Interactions among Subunits of the Oligosaccharyltransferase Complex*

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The mammalian oligosaccharyltransferase (OST) is an oligomeric complex composed of three membrane proteins of the endoplasmic reticulum: ribophorin I (RI), ribophorin II (RII), and OST48. In addition, sequence homology between the Ost2 subunit of the yeast OST complex and Dad1 (defender against apoptotic death) suggests that Dad1 may represent a fourth subunit of the mammalian OST complex. In attempts to elucidate the structural organization of this complex, we have studied the interactions among its subunits. Using the yeast two-hybrid system, we have shown that the luminal domains of RI and RII (RIL and RIIL, respectively) interacted with the luminal domain of OST48 (OST48L), but no direct interaction was observed between RIL and RIIL. These results were confirmed by biochemical assays. Deletion analyses using the yeast two-hybrid system showed that subdomain of RIL or RIIL adjacent to the respective transmembrane domains interacted with OST48L. Of the three equal length subdomains of OST48L, the one at the N terminus and the one next to the transmembrane domain interacted with RIL. None of these three subdomains of OST48L interacted with RIIL. The yeast two-hybrid assay also revealed affinity between the cytoplasmically located N-terminal region of Dad1 and the short cytoplasmic tail of OST48, thus placing Dad1 firmly into the OST complex. In addition, we found a homotypic interaction between the cytoplasmic domains of RI, which may play a role in the formation of the oligomeric array formed by components of the translocation machinery.

During their translocation into the lumen of the rough endoplasmic reticulum, polypeptides made on membrane-bounded polysomes may be cotranslationally modified by N-glycosylation (1, 2). During this process, the oligosaccharyltransferase catalyzes the transfer of high mannose oligosaccharides, which are preassembled on lipid-anchored dolicholpyrophosphate moieties, to certain asparagine residues facing the lumen of the ER (3). The mammalian OST was first isolated by incubating high salt-extracted dog pancreas rough microsomes with non-ionic detergents, followed by purification on sucrose density gradients and by ion exchange chromatography (4). It was found that the OST forms an oligomeric complex that sediments in a sucrose gradient as a 10 S particle. This complex was composed of three integral membrane proteins, of which ribophorin I (RI) (5, 6) and OST48 (7) are type I transmembrane proteins with most of their polypeptide chains facing the lumen of the ER (Fig. 1). The third member of the complex, ribophorin II (RII), also has a large luminal domain, but the disposition of the transmembrane and cytoplasmic domains is less clear: although this region bears three hydrophobic domains (6, 8), only the one most proximal to the N terminus is of sufficient length and hydrophobicity to function as a typical transmembrane domain and RII may, therefore, also have a type I disposition, like RI and OST48 (Fig. 1). The OST complex was later purified from other mammalian species (9, 10), as well as from yeast (11). The yeast OST complex contains apparently six subunits (11–16), and the mammalian one included originally only three subunits (4). On the basis of homology to the yeast OST proteins, it was possible to identify another mammals protein, Dad1 (defender against apoptotic death) was originally cloned with the yeast OST complex (11, 16), Dad1 represented a possible fourth subunit of the mammalian OST complex. This conclusion was most recently confirmed when Dad1 was shown to copurify with RIL, RII, and OST48 (18). Dad1 is a small hydrophobic protein with a cytoplasmically located N terminus and up to three transmembrane domains (2) (Fig. 1). As is true for the other subunits of the OST, the precise role of Dad1 in N-glycosylation is not known.

In native rough microsomal membranes, components of the OST complex such as the ribophorins are in close proximity to membrane-bound ribosomes, since they can be chemically cross-linked to the 60 S ribosomal subunit (19). Furthermore, site-specific antibodies directed against the cytoplasmic domain of RI, but not those against the luminal domain of the protein, inhibited the targeting to microsomal membranes of ribosome-nascent chain complexes formed in a cell-free translation mixture (20). These results suggest that the ribophorins are also adjacent to the polypeptide translocation site. This conclusion is in agreement with recent studies, showing that during the purification of the Sec61p complex, in which dog pancreas rough microsomes were extracted with certain detergents at different ionic strength, the subunits of the OST complex, RI and RII and OST48, remained tightly associated with the Sec61p complex (21). The Sec61p complex, which is composed of an α, β, and γ subunit, is a key component of the translocation apparatus since it not only forms the translocation pore but has also ribosome binding activity (21, 22).

It may be expected that the enzymatic activity of the OST...
complex is dependent on the integrity of the oligomeric assembly of its subunits. The long range goal of our studies is to understand the contribution of each OST protein to the overall function of the complex, which is likely to be related to the spatial relationship among the components of OST complex. We present here the first information about specific interactions among the OST subunits. Using the yeast two-hybrid approach in conjunction with in vitro binding studies, we demonstrated that the luminal domain of OST48 interacts with the luminal domains of RI and RII, but we detected no direct interactions between RIL and RIIL. Furthermore, we found that the cytoplasmically located N-terminal region of Dad1 interacts with the cytoplasmic tail of OST48 and that cytoplasmic domains of RI interact with each other in a homotypic fashion.

**EXPERIMENTAL PROCEDURES**

**Yeast Vectors and Plasmid Construction**—The vectors pEG202 and pJG4-5 used in the yeast two-hybrid assay were obtained from Drs. Russ Finley and Roger Brent (Massachusetts General Hospital, Boston) (23). The rat RI and RII cDNAs were cloned in this laboratory by Harnik-Ort et al. (5) and Pirozzi et al. (8), respectively. The dog OST48 cDNA was a gift of Dr. Reid Gilmore (University of Massachusetts Medical School, Worcester) (7). Full-length human Dad1 cDNA was amplified from a HeLa cDNA library (provided by Dr. Li Zhang, New York University Medical Center, New York) using the following primers for PCR: sense strand primer, 5′-GGG TGG GTA TCG GTG TCT-3′; and antisense strand primer, 5′-CCC GAC TCA GCC AAG AAA GGT CAT GAC-3′. To make either bait or prey plasmids encoding the constructs shown in Table I, we generated cDNAs encoding these domains by PCR using full-length cDNAs as templates and the respective primers that provided also EcoRI and XhoI restriction sites. All fragments were ligated into the EcoRI and XhoI sites of pEG202, downstream of the LexA DNA binding domain, and into the same sites of pG4-5, downstream of B42/HA. Transformation of bacteria and preparation of plasmids were done according to standard molecular biology techniques (24). To generate the construct encoding a single copy of the cytoplasmic domain of OST48 (OST48C1, amino acids 406–414), two oligonucleotides (sense strand oligonucleotide 5′-AATTC CAC ATG AAG GAG AAG GAG AAA TCT GAC GTA GGA TCC C 5′ and antisense strand oligonucleotide 5′-TCTAG GTC TAC C GA TCA GCC AAC AAA GGT CAT GAC-3′) were synthesized. Since OST48C1 is encoded by only 27 nucleotides, it is impossible to check the insert through enzyme digestion of the cloned constructs. We, therefore, introduced a BamHI restriction site after the stop codon. To generate three repeats of OST48C1 (OST48C3), synthetic oligonucleotides (sense strand oligonucleotide 5′-AATTC CAC ATG AAG GAG AAG GAG AAA TCT GAC GTA GGA TCC C 5′ and antisense strand oligonucleotide 5′-TCTAG GTC TAC C GA TCA GCC AAC AAA GGT CAT GAC-3′) were synthesized. Sense strand and antisense strand oligonucleotides were phosphorylated and annealed as described previously (24) and ligated into same sites of pEG202 and pG4-5 as described above. To subclone OST48L into pAA, a plasmid that contains LEU2 as a selectable marker (gift of Dr. Eric Chang, New York University, New York) (25), the PCR-generated cDNA encoding OST48L was inserted in frame between Nhel and Sall sites of pAA. All constructions were confirmed by sequencing on the 373 DNA sequencer (ABI, Foster City, CA) according to the manufacturer’s protocols.

**Yeast Transformation**—The yeast strain EGY48 (MATa ura3-52 his3 trpl LexAop-LEU2) was provided by Drs. Russ Finley and Roger Brent (23, 24), was used for transformation. The techniques used to apply the yeast two-hybrid system were previously described (26). The yeast transformants were grown at 30 °C for 3 days on SD plates that contain 2% dextrose and a mixture of amino acids that lacks histidine and tryptophan as well as uracil. The colonies were then transferred to X-gal plates that contain 2% galactose and 1% raffinose, an amino acid mixture that lacks histidine, tryptophan, and leucine as well as uracil, and then cultured further at 30 °C for another 3 days.

**RESULTS**

**Interactions of the Luminal Domains of Both RI and RII with the Luminal Domain of OST48 Were Detected by the Yeast Two-hybrid System**—Fig. 1 shows a schematic representation of four mammalian OST subunits. To detect potential interactions among the luminal domains of these OST subunits, we subcloned the cDNAs encoding RIL1-1,115, RIIL1-1,516, or OST48L1-1,865 (see Fig. 1) into the cloning site of yeast plasmids pEG202 (27) and pJG4-5 (28) containing the β-galactosidase reporter plasmid pSH18-34 were then transformed with different pairs of plasmids, such as pEG202-OST48L/pJG4-5-RIL, pEG202-OST48L/pJG4-5-RIIL, and pEG202-RIL/pJG4-5-RIL, to express LexA and B42 fusion proteins. As detected on the X-gal plate shown in Fig. 2A,
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The Interactions between Different Subunits of the OST Complex Can Be Confirmed Biochemically—To confirm the interactions between OST48L and RIL or RIIL by an independent approach, we established a biochemical assay. In this assay, GST or a GST-OST48L fusion protein expressed in the bacteria (Fig. 3A) was incubated with[^35S]methionine-labeled RIL or RIIL which was synthesized in an in vitro transcription/translation system (Fig. 3B). After extensive washing with buffer containing 0.5% Triton X-100 and 1 mM dithiothreitol, bound proteins were eluted with free glutathio-
A fusion protein expressed in bacteria was purified using glutathione-agarose beads, subjected to SDS-PAGE, and stained with Coomassie Blue. cDNAs encoding RI or RIIL was incubated with GST or GST-OST48L fusion protein and eluted with free glutathione. Samples were analyzed by SDS-PAGE followed by autoradiography (for details, see “Experimental Procedures”).

For the observed interactions with OST48L, we again used the yeast two-hybrid assay and expressed fusion proteins with B42. For further details, see legend to Fig. 2A. B, that the fusion proteins used in A were expressed at comparable levels was confirmed by Western blot analysis. For details see legend to Fig. 2B.

To localize the regions within OST48L that are responsible for interacting with RI and RIIL, we divided OST48L into three subdomains of equal length, OST48L, OST48L, and OST48L, and tested them for interactions with RI and RIIL in the yeast two-hybrid system. We observed that the one-third portion at the N terminus of OST48L (OST48L) and another portion next to the transmembrane domain of OST48L (OST48L) interacted with RIIL, but no interaction was seen between the middle region (OST48L, j) and RIIL (Fig. 4A; Table I). On the other hand, none of the three subdomains of OST48L interacted with RIIL (data not shown), suggesting that either the RIIL-binding sites within OST48L were destroyed by the deletions or that the entire structure of OST48L is required for its interaction with RIIL. In these experiments, Western blot analysis using anti-HA tag antibody showed that subdomains of RI, RIIL, and OST48L fused with B42/HA were expressed at comparable levels (Fig. 4B, for details, see also legend to Fig. 2B).

The Yeast Two-hybrid System Allowed Us to Detect Interactions between the Cytoplasmic Domain of OST48 and the N-terminal Tail of Dad1, as Well as Homotypic Interactions of the Cytoplasmic Domains of RI—We have also tested whether the cytoplasmic domains of RI, RIIL, and Dad1 interact with each other. The cDNAs encoding the cytoplasmic domains of RI and RIIL (RIC and RIIC, respectively) and the cytoplasmically located N-terminal domain of Dad1 (Dad1) were subcloned into pEG202 and pJG4-5 at the same sites as described above. Since the cytoplasmic domain of OST48L is only 9 amino acids long, we initially inserted a cDNA encoding three repeats of this domain (OST48C3) into pEG202 and pJG4-5. By expressing pairwise combination of these constructs in the yeast two-hybrid system, we found that OST48C3 interacted with

The interactions revealed in the yeast two-hybrid system (Fig. 2A). Although only about 1% of the labeled RI and RIIL added to the incubation mixture bound specifically to GST-OST48L, this result was highly reproducible (three independent experiments). It is not entirely surprising that the level of specific binding is low, since the proper folding of the in vitro synthesized proteins as well as of the GST-OST48L fusion protein made in bacteria is a prerequisite for this assay to function.

Characterization of Subdomains within RI, RIIL, and OST48L Involved in Interactions among the OST Subunits—To determine which regions within RI and RIIL are responsible for the observed interactions with OST48L, we used the yeast two-hybrid assay and expressed fusion proteins with subdomains of RI and RIIL. Initially, we tested for the interaction of OST48L with either the N-terminal halves of RI and RIIL (RI1–207 and RI1–258, respectively; see Fig. 1) or the C-terminal halves of RI and RIIL (RI208–415 and RI259–516, respectively; see also Fig. 1). We found that the C-terminal halves of RI (RI208–415) and RIIL (RI259–516) are mainly responsible for the interactions with OST48L (Fig. 4A, a and e; Table I), whereas the N-terminal halves do not interact with OST48L (Fig. 4A, a and e; Table I). To define even more narrowly the interacting domains within RI (RI208–415 or RI259–516), we generated the deletion mutants RI208–311, RI312–415, RI259–387, and RI388–516 (see also Table I). Results shown in Fig. 4A revealed that RI312–415 (d) and RI388–516 (h), which are adjacent to the respective transmembrane domains of RI and RIIL, have high affinity for OST48L, whereas essentially no interactions were observed between OST48L and RI208–311 (c) or RI259–387 (g) (see also Table I). In the set of liquid assays where OST48L was expressed as a LexA fusion protein, the background was rather high, and β-galactosidase activity units of up to 18 were observed.

To localize the regions within OST48L that are responsible for interacting with RI and RIIL, we divided OST48L into three subdomains of equal length, OST48L, OST48L, and OST48L, and subjected to SDS-PAGE, and stained with Coomassie Blue. The translation mixtures were analyzed by SDS-PAGE followed by autoradiography.
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Dad11–28 (see Table I). Using the yeast two-hybrid system, HA-RIC, B42-HARIIC, and B42-HA-Dad11–28 was confirmed by Western blot analysis (for detail, see legend to Fig. 2B). The Seyg two-hybrid system has been used successfully to characterize interactions of nuclear and/or cytoplasmic proteins and the cytoplasmic or extracellular domains of membrane proteins from a variety of organisms (30–33). In the yeast two-hybrid system, + + , the B42-HA fusion proteins with OST48C3, RIIC, and RIC were not expressed in yeast cells as LexA or B42 fusion proteins. The possible interactions of four pairs were tested in the yeast two-hybrid system as indicated in the figure. For further details, see legend to Fig. 2A. B, the expression of all B42 fusion proteins including B42-HA-OST48C3, B42-HA-OST48C3, B42-HA-RIIC, B42-HARIIC, and B42-HA-Dad11–28 was confirmed by Western blot analysis (for detail, see legend to Fig. 2B).

Dad11–28 (Fig. 5A, a; Table I). The possibility that OST48C3 by itself contains a transcriptional activating domain, leading to a false positive result, was ruled out by exchanging the inserts between the two plasmids (data not shown). We found consistently the β-galactosidase activity on the X-gal plate higher (+ + , Fig. 5A) than expected from the results of the liquid assay (10 units, Table I). An interaction of apparently similar strength was obtained when only one copy of the cytoplasmic domain of OST48 (OST48C1) was tested for interaction with Dad11–28 (see Table I). Using the yeast two-hybrid system, apparently no interaction was observed between RIC and Dad11–28 or between RIC and OST48C3, since results in both the X-gal plate assay and the liquid assay were negative (Fig. 1, 5A, b and c; Table I). Similarly, RIIC does not interact with RIC, Dad11–28, or OST48C3 (data not shown). That the fusion protein LexA-RIIC encoded by pEG202-RIIC did enter the nucleus was demonstrated by the repression assay since the expression of LexA-RIIC in yeast cells blocked synthesis of the lacZ gene product (data not shown). We also tested for homotypic interactions between the cytoplasmic domain of RI by co-expressing RIC in the yeast cells as LexA and B42/HA fusion proteins (Fig. 5A, d; Table I). A weak but consistently positive reaction was observed on the X-gal plate. Based on the rate of color development, the homotypic interaction is not as strong as the interaction between OST48C3 and Dad11–28 (compare Fig. 5A, a–d). As in previous experiments, we demonstrated that the B42-HA fusion proteins with OST48C3, RIC, RIIC, and Dad11–28 were expressed at comparable levels by performing Western blots (Fig. 5B).

DISCUSSION

The yeast two-hybrid system has been used successfully to characterize interactions of nuclear and/or cytoplasmic proteins and the cytoplasmic or extracellular domains of membrane proteins from a variety of organisms (30–33). In the experiments described here, the yeast two-hybrid system was employed for the first time to detect interactions between protein domains that are normally within the ER lumen, which is topologically equivalent to the extracellular space. In contrast to the cytosol and the nucleoplasm, the lumen of the ER is characterized by high calcium concentrations. It is also the site where RIL and RIIL are normally N-glycosylated (1). Since the yeast two-hybrid test functions in the nucleoplasm of yeast cells, which is not a high calcium environment, it appears that interactions between the luminal domain of OST48 and those of RI and RIIL are not dependent on the presence of high calcium levels. Likewise, it appears that RI and RIIL, which are glycosylated in the native state, but not in the yeast two-hybrid system, do not need to be glycosylated to interact with OST48. Evidence that N-glycosylation of RIIL may not be required for its proper oligomerization and functioning is provided by the fact that, in vivo, only about half of the molecules are glycosylated (6).

As mentioned in the Introduction, the oligosaccharyltransferase activity was first purified from canine pancreas rough microsomal membranes, and initially three subunits RI, RII, and OST48 were identified as part of an oligomeric complex (4). Later the corresponding yeast enzyme was isolated and shown to contain Ost1p, Swp1p, Wbp1p, and Ost2p (which are homologous to the mammalian OST subunits RI, RII, OST48, and Dad1, respectively) as well as two additional polypeptides (Ost3p and Ost5p). The first four subunits are essential for viability of haploid yeast cells and are required for oligosaccharyltransferase activity as demonstrated by in vivo and in vitro assays (11–13, 16, 34). It was observed that Ost2p, a subunit of the yeast complex, showed 40% sequence identity with the mammalian protein Dad1 as described before. Dad1 was characterized as an integral membrane protein that suppresses apoptotic cell death and a specific temperature-sensitive mutation in the corresponding gene triggered apoptosis at the non-permissive temperature (17). Taken together, these findings suggested that Dad1 is a fourth subunit of the mammalian OST complex, which was recently confirmed by biochemical means (18). It appears therefore that underglycosylation of certain proteins caused by the temperature-sensitive point mutation in the dad1 gene results in apoptosis. The notion that inhibition of N-glycosylation may indeed lead to apoptosis was recently demonstrated by treating human promyelocytic HL-60 cells with tunicamycin. It was shown that this drug, which interferes with the synthesis of dolichol-linked oligosaccharide, triggers the apoptotic death of these cells (35).

In the present study we provide evidence that Dad1 interacts with OST48 via cytoplasmically exposed domains, which strongly supports the notion that Dad1 is indeed a fourth subunit of the mammalian oligosaccharyltransferase complex.

The finding that the cytoplasmic domains of RI interact with each other in a homotypic fashion is not completely unexpected. Our previous analysis of the conformation of the RI sequence revealed that the cytoplasmic portion of RI is likely to form α-helical domains (5), and helical wheel analysis provided evidence for two short heptad repeats (amino acids 527–537 and 572–583). The formation of leucine zipper-like structures may, therefore, interconnect adjacent oligosaccharyltransferase complexes. This type of interaction may be part of the mechanism that integrates the OST complex into a proteinaceous network formed by the components of translocation apparatus. The existence of such a proteinaceous network has been inferred from our previous results showing that the components of the translocation apparatus, including membrane-bound ribosomes, form large oligomeric arrays that define the domain of the rough endoplasmic reticulum and are physically excluded from small transport vesicles that bud from the transitional elements of the ER (1, 19, 36). Since the oligosaccharyltransferase complex is located in the immediate vicinity of the protein...
translocation channel (19, 20) and tightly bound to the Sec61p complex (21), interactions among these components would provide a mechanism to segregate the components of the translocation apparatus, including the OST complex, to the rough domain of the ER (36). Since the cytoplasmic domain of RI is not conserved in its yeast homologue Ost1, the homotypic interaction between RIC may be a unique feature of mammalian cells.

Our findings concerning the interactions between the mammalian OST subunits, OST48 and RII, are also supported by genetic tests and chemical cross-linking studies performed on the yeast OST. te Heesen et al. (12) have shown that overexpression of SWP1 results in the suppression of the temperature-sensitive phenotype of the WBP1-2 mutation and that Swp1p can be cross-linked to Wbp1p when yeast membrane fractions, solubilized with Triton X-100, are treated with a reversible cross-linking reagent (12). A sequence comparison of RII with its yeast homologue Swp1p reveals that the Swp1p represents a truncated form of RII lacking about two-thirds of the N-terminal portion of the luminal domain (13). The conserved luminal domain of Swp1p is homologous to the subdomain of RII (RII398–516) that is responsible for the interaction with OST48L. By analogy to our findings on the mammalian oligosaccharyltransferase complex, this portion of Swp1p is expected to interact with the luminal domain of Wbp1. Support for the observed interaction between Dad1 and OST48 was also obtained for the yeast homologues. Silberstein et al. (16) demonstrated that the OST2 gene is a suppressor of the WBP1-2 mutation and that overexpression of Ost2p increases the stability of Wbp1p and Swp1p (16). These results suggest a direct physical interaction among Wbp1, Swp1, and Ost2, which is analogous to the interactions that occur among the mammalian homologues OST48, RII, and Dad1. Although the sequence homology between the mammalian OST subunits and their yeast counterparts is not very high (22–28% identity for RII, OST48, and RI and 40% for Dad1) (2), the near neighbor relationships between the subunits of the OST complexes of these phylogenetically distant species appear to be well conserved. This may be expected, since the reactions catalyzed by the yeast or the mammalian enzyme complexes are identical. A more detailed structural analysis of both OST complexes may, in fact, allow us to identify functionally important domains within the respective subunits.

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