The 48-kDa Subunit of the Mammalian Oligosaccharyltransferase Complex Is Homologous to the Essential Yeast Protein WBPl*

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Oligosaccharyltransferase has been purified from canine microsomal membranes as a protein complex with three nonidentical subunits of 66, 63/64, and 48 kDa. The 66- and 63/64-kDa subunits were found to be identical to ribophorins I and II, respectively. The ribophorins are integral membrane glycoproteins that were previously shown to be localized exclusively to the rough endoplasmic reticulum. The 48-kDa subunit (OST48) of the oligosaccharyltransferase complex is not a glycoprotein and is not recognized by antibodies to either ribophorin. Here, we describe the characterization of a cDNA clone that encodes OST48. Like ribophorins I and II, OST48 was found to be an integral membrane protein, with the majority of the polypeptide located within the lumen of the endoplasmic reticulum. OST48 does not show significant amino acid sequence homology to either ribophorin I or II. A 45-kDa integral membrane protein, designated WBPl, from the yeast Saccharomyces cerevisiae was found to be 25% identical in sequence to OST48. Recently, WBPl was shown to be essential for in vivo and in vitro expression of oligosaccharyltransferase activity in yeast. We conclude that OST48 and WBPl are homologous gene products.

Asparagine-linked glycosylation of proteins occurs within the lumen of the rough endoplasmic reticulum (RER) during or shortly after transport of the nascent polypeptide across the membrane bilayer (1,2). The enzyme oligosaccharyltransferase catalyzes the transfer of a high mannose oligosaccharide (GlcNAcMan,Glc) onto asparagine acceptor sites within an Asn-X-Ser/Thr consensus motif, with X denoting any amino acid with the exception of proline (3). The dolichol-linked oligosaccharide donor ( dolichol-P-P-GlcNAcMan-Glc) for the transferase reaction is reassembled by the addition of monosaccharides to dolichol phosphate by the sequential action of a series of membrane-bound glycosyltransferases (3).

Oligosaccharyltransferase has been purified from canine pancreas microsomal membranes as a protein complex consisting of three polypeptide subunits: ribophorins I and II and a 48-kDa polypeptide (4). Oligosaccharyltransferase activity, as well as the protein complex consisting of ribophorins I and II and the 48-kDa polypeptide, can be immunodepleted from partially purified enzyme preparations by antibodies that recognize the cytoplasmic domain of ribophorin I (4). Ribophorins I and II are integral membrane glycoproteins of the rough, but not smooth, endoplasmic reticulum (5) that can be cross-linked to endogenous membrane-bound ribosomes (6). A functionally significant relationship between the ribophorins and ribosomes engaged in protein translocation was suggested by the intriguing discovery that the RER contains roughly equimolar amounts of the ribophorins and membrane-bound ribosomes (7). Although several subsequent reports suggest that the ribophorins do not participate directly in ribosome binding (8,9), antibodies directed against the cytoplasmic domain of ribophorin I inhibit protein translocation by interfering with ribosome targeting to the membrane (10). Ribophorin I, and by analogy the oligosaccharyltransferase complex, is presumably located in the immediate vicinity of the protein-conducting channels through which polypeptides are transported across the RER (11).

The protein sequences of ribophorins I and II have been deduced by analysis of rat and human cDNA clones (12-14). Both ribophorins contain an amino-terminal cleavable signal sequence for initiating translocation across the RER (15). The membrane-spanning segments of ribophorins I and II are located toward the carboxyl terminus of the polypeptide; hence, the majority of both proteins is located within the lumen of the RER (12,13). The nonglycosylated 48-kDa subunit of the oligosaccharyltransferase (OST48) was not recognized by antibodies to ribophorins I and II, suggesting that OST48 was not derived from either ribophorin by prolyl-lys (4).

The finding that the oligosaccharyltransferase is a protein complex rather than a single polypeptide raised several questions concerning the structure of the complex and the function of the individual subunits. The membrane-spanning segment of ribophorin I contains a sequence that matches a proposed recognition element for the dolichol moiety of the lipid-linked oligosaccharide substrate of the enzyme (4,16). To date, biochemical or genetic data supporting a direct interaction between the proposed dolichol recognition element and a lipid-linked oligosaccharide have not been presented for any glycosyltransferase, so the functional significance of such a site within ribophorin I remains to be evaluated. Here, we describe the isolation of a cDNA clone that encodes the 48-kDa subunit of the oligosaccharyltransferase. Analysis of the amino acid sequence of OST48 revealed hydrophobic segments near the amino and carboxyl termini of the deduced protein sequence. The former hydrophobic sequence resembles a typical RER signal sequence, whereas the latter sequence resembles a membrane-spanning segment or stop-transfer sequence. The sequence of OST48 did not show

23658
DNA Sequencing—PstI-PstI and BamHI-PstI subclones in M13mp18 and M13mp19 were prepared from plasmid pOST48-11B. DNA sequencing of both strands of pOST48-11B was by the dideoxy chain termination method using deoxyadenosine 5’-3’[35S]triphosphate (Du Pont-New England Nuclear). Sequences (United States Biologicals) (24), and either single-stranded M13 templates (21) or double-stranded pTEX plasmid templates (24). The M13 universal primer and synthetic oligodeoxynucleotides complementary to internal sequences were used as sequencing primers. Oligonucleotides were synthesized using an Applied Biosystems Model 392 DNA/RNA synthesizer. The sequence shown in Fig. 2 is a composite of the sequence of the complete 1.6-kb insert in pPOST48-11B (nucleotides 19-1597) and the 5’-sequence from the 1.6-kb insert in pPOST48-2 (nucleotides 1-18). Both strands of the cDNA insert in pPOST48-11B were sequenced. Both strands of pPOST48-2 were sequenced between the 5’-multiple cloning sequence and the first internal sequencing primer. DNA sequence analysis and protein sequence comparisons were done with MacVector (BDH), Microgenie™ (Beckman Instruments), and DNA Star software programs.

**RESULTS**

cDNA Cloning of OST48—Oligosaccharyltransferase was purified from canine pancreas microsomal membranes as a complex consisting of three polypeptide subunits (4). Preparative polyacrylamide gel electrophoresis in the presence of SDS resolved the 48-kDa subunit (ribophorin I) and 63/64-kDa (ribophorin II) subunits. After electrophoretic transfer to a nitrocellulose membrane, the 48-kDa subunit was localized by staining the nitrocellulose sheet with Ponceau S. Internal tryptic peptides released by in situ digestion of the immobilized 48-kDa subunit with trypsin were resolved by gas-phase sequencing (Table I). Together, the four peptides correspond to ~16% of the OST48 protein. Two degenerate oligonucleotide PCR primers were synthesized based on the amino acid sequence of the largest tryptic peptide (Fig. 1A). We obtained a PCR product of the anticipated size (91 base pairs) using plasmid DNA from a MDCK cell cDNA library as the template. The PCR product was eluted from a polyacrylamide gel and cloned into M13mp18 for DNA sequencing and for preparation of hybridization probes. DNA sequencing

### Table 1

| Peptide | Amino acid sequence
|---------|---------------------|
| 1       | YSGSYNGYLAVALSR    |
| 2       | GQGKQDNPPLDILT     |
| 3       | SVPFD*DIQLEFY       |
| 4       | VGETAPPNNT*TDLVEYSIVIEQL |

*Residues denoted by asterisks were not obtained in sufficient yield to assign an amino acid.
confirmed that the 91-base pair band was an authentic amplification product of an OST48 cDNA (Fig. 1A). A cDNA encoding the 48-kDa subunit of the oligosaccharyltransferase was obtained by colony hybridization screening of the MDCK cell cDNA library using a single-stranded hybridization probe derived from the PCR product. Two hybridization-positive clones were identified in the initial screen. The size of the cDNA insert was estimated by agarose gel electrophoresis after digestion of positive plasmids with restriction enzymes. Five additional positive colonies were obtained that harbored plasmids containing inserts of -1.6 kb. The complete sequence of the pOST48-11B cDNA insert corresponds to nucleotides 1-18 in Fig. 2. Assuming that the cellular RNA contains a typical poly(A) tract of -200 nucleotides, we can conclude that 49,631. In addition to the first AUG codon (nucleotides 4-6), there is a second in-frame AUG codon near the 5'-end of the cDNA insert (nucleotides 22-24). The context of both AUG codons is favorable for initiation of translation in that purines are present at positions -3 and +4 relative to the A of the AUG codon (31). Assuming that the 5'-sequence obtained from pOST48-2 is derived from an authentic OST48 mRNA, then it is likely that initiation of translation occurs at the AUG codon that is closest to the 5'-end of the mRNA (31). Exceptions to this rule occur when the first AUG codon is located within 10 nucleotides of the mRNA cap site. Further analysis will be required to determine the length of the 5'-noncoding region of the OST48 mRNA.

Northern blot analysis using the antisense strand of the PCR product as a probe revealed a mRNA of ~17 kb in the total RNA isolated from COS, Chinese hamster ovary, and MDCK cells (Fig. 3, lanes a, b, and d, respectively). OST48 mRNA of a similar size was detected in poly(A)-selected mRNA isolated from canine pancreas (lane c). The migration position of a 1.7-kb T7 RNA polymerase transcription product of the OST48 cDNA is designated by the asterisk. The 3'-noncoding segment of the cDNA insert in pOST48-11B contained 40-45 adenosine residues in addition to those shown in Fig. 2. Assuming that the cellular RNA contains a typical polyadenosine tract of ~200 nucleotides, we can conclude that
the cDNA sequence determined here is derived from a nearly full-length mRNA.

The locations of the four tryptic peptides from which amino acid sequence was obtained are indicated in Fig. 2 (solid underlining). The agreement between the cDNA-derived sequence and the peptide sequence data was exact for all amino acid residues within the tryptic peptides that were unambiguously assigned. Hydropathy analysis using the method of Kyte and Doolittle (32) revealed the presence of two major hydrophobic protein segments, which are indicated by the negative hydropolarity values in the graph in Fig. 2. The amino-terminal hydrophobic segment located between residues 15 and 31 resembles a cleavable signal sequence for initiating translocation across the endoplasmic reticulum (33, 34). Eukaryotic signal sequences range in length from 13 to 36 amino acids and are composed of three structural regions: an amino-terminal basic region (n region), a central hydrophobic segment (h region), and a more polar carboxy-terminal region (c region) (33). These three regions are readily identified within the first 31 residues of OST48. Signal peptidase cleavage sites are located in the c region, with small uncharged amino acids located at positions −1 and −3 relative to the processing site (33). The signal peptidase processing site in OST48 is predicted to be between residues 31 and 32 using the weight-matrix method of von Heijne (34). The calculated Mₐ of 46,141 for the processed protein is in reasonable agreement with the Mₐ of 48,000 estimated from mobility on SDS-polyacrylamide gels. A second nonpolar segment located between residues 417 and 436 is of sufficient length and hydrophobicity to be a membrane-spanning segment. Based upon this arrangement of hydrophobic segments, we would anticipate that the majority of OST48 is located within the endoplasmic reticulum lumen, with residues 437–445 located in the cytoplasm. The predicted cytoplasmic domain of OST48 is 9 amino acids in length and is highly charged. Membrane proteins that contain a single spanning segment and a carboxy-terminal cytoplasmic domain are typically designated as type I integral membrane proteins. OST48 does not contain an Asn-X-Ser/Thr consensus site for Asn-linked glycosylation, consistent with our previous observation that the 48-kDa subunit of the oligosaccharyltransferase complex is not glycosylated (4).

In Vitro Membrane Integration of OST48—The membrane orientation of OST48 was investigated by in vitro translation of an OST48 mRNA transcript. The cDNA inserts from plasmids pOST48-11B and pOST48-2 were subcloned into pGEM-4 to allow transcription of mRNA with T7 RNA polymerase. Translation of the mRNA transcripts in a rabbit reticulocyte lysate translation system yielded mRNA-dependent translation products with apparent Mₐ values of 50 (Fig. 4A, lanes a and c). The translation product encoded by the OST48-2 RNA transcript migrated slightly less rapidly than that encoded by the OST48-11B transcript, consistent with the presence of 6 additional amino-terminal amino acids. As expected, both AUG codons appear to be adequate for initiation of translation in the in vitro system. The inclusion of canine pancreas microsomal membranes increased the mobility of the translation product, consistent with proteolytic removal of an amino-terminal signal sequence (lanes b and d). The processed forms derived from the two precursors had identical mobilities, indicating that the 6 amino-terminal residues encoded by the pOST48-2 insert were not essential for the function of the signal sequence. The processed form of the in vitro translation product co-migrated with the Coomassie Blue-stained 48-kDa subunit of the oligosaccharyltransferase complex (data not shown).

Translation products produced in the absence and presence of microsomal membranes were subjected to extraction with sodium carbonate (pH 11.5) to determine whether OST48 behaves as an integral membrane protein or as a peripheral membrane protein or luminal content protein (Fig. 4A, lanes e–h). Luminal content proteins, as well as peripheral mem-

![Fig. 3. Northern blot analysis of RNA. Total RNA (30 µg) isolated from COS (lane a), Chinese hamster ovary (lane b), or MDCK (lane d) cells or 5 µg of canine pancreas poly(A) RNA (lane c) was denatured with glyoxal, electrophoresed, blotted, and hybridized as described “Experimental Procedures.” Molecular sizes (in kilobases) of RNA markers are shown. The migration position of a 1.7-kb mRNA the cDNA sequence determined here is derived from a nearly full-length mRNA.](image-url)
brane proteins, are extracted from RER-derived vesicles by sodium carbonate because the membrane vesicles are converted into sheets (35). The precursor form of OST48 (pOST48) was recovered in the supernatant (S) fraction irrespective of whether membranes were included in the \textit{in vitro} translation reaction (lanes e and g). A significant fraction of OST48 sedimented with the membrane pellet (P) after alkaline extraction (lane h). The incomplete recovery of proteolytically processed OST48 in the pellet fraction suggests that the \textit{in vitro} integrated form of OST48 may be less stably associated with the membrane bilayer than several other integral proteins that have been analyzed by this procedure (30, 36). Nonetheless, the selective recovery of OST48 in the pellet fraction supports the identification of the carboxy-terminal hydrophobic sequence of OST48 as a membrane-spanning segment.

The \textit{in vitro} translation products were digested with proteases to examine the membrane orientation of \textit{in vitro} integrated OST48 (Fig. 4B). Trypsin was initially selected for these protease protection experiments due to the presence of 3 leucine residues within the putative 9-amino acid cytoplasmic domain of OST48. Although no increase in migration of OST48 was observed following trypsin digestion (lanes d and e), cleavage at all but the most amino-terminal of these three sites would probably not cause a detectable alteration in the mobility of OST48 on an SDS-polyacrylamide gel. Because an incomplete digestion of OST48 in detergent-permeabilized vesicles was routinely observed (lane f), additional protease protection experiments were conducted using proteinase K. As expected, the 50-kDa precursor form of OST48 was completely digested by proteinase K when translated in the absence (lanes a–c) or presence (lanes d, g, and h) of microsomal membranes. The \textit{in vitro} integrated form of OST48 was protected from digestion by proteinase K (lane g) unless the membranes were permeabilized with detergent (lane h). Taken together, the results shown in Figs. 2 and 4 support the conclusion that OST48 is a type I integral membrane protein with a very small cytoplasmic domain, a single membrane-spanning segment, and a large luminal domain.

Homology between OST48 and WBPl—A search of DNA (GenBank™ and EMBL) and protein (National Biomedical Research Foundation) sequence data bases disclosed a homology between OST48 and WBPl, a 45-kDa membrane protein from the yeast \textit{Saccharomyces cerevisiae}. The WBPl gene was initially cloned as a possible nuclear pore protein (17). The WBPl gene encodes the 48-kDa protein present in the oligosaccharyltransferase preparation. First, the protein sequence encoded by the cDNA clone contains the sequences of three tryptic peptides in addition to the one used to design the PCR primers. Second, the \textit{in vitro} synthesized and membrane-integrated form of OST48 co-migrates with purified OST48 on SDS-polyacrylamide gels. Finally, the absence of consensus sites for Asn-linked glycosylation and the presence of a membrane-spanning segment are consistent with the available data concerning OST48.

**FIG. 5. Sequence alignment between 48-kDa subunit of oligosaccharyltransferase (OST48) and 45-kDa yeast protein (WBPl).** Identical amino acids are \textit{boxed}. Conservative replacements are designated by \textit{colons} using the following similarity rules: $G = A, T = C; R = A, G, T; N = A, C, G, T; Y = R, D; W = A, R; M = F, L, I, V; F = F; E = D; Q = N; K = R, H; D = E; Q = N; M = I = L; V = V; \text{ and } F = W = Y$. Gaps are indicated by \textit{dashes}. Sequence alignment was performed using the \textit{Amino Acid Align} (AANW) program from DNA Star. Within the overlapping region of 433 amino acids, the two sequences are 24.6% identical and 50% homologous. Two consensus sites in WBPl for Asn-linked glycosylation are \textit{underlined}. Signal peptidease cleavage sites are indicated by \textit{arrows}. The boundaries of the predicted membrane-spanning segments of OST48 and WBPl are indicated by \textit{asterisks}. The boundaries of the WBPl membrane-spanning segment are taken from te Heesen et al. (17).
Hydropathy analysis of OST48 revealed the presence of two hydrophobic segments located near the extreme amino and carboxyl termini of OST48. The amino-terminal hydrophobic segment was shown by in vitro translation experiments to function as a cleavable signal sequence for initiating translocation across the endoplasmic reticulum. The amino terminus of endogenous canine OST48 corresponds to serine 32. Thus, the authentic signal peptide cleavage site for OST48 agrees with the predicted signal peptide cleavage site. The in vitro integration experiments suggest that the second hydrophobic segment functions as a membrane-spanning segment. Although further experimentation will be required to analyze the membrane topology of endogenous canine OST48, the current evidence suggests that OST48 is a type I integral membrane protein with the majority of the predicted 9-amino acid cytoplasmic domain of OST48 is present in many tissues from different vertebrate species (13, 37). The conclusions that have been drawn from the analysis of WBPI are in good agreement with our findings concerning the mammalian oligosaccharyltransferase complex. The finding that overexpression of the WBPI protein does not increase enzyme activity suggests that the yeast oligosaccharyltransferase is a protein complex (38). A compelling argument supporting a role for OST48 (WBPI) in Asn-linked glycosylation has now been provided by the fact that two entirely independent approaches led to the identification of this protein as a subunit of the oligosaccharyltransferase complex. Current studies in our laboratory are directed toward the purification of the yeast oligosaccharyltransferase complex. It will be of interest to determine whether the yeast enzyme has subunits that are homologous to the ribophorins.

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