT GTC AAG TTT TCC AGT GGT TAT TAT GAC TTT TCT

     Val Lys Val Glu Gly Asp Asn Arg Tyr Ile Ala Asn Thr Val Glu Leu Arg Val Lys Ile Ser Thr Glu Val Gly Ile Thr Asn Val Asp Leu Ser Thr Val Asp 385
1083 GTC AAA GTT GAG GGT GAC AAT CGT TAT ATT GCA AAT ACT GTA GAG CTT AGA GTC AAG ATT TCC ACT GAA GTT GGC ATC ACA AAT GTT GAT CTC TCC ACT GTG GAT

     Lys Asp Gln Ser Ile Ala Pro Lys Thr Thr Arg Val Thr Tyr Pro Ala Lys Ala Lys Gly Pro Phe Ile Ala Asp Ser Pro Gln Asn Phe Ala Leu Phe Phe Gln 420
1188 AAG GAT CAG AGC ATT GCA CCC AAA ACC ACA CGG GTG ACC TAC CCA GCC AAG GCC AAG GGC CCT TTC ATC GCA GAC AGC CCC CAG AAC TTT GCT TTA TTC TTC CAG

     Leu Val Asp Val Asn Thr Gly Ala Glu Leu Thr Pro His Gln Thr Phe Val Arg Leu His Asn Gln Lys Thr Gly Gln Glu Val Val Phe Val Ala Glu Pro Asp 455
1293 CTG GTA GAT GTG AAC ACT GGT GCT GAA CTC ACC CCT CAC CAG ACA TTT GTC CGA CTC CAC AAC CAG AAG ACT GGC CAG GAA GTG GTG TTT GTC GCA GAG CCA GAC

     Ser Lys Asn Val Tyr Arg Phe Glu Leu Asp Thr Ser Glu Arg Lys Ile Glu Phe Asp Ser Ala Ser Gly Thr Tyr Thr Leu Tyr Leu Ile Ile Gly Asp Ala Thr 490
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     Leu Lys Asn Pro Ile His Trp Asn Val Ala Asp Val Val Ile Arg Phe Pro Glu Glu Asp Ala Pro Ser Thr Val Leu Ser Lys Asn Leu Phe Thr Ala Lys Gln 525
1503 TTG AAG AAC CCA ATC CAC TGG AAT GTG GCT GAT GTT GTC ATC AGG TTC CCA GAA GAA GAT GCT CCG TCG ACT GTC CTG TCC AAG AAC CTT TTC ACT GCC AAA CAG

     Glu Ile Gln His Leu Phe Arg Asp Pro Glu Lys Arg Pro Pro Thr Val Val Ser Asn Thr Phe Thr Gly Leu Ile Leu Ser Pro Leu Leu Leu Leu Phe Ala Leu 560
1608 GAA ATT CAG CAT CTG TTC CGC GAC CCT GAG AAG AGG CCT CCC ACG GTG GTG TCC AAT ACA TTC ACT GGC CTG ATC CTC TCG CCC TTG CTT CTG CTC TTT GCT CTG

     Trp Ile Arg Ile Gly Ala Lys Ile Ser [Asn Phe Thr] Phe Gly Leu Thr Ile Ile Phe His Leu Gly His Ala Met Leu Ala Met Tyr Val Tyr Trp Thr Gln Leu 595
1713 TGG ATC CGG ATT GGT GCC AAA ATC TCC AAC TTC ACT TTT GGC CTA ACG ATT ATA TTT CAC CTG GGT CAT GCT ATG CTN GCT ATG TAT GTC TAC TGG ACT CAA CTC

     Asn Met Phe Gln Thr Leu Lys Tyr Leu Ala Ile Leu Gly Ser Val Thr Phe Leu Ala Gly Asn Arg Met Leu Ala Gln Gln Ala Ile Lys Arg Thr Ala His  *  629
1818 AAC ATG TTC CAG ACC CTG AAA TAC CTG GCC ATC CTG GGC AGT GTG ACA TTT TTG GCT GGC AAT CGG ATG CTG GCC CAG CAG GCA ATC AAG AGA ACA GCA CAT TAG

1923 CTC CAG AAA AAG GAA GGA GAT TCT GAA ACC TGA AGT GAA TAT CAA GAC AAG GAG TCA AGA ACA ATT CAC NGT TTG AAA AGA GGG ATT TTT AAA AAG TTT ATT TAA
2028 AAA GAA AAA AGT TAA AAT TGT GGT TAT ACT TTT GCT TGT ATT TCT ATT TCC CTG ACA CAG AGC AGA TAG ATA CCT GTG AGC CCA GAT CCT CTC TTC TTT GGC ATG
2133 GTG CCC TAG CAA GAT GCT GTG AAT ATT CTA ACT TAC CCA CAT GTT GCG TTG AAA AGT TGA AAT CTG TAA TTA AGC TAT TAT GGA ATA AAA AGA ATA ACA GGA ACA
2238 AAG AAA AAA AAA AAA AAA AAA
```

Figure 1. Continued

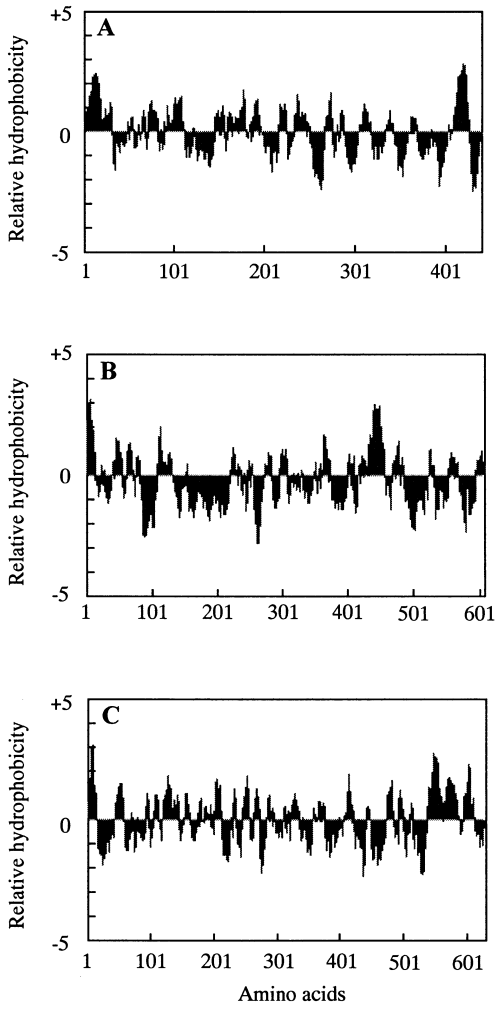

Figure 2. Hydrophobicity profiles for pig OST48, ribophorin I and ribophorin II. The hydrophobicity profiles were calculated according to Kyte and Doolittle (1982) with a window setting of seven amino acids. Numbers on the horizontal axis designate amino acid positions according to the ORFs in Figure 1 (**A**) OST48; (**B**) ribophorin I; (**C**) ribophorin II.

because a proline in the Xaa position of the Asn-191-sequon and on the C-terminal side of the Asn-221 consensus sequence (see Figure 1B) are both known to impair glycosylation [2,3]. Hydrophobicity analysis of the ~68.7 kDa polypeptide revealed a highly apolar amino acid sequence at the N-terminus displaying typical features for a cleavable signal sequence and a second hydrophobic domain between Pro-440 and Val-458 most likely used for transmembrane anchoring (Figure 2B). Thus ribophorin I possesses a type I membrane topology similar to OST48, with a ~15 kDa C-terminal domain being directed towards the cytosol and the bulk of its N-terminal domain (~52 kDa) in the lumen. The postulated type I membrane classification fits with previous observations that the N-terminal signal sequence is cleaved and that mature ribophorin I is mono-N-glycosylated, since both structural modifications are known to take place in the ER lumen [14].

Ribophorin II

On screening of the oligo-dT-primed cDNA library from pig muscle using a degenerate 53 bp oligonucleotide probe derived from human ribophorin II, several independent ribophorin II-specific λ-clones were isolated, the longest carrying a 2259 bp insert. This insert contained a 1890 bp ORF between $A^{33}TG$ and TAG^{1923}, encoding a polypeptide of 629 amino acids with a calculated molecular mass of ~69.3 kDa (Figure 1C). The 3′-UTR of the 2259 bp cDNA sequence terminated in an 18 residue polyA-sequence. Ribophorin II contains a cleavable signal sequence at its N-terminus but differs from OST48 and ribophorin I in having three adjacent stretches of hydrophobic amino acid domains close to the C-terminus (Figure 2C). Thus ribophorin II may be associated with the membrane as a type I or a type IV protein depending on whether and which of these apolar sequences are used for membrane anchoring. It remains, however, to be shown which is correct. The ~69.3 kDa sequence contains two potential N-glycosylation sites located at Asn-570 and Asn-106. Independently of whether ribophorin II possesses type I or type IV topology, Asn-570 is thought to be cytoplasmically located, with Asn-106 on the luminal side and thus probably glycosylated [14].

Sequence homology

The amino acid sequences of pig OST48, ribophorin I and ribophorin II were compared with those of OST subunits from other species. The amino acid sequence of pig OST48 is 94.3% and 93.3% identical with dog [17] and human [9] OST48, respectively. A high degree of sequence identity is also found for pig ribophorin I and pig ribophorin II when compared to the rat (RI: 93%; RII: 87.3%) and human proteins (RI: 89.9%; RII: 86.6%) [14–16]. Observed differences include mainly conservative or single exchanges, indicating considerable conservation between species.

In vitro translation of OST subunits

In order to characterize the subunit polypeptides encoded for by the ORFs, the cDNAs specific for OST48, ribophorin I and ribophorin II were subcloned into the mammalian expression vector pcDNA3 (see Methods). The resulting vector constructs (pcOST48, pcRI and pcRII) were then used to program a TNT-T7 coupled transcription/translation system (Promega), followed by SDS-PAGE analysis of [^{35}S]methionine-labelled translation products. The results summarized in Figure 3 show that, in the presence of pcOST48 (lane 1), pcRI (lane 2) and pcRII (lane 3), distinct polypeptides are synthesized having molecular masses of ~50 kDa, ~66 kDa and ~61 kDa, respectively, which differ, however, from the calculated masses, a phenomenon frequently observed with SDS gels. The size of the translation products corresponded with the molecular masses previously determined for the purified subunit proteins, confirming specificity and correctness of the vector constructs [6,10].

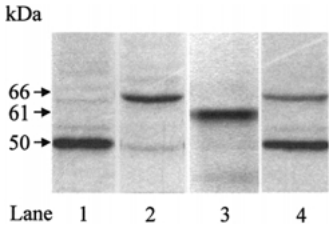

Figure 3. *In vitro* transcription/translation of pig OST subunits. Aliquots of the cell-free TNT-T7 transcription/translation system were programmed in the presence of [^{35}S]methionine with either pcOST48 (lane 1), pcRI (lane 2), pcRII (lane 3) or pcOST48-RI encoding both OST48 and ribophorin I (lane 4), followed by SDS-PAGE separation of [^{35}S]labelled translation products and autoradiography.

In addition to pcOST48, pcRI and pcRII, an expression vector was constructed containing the cDNA of both OST48 and ribophorin I (pcOST48-RI). The rationale for pcOST48-RI synthesis was to ensure that after COS-1 cell transfection both subunit proteins are over-expressed in the same cell. As expected, incubation of the TNT system with pcOST48-RI yielded two translation products (Figure 3, lane 4), whose molecular masses were identical with those of the polypeptides synthesized in the presence of either pcOST48 (lane 1) or pcRI (lane 2). This indicated that the OST48 and ribophorin I sequence is correctly encoded by pcOST48-RI and that both subunits are synthesized efficiently.

COS-1 cell expression and characterization of OST-subunits

In order to investigate which of the OST subunits are responsible for catalytic activity, COS-1 cells were transfected with pcOST48, pcRI and pcRII, respectively. Detergent extracts of the cells were then analysed by SDS-PAGE/immunoblotting and the over-expressed proteins tested for OST activity using Dol-PP-[^{14}C]GlcNAc$_2$ and N-benzoyl-Asn-Gly-Thr-N-methylamide as the substrates [4]. The results summarized in Figure 4 show that after transfection with pcOST48, a $\sim$48 kDa polypeptide is over-expressed and specifically immunostained by an anti-OST48-antibody (lane 2). The molecular mass of this product, which is not seen in control cells under the loading conditions used (lane 1), corresponded with that of the OST48 polypeptide calculated from its ORF, taking into account signal peptide cleavage. Furthermore, its apparent molecular mass was identical with that of an anti-OST48-antibody-positive protein from pig liver microsomes (not shown). Despite over-expression of the OST48 protein, no increase in OST activity could be detected in transfected cells. Similarly, negative results were obtained after COS-1 cell transfection with either the pcRI or the pcRII vector, although in these cases, over-expression of ribophorin I and ribophorin II was difficult to demonstrate reliably by immunoblot analysis due to the high endogenous level of both proteins. The expression of inactive subunits could mean either that (i) OST48, ribophorin I and ribophorin II are not directly involved in OST catalysis although this is highly unlikely; or (ii) the over-expressed polypeptides are essential for catalysis but are unable to fold correctly; or (iii) the expression of OST activity requires the presence of other subunit components.

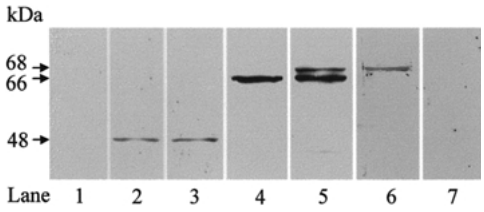

Figure 4. Immunoblot analysis of pig OST subunits. Detergent extracts were prepared from control cells (lanes 1, 4 and 7) and COS-1 cells transfected with pcOST48 (lane 2) or pcOST48-RI-myc (lanes 3, 5 and 6) and aliquots of the cell lysates containing $\sim$15 μg protein, were analysed by SDS-PAGE and immunoblotting. Over-expressed subunit proteins were stained using either anti-OST48-antibodies (lanes 1–3), anti-ribophorin I-antibodies (lanes 4 and 5) or anti-myc-antibodies (lanes 6 and 7) for detection. Endogenous OST48 is only detectable on gels after over-loading with protein.

From previous observations, which showed that a catalytically active OST enzyme preparation purified from pig liver consisted of OST48 and ribophorin I, the inference can be drawn that these two subunits are involved in catalysis [10]. In order to examine this possibility in more detail, COS-1 cells were co-transfected with pcOST48 and pcRI, or transfected with a vector plasmid containing both the cDNA for OST48 and ribophorin I (pcOST48-RI). Since, due to the high level of endogenous ribophorin I, over-expression of this subunit was difficult to demonstrate, an additional pcDNA3-derived vector was constructed which encoded for both OST48 and ribophorin I tagged at its C-terminus with a myc-specific sequence motif (pcOST48-RI-myc). This structural modification was expected to facilitate discrimination between endogenous ribophorin I and its over-expressed (hybrid) form by using an anti-myc antibody. The result of a typical experiment using the pcOST48-RI-myc vector for transfection is shown in Figure 4. As can be seen, the anti-OST48 antibody and the anti-myc antibody immunostained a $\sim$48 kDa (lane 3) and a $\sim$68 kDa (lane 6) protein specifically. Both protein bands cannot be detected in control cells (lane 1 and 7). The $\sim$68 kDa ribophorin I hybrid protein which is slightly larger than endogenous ribophorin I due to the attached myc-peptide tag, is also recognized by the anti-ribophorin I-antibody (upper band, in lane 5). This shows that both OST48 and the ribophorin I myc-hybrid protein are efficiently over-expressed, and that the ribophorin I sequence is encoded correctly by the pcOST48-RI-myc vector cDNA. Because this vector plasmid was derived from pcOST48-RI, we assume that ribophorin I is also co-expressed with OST48 after COS-1 cell transfection with pcOST48-RI.

The OST activity measured under standard assay conditions in detergent extracts of COS-1 cells co-expressing

Table 2. Relative transferase activity in COS-1 cells over-expressing OST subunits. COS-1 cells were transfected with either pcOST48, pcRI, pcRII, pcOST48-RI or pcOST48-RI-myc or co-transfected with pcOST48 and pcRI, followed by determination of the OST activity in detergent extracts using a standard assay (see Methods). The relative OST activities represent mean values of six independent transfection experiments with 100% relative activity in control cells. The numbers are calculated based on initial glycosylation rates, followed by normalisation using the protein concentration

Expression vector	*Relative OST activity (%)*
pcDNA3 (control)	100
pcOST48	103 ± 12
pcRI	100 ± 4
pcRII	100 ± 6
pcOST48-RI or pcOST48-RI-myc or pcOST48 plus pcRI	125 ± 15*

*No further increase was seen when OST48, ribophorin I and ribophorin II were co-expressed.

OST48/ribophorin I or OST48/ribophorin I-myc are summarized in Table 2. For comparison, the Table also includes the results of activity measurements obtained after over-expression of OST48, ribophorin I and ribophorin II alone. The numbers given represent mean values for six independent transfection experiments, normalized against control cells. The relative OST activity in control cells is not altered after over-expression of either OST48, ribophorin I or ribophorin II. On the other hand, co-expression of OST48 and ribophorin I or OST48 together with myc-tagged ribophorin I was found to result in an approximately ~25% increase of OST activity compared with the activity of control cells. Although rather moderate, this increase in OST activity was reproducible and did not depend on whether COS-1 cells were transfected with either pcOST48 and pcRI, pcOST48-RI, or pcOST48-RI-myc. Co-expression of OST48 with ribophorin I and ribophorin II produced no further increase. These observations indicate that both OST48 and ribophorin I may indeed represent the catalytic subunits in the hetero-oligomeric OST complex.

The double-lysine motif in the cytosolic peptide domain of the OST48 polypeptide confers ER-residency

The OST complex is associated with the ER membrane since asparagine-linked glycosylation is a co-translational event. In order to examine whether OST48 and ribophorin I polypeptides contain structural information mediating ER residency, or whether ER retention requires their interaction with other components of the OST complex, COS-1 cells were transfected using either pcOST48, pcRI-myc or pcOST48-RI-myc, followed by analysis of the subcellular location of the over-expressed proteins by immunofluorescence microscopy. Immunostaining of OST48 and myc-tagged ribophorin I was carried out using polyclonal rabbit antibodies against OST48 and a monoclonal mouse anti-myc-antibody. Protein-antibody complexes were tagged using fluorescently labelled rabbit- and mouse-specific anti-IgG-antibodies, thus allowing differential staining of both OST48 and myc-tagged ribophorin I in the same cell. Typical fluorescence patterns, obtained from detergent-permeabilized COS-1 cells transfected with the pcOST48-RI-myc double vector, are shown in Figure 5A and B. Both the anti-OST48- (A) and the anti-myc-antibody (B) reacted similarly to give a diffuse reticular network staining,

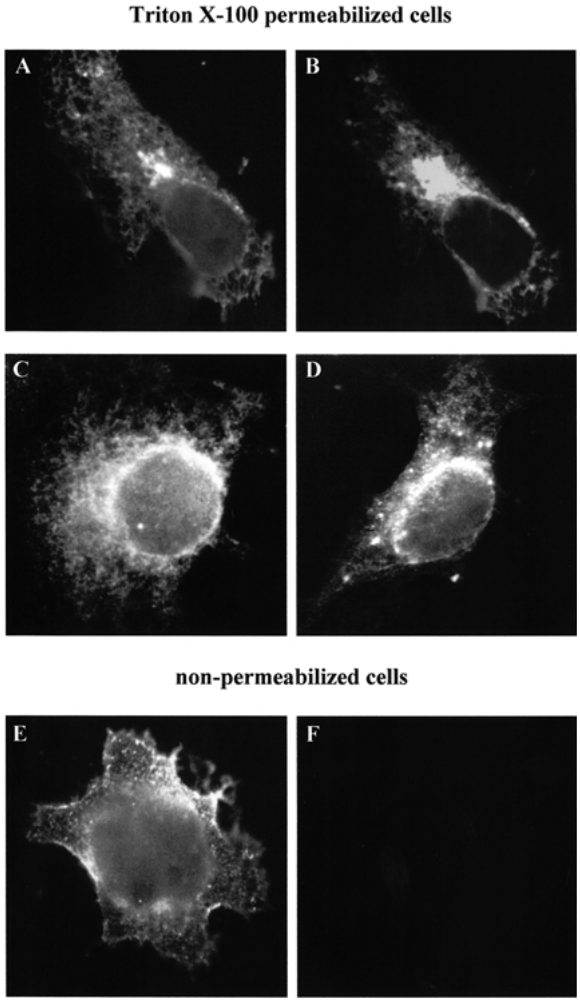

Figure 5. Immuno-localisation of OST48, OST48-Leu and myc-tagged ribophorin I. COS-1 cells were transfected with pcOST48-RI-myc (**A, B**), pcOST48 (**C, F**), pcRI-myc (**D**) or pcOST48-Leu (**E**) and fixed either with formaldehyde (**E, F**) or fixed and then permeabilised with Triton X-100 (**A–D**). Immunostaining was carried out as described by Bieberich and Bause (1995) using mouse anti-myc-antibodies (**B, D**) and rabbit anti-OST48-antibodies (**A, C, E, F**), followed by detection of antigen-antibody complexes with goat anti-mouse-IgG-(**B, D**) and goat anti-rabbit-IgG-antibodies (**A, C, E, F**) tagged with a fluorescein (DTAF) and rhodamine (TRITC) label, respectively. Differential staining for OST48 and myc-tagged ribophorin I in the same cell is shown in (**A**) and (**B**). Plasma membrane staining for OST48-Leu is only observed in non-permeabilised cells in the absence of co-expressed ribophorin I (**E**).

typical for endoplasmic reticulum structures. No immunostaining was detectable with either antibody after COS-1 cell transfection with pcDNA3. These observations show that OST48 and myc-tagged ribophorin I are co-expressed as ER-resident proteins. A similar fluorescence pattern was obtained after COS-1 cell transfection with either pcOST48 (Figure 5C) or pcRI-myc (Figure 5D), indicating that both OST48 and ribophorin I carry information for ER-localisation.

OST48 is a type I transmembrane protein containing a double-lysine motif at the $-3/-5$ position of its cytosolic domain (see Figure 1A). In order to investigate whether this sequence motif could be involved in ER localisation, the lysine residue at the -3 position was substituted by leucine and the corresponding vector construct (pcOST48-Leu) used for COS-1 cell transfection. As shown in Figure 5E, non-permeabilised COS-1 cells over-expressing the OST48-Leu mutant protein exhibit an intense immunofluorescent labelling of the plasma membrane which is not seen after COS-1 cell transfection with pcOST48 (Figure 5F). We conclude from this observation that the double-lysine motif is able to confer ER localisation on the OST48 protein. Although expressed in COS-1 cells as an ER-resident protein (Figure 5D), the sequence of ribophorin I lacks any recognizable motifs known to mediate ER retention or retrieval. This indicates that ribophorin I must be retained in the ER by a different mechanism, most likely involving the luminal domain [31]. Cell surface staining was not detectable when the OST48-Leu mutant was over-expressed together with ribophorin I or myc-tagged ribophorin I. Thus retention of OST48-Leu in the ER by interaction with ribophorin I suggests in turn that the double-lysine motif has no primary function for the ER localisation of this subunit.

Conclusions

This paper describes the cDNA cloning, COS-1 cell expression and the functional characterization of the subunits of the pig OST complex. The results show that co-expression of OST48 together with ribophorin I, or the myc-tagged ribophorin I variant, resulted in a modest ($\sim 25\%$) but reproducible increase of OST activity not seen after COS-1 cell expression of either OST48, ribophorin I or ribophorin II alone. We conclude from this observation that both OST48 and ribophorin I may be essential for OST activity and thus represent the catalytic subunits in the hetero-oligomeric protein complex. This conclusion is consistent with previous findings that a catalytically active OST preparation, purified from pig liver, consisted of only these two subunits [10]. We do not yet know whether only one of the two subunits is involved in transferase catalysis and substrate binding, or whether peptide glycosylation requires the concerted action of both OST48 and ribophorin I. In this context it is worth pointing out that ribophorin I contains a highly hydrophobic (transmembrane) domain which could serve as binding site for the Dol-PP-activated oligosaccharide substrate similar to the putative dolichol recognition sequence found in yeast glycosyltransferases [32]. By contrast, Pathak *et al*. suggest using inhibition and competition experiments that Wbp lp, the yeast equivalent of mammalian OST48, is involved in catalysis as well as in glycolipid binding [33]. The observation that OST48 and ribophorin I are double-labelled after incubation of the pig liver enzyme in the presence of an inhibitor peptide and Dol-PP-[^{14}C]oligosaccharides would, on the other hand, favour a concerted contribution to catalysis exerted by both [12].

Several reasons can be put forward for the low level ($\sim 25\%$) of over-expressed OST activity: (i) the high background activity in COS-1 cells together with the transfection yield (generally between 10 and 20%), interferes with the measured activity; (ii) the dimeric OST complex expressed in COS-1 cells is catalytically active but unstable. Taking into account the experimental conditions under which cells were cultured (48 h at 37°C), this possibility appears not unlikely because the dimeric OST48/ribophorin I complex, isolated from pig liver [10] was found to lose activity rapidly after separation of other subunits ($\sim 50\%$ loss on 3–5 h storage at 4°C) (unpublished results). This indicates that other subunit components, although not directly involved in catalysis, may stabilize transferase activity; (iii) the over-expressed OST48 and ribophorin I polypeptide chains fail to fold correctly in the absence of other subunits. This would point to polypeptide folding as well as subunit association into the membrane-bound OST complex requiring both temporally and spatially controlled interactions between individual subunits. Support for this interpretation is provided by recent studies on glucosidase II [23]. This processing enzyme is associated with the membrane as a dimeric protein complex consisting of a large catalytic α-subunit and a smaller β-subunit, the latter containing an ER-retrieval signal of the HDEL type [30]. Whereas the α-subunit was purified from pig liver as a distinct and catalytically active protein, expression of glucosidase II activity in COS-1 cells was found to be linked to the presence of both the α- and the β-subunits [23, 34]. This indicates that the β-subunit is needed for and actively involved in α-subunit maturation, although it appears not to be required once the α-subunit has adopted its catalytically active conformation.

The OST48 polypeptide, which is expressed in COS-1 cells as an ER-resident protein, contains a double-lysine motif in the $-3/-5$ position of its cytosolic domain. The observation that after replacement of the lysine at the -3 position by leucine the OST48-Leu mutant is expressed on the cell surface indicates that this sequence motif may be operative in ER localisation and, furthermore, that the OST48 polypeptide lacks additional structural information conferring ER-retention. This implies that OST48 is retained within the ER either by the double-lysine motif, or by interaction with other subunits of the OST complex which do not leave from the ER. We favour the latter possibility because the OST48-Leu mutant is retained in the ER when co-expressed with ribophorin I. Similar observations have been made for rat

ribophorin I and a dog OST48 mutant in which the two lysines have been replaced by serine, after co-expression in HeLa cells [22]. This suggests that the double-lysine motif is not relevant for OST48 localisation except in so far as it may serve as a retrieval signal for molecules which have escaped the ER lumen.

Acknowledgements

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