Dimeric organization of the yeast oligosaccharyl transferase complex

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The enzyme complex oligosaccharyl transferase (OT) catalyzes N-glycosylation in the lumen of the endoplasmic reticulum. The yeast OT complex is composed of nine subunits, all of which are transmembrane proteins. Several lines of evidence, including our previous split-ubiquitin studies, have suggested an oligomeric organization of the OT complex, but the exact oligomeric nature has been unclear. By FLAG epitope tagging the Ost4p subunit of the OT complex, we purified the OT enzyme complex by using the nondenaturing detergent digitonin and a one-step immunoaffinity technique. The digitonin-solubilized OT complex was catalytically active, and all nine subunits were present in the enzyme complex. The purified OT complex had an apparent mass of ≈ 500 kDa, suggesting a dimeric configuration, which was confirmed by biochemical studies. EM showed homogenous individual particles and revealed a dimeric structure of the OT complexes that was consistent with our biochemical studies. A 3D structure of the dimeric OT complex at 25-Å resolution was reconstructed from EM images. We suggest that the dimeric structure of OT might be required for effective association with the translocon dimer and for its allosteric regulation during cotranslational glycosylation.

electron microscopy | membrane protein purification | protein N-glycosylation

After translocation across the endoplasmic reticulum (ER) membrane, newly synthesized polypeptides are N-glycosylated by the oligosaccharyl transferase (OT) enzyme complex in the lumen of the ER (for recent reviews on OT, see refs. 1 and 2). The catalytic activity of the OT complex involves transfer of an oligosaccharide chain from the donor substrate, dolichol-P-P-oligosaccharide, to certain -Asn-X-Ser/Thr- (-N-X-S/T-) sites in the nascent polypeptide chains. The OT enzyme complex is a multisubunit protein complex. In yeast, OT consists of nine different subunits, all of which contain one or more predicted transmembrane segments. In yeast, five of these proteins, Swp1p, Wbp1p, Ost2p, Ost1p, and Stt3p, are encoded by essential genes; four others that are not essential, Ost3p, Ost4p, Ost5p, and Ost6p, are also part of the OT complex. All yeast OT subunits have been cloned and sequenced (3–8), and the structure of one of them, Ost4p, has been elucidated by solution NMR (9). Homologs of all of the yeast OT subunits have been identified in higher eukaryotic organisms (3–6).

Although the OT enzyme complex catalyzes one of the most crucial and abundant posttranslational protein modifications, leading to the biosynthesis of an important class of macromolecules in the cell (namely, N-glycoproteins), very little information about its mechanistic aspects is available. Studies in the last two decades have provided clues to the possible functions of the OT subunits in the N-glycosylation reaction, but only recently has it been established that one of the OT subunits, Stt3p, bears the active site of the OT reaction (10–12). Ost3p and Ost6p are believed to perform redundant functions in the OT reaction because the two proteins exhibit sequence similarity and strikingly similar hydropathy plots (13). It has been postulated that two isoforms of the OT complex may exist in the yeast ER

membrane, which are marked by the presence of either Ost3p or Ost6p (14–16). Ost3p and Ost6p may determine the association of the two isoforms with the two translocon complexes: namely, the Sec61 and Ssh1 complexes, respectively (15). Ost4p is proposed to be responsible for recruiting Ost3p or Ost6p into the OT complex (16). Other studies have indicated that the donor substrate may be recognized by Wbp1p (17–19). Recently, it has been reported that the luminal domain of Wbp1p may possess the divalent metal ion-binding site (20), which has been shown to be essential for the catalysis of the OT reaction (21). Because Ost1p has been found to cross-link with a number of proteins, irrespective of their glycosylation status (22–24), it is proposed that the luminal domain of Ost1p may be involved in funneling the newly synthesized polypeptides into the active site of the OT reaction (24). However, it is important to note that with the exception of Stt3p, the contributions of the other OT subunits in the N-glycosylation reaction are highly speculative and based on indirect evidence. A possible conformational arrangement of the yeast OT complex has been proposed based on studies using a chemical cross-linker and the split-ubiquitin two-hybrid technique (22, 25). Other researchers have proposed the presence of three subcomplexes within the OT complex (26). Steady-state kinetic experiments have suggested an allosteric regulatory mechanism for the binding of the donor substrate (27). However, to date, no structural studies of the OT complex have been reported that could allow us to test these mechanistic or conformational proposals. In this report, we describe a one-step immunoaffinity chromatographic purification procedure for the OT complex. We show that all known nine subunits are present in the purified OT complex. Our biochemical analyses suggest a dimeric structure of the digitonin-solubilized yeast OT complex. We further report the structural analysis of the yeast OT complex by EM and single-particle image reconstruction. The structural result is in agreement with our biochemical studies.

Results and Discussion

Immunoaffinity Purification of the OT Complex. The OT complex is composed of nine subunits, so it was important to confirm the presence of all subunits in the purified preparation. Because we have antibodies to all of the OT subunits except Ost4p, Ost3p, and Ost6p, we generated a yeast strain in which genes encoding Ost4p, Ost3p, and Ost6p were epitope-tagged. We chose to epitope tag the C terminus of these three subunits because it has been previously demonstrated that the C-terminal tagging of these subunits does not lead to any growth defect (22, 26, 28, 29). We tagged the chromosomal copies of these three subunits and did not express them on an overexpression plasmid, thereby

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Abbreviations: OT, oligosaccharyl transferase; HRP, horseradish peroxidase; HA, hemagglutinin; ER, endoplasmic reticulum.

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eliminating the possibility of artifactual complex formation. The genes encoding Ost4p, Ost3p, and Ost6p were 3FLAG-, triple hemagglutinin (3HA)-, and 13MYC-tagged, respectively. Because Swp1p, Ost6p, and Ost3p are known to be of similar mobility on SDS/PAGE gels (13), addition of 13MYC and 3HA at the C termini of Ost6p and Ost3p increased their molecular mass to ≈ 60 and ≈ 37 kDa, respectively, allowing their easy identification by SDS/PAGE.

Because all components of the OT complex are transmembrane proteins that are residents of the ER membrane, it was important to solubilize the microsomes in an appropriate detergent that did not denature any of the subunits. Digitonin has been the detergent of choice of many researchers for the purification of the active OT complex (4, 30, 31), although others have reported purification of catalytically active OT complexes by use of detergents such as Nikkol and Nonidet P-40 (13, 14, 32). Although a comparative study of OT activity in various detergents has not been reported yet, digitonin seems to be an ideal detergent for the purification of the OT complex because we found that the digitonin-solubilized microsomal extract retains high OT activity even after storage for several days at 4°C (data not shown).

The OT complex has been purified from various mammalian and yeast sources by using a combination of chromatographic techniques (3–8, 18, 27, 30, 31, 33, 34), and other studies have used the immunoaffinity technique (13, 27, 34, 35). The purification of the OT complex was first carried out from canine pancreatic microsomes as a complex of three polypeptides: ribophorin I, ribophorin II, and OST48 (mammalian homologs of Ost1p, Swp1p, and Wbp1p, respectively) (3, 33). A similar composition was then reported for the avian, human, and pig OT complexes (6–8, 31). In early studies, the purification of the yeast enzyme complex was carried out, which indicated that the complex might be composed of four to six subunits (18, 30, 34, 35). In a later study, all subunits were reported to be present in the purified OT complex based on their migration on SDS/PAGE gels (27). However, the presence of some of the OT subunits could not be confirmed in this study by Western blotting, presumably because of the unavailability of antibodies against Ost3p, Ost4p, and Ost5p. Recently, the purification of the isoforms of the mammalian OT complex was reported, but the presence of homologs of Ost4p and Ost5p was not tested (4), again presumably because of an unavailability of antibodies. The purification of the yeast OT complex that detected all subunits except Ost4p has also been reported (13, 35). Because it has been previously demonstrated that, after solubilization with digitonin, one can coprecipitate all of the other OT subunits by immunoprecipitating one OT subunit (26), we decided to employ an immunoaffinity technique for the purification of the OT complex.

Having solubilized the microsomes in digitonin buffer, we immunoprecipitated Ost4p by using an anti-FLAG affinity gel. Analysis of the precipitate by Western blotting revealed that all of the other OT subunits coprecipitated along with Ost4p (Fig. 1A). We used a 3XFLAG peptide to elute the OT complex in its native form from the anti-FLAG beads. The eluant was analyzed under nondenaturing conditions by using blue native gels. Blue native gels are believed to provide reliable estimates of the molecular mass of protein complexes (36). After silver staining of the blue native gel, a protein complex with an molecular mass of ≈ 500 kDa was observed (Fig. 1B). Western blots of the blue native gel using antibodies against Stt3p and Ost1p or an anti-FLAG mAb (Fig. 1C) revealed that the three antibodies recognized the 500-kDa protein complex, suggesting that this band corresponds to the OT complex. We further separated the components of the OT complex by SDS/PAGE followed by silver staining as seen in Fig. 1D. We also analyzed the sample by Western blot, which was probed with antibodies specific to all nine subunits of the OT complex (Fig. 1E). On comparison of

Fig. 1. Biochemical characterization of the immunopurified yeast OT complex. (A) All OT subunits coprecipitate along with Ost4p when microsomes are solubilized in digitonin buffer. The OT complex was immobilized on anti-FLAG beads as described in *Materials and Methods*. After washing with wash buffer, the anti-FLAG beads (20- μ l settled bed) were resuspended in SDS/PAGE sample buffer (200 μ l) containing 10% 2-mercaptoethanol and incubated at 55°C for 20 min. The supernatant was collected by centrifugation. Twenty microliters of the sample was applied per lane on SDS/PAGE gels. After electroblotting, the blots were probed with polyclonal antisera specific to Ost1p, Ost2p, Ost5p, Stt3p, Wbp1p, or Swp1p to identify the respective proteins. The blots were also probed with polyclonal anti-HA or anti-Myc or with monoclonal anti-FLAG antibodies to identify the Ost3-HAp, Ost6-Mycp, and Ost4-FLAGp subunits. (B) Analysis of purified OT on a blue native gel. After eluting the OT complex from anti-FLAG beads by using 3XFLAG peptide, 20 μ l of the eluant was mixed with blue native gel sample buffer and applied on the gel, which was stained with silver reagent. (C) Western blot after blue native gel of purified OT using the indicated antibodies. Five microliters of eluant was applied per lane on blue native gel for electroblotting. (D) Silver-stained SDS/PAGE of purified OT. The eluant (20 μ l) was applied in one lane for SDS/PAGE. The gel was stained with silver reagent. The star indicates two bands that were identified as proteolytic C-terminal fragments of Wbp1p (data not shown). (E) Western blotting after SDS/PAGE of purified OT using polyclonal antibodies against Stt3p, Myc epitope, Ost1p, Wbp1p, HA epitope, Swp1p, Ost2p, and Ost5p. A monoclonal M2 antibody against FLAG epitope was used to stain Ost4p. Two microliters of eluant was applied per lane on SDS/PAGE gel for electroblotting. Molecular mass markers are indicated. For immunoblotting, primary and HRP-coupled secondary antibodies were used at a dilution of 1:3,000. The HRP-conjugated secondary antibodies were visualized with the LumiGLO kit (Kirkegaard & Perry Laboratories, Gaithersburg, MD).

the silver-stained gel and Western blots (Fig. 1D and E), we found that all of the subunits of the OT complex were present in the purified sample, and bands corresponding to each subunit were easily visible on silver-stained gels. No other extra band was visible on SDS/PAGE gel, suggesting that the one-step immunoaffinity procedure yields pure OT complex.

Fig. 3. Individual OT particles are dimers as revealed by EM. (A and C) Raw electron micrographs of OT particles embedded in uranyl acetate stain and in vitreous ice, respectively. (Scale bar, which applies to A and C: 100 nm.) (B and D) Selected 2D class averages of OT particle images in stain and in vitreous ice, respectively. (Scale bar: 20 nm.) (E) Class averages with a near in-plane twofold symmetry axis (7) and a near twofold symmetry axis perpendicular to the plane (3) and their corresponding symmetrized images in 2 and 4, respectively.

particles such as ribosomes or viruses that cannot be fully embedded in heavy metal stain, the OT complexes could be fully stained because of their relatively small size, resulting in well preserved structural features.

A near-mirror symmetry was found in a number of class averages, as indicated by dashed red lines across the middle of two images in Fig. 3*B*, suggestive of an in-plane twofold axis of these particles as positioned on the supporting carbon film. One such image is shown in Fig. 3*E1* at a larger scale, and its mirror-symmetrized image is shown in Fig. 3*E2*. One class average displayed a near-twofold symmetry (Fig. 3*E3*), indicating a close to vertical orientation of a dimeric object on the carbon film. The twofold symmetrized version of the image is shown in Fig. 3*E4*. Based on the overall size of the OT particles and the presence of the apparent mirror symmetry and the near-twofold symmetry in some of the class averages, we conclude that the OT complex has a dimeric configuration, which is in agreement with the biochemical studies discussed above.

Structural Description of the 3D EM Map of the OT Complex. A 3D map of the OT complex was obtained from 4,900 negatively stained particles by 3D image reconstruction. The structure was estimated to have a resolution of 25 Å. Fig. 4 shows six surface-rendered views of the 3D reconstruction. Because the vast majority of oligomeric membrane proteins have their symmetry axes perpendicular to the membrane, the twofold axis of the OT dimer is highly likely to be arranged in the same way. The orientation of the structure with respect to the lipid bilayer has yet to be determined. We tentatively orient the larger end of the structure along the symmetry axis toward the ER lumen and the smaller end toward the cytosol, based on the fact that all nine OT

Fig. 4. 3D EM map of the dimeric OT complex at 25-Å resolution. The map is rendered as various surface views. *B* and *C* are 40° and 90° rotated views from *A* around a vertical axis (red line), respectively. *D* is a 90° rotated view from *A* around a horizontal axis (red line). *E* and *F* are 90° and 180° rotated views from *D* around a vertical axis (red line), respectively. Assignment of orientation (lumen versus cytosol and top, side, and bottom views) and transmembrane portion (two parallel dashed white lines) are tentative. The blue arrows and ellipses represent the twofold axis of the OT dimer. The vertical height of the OT as shown in *A–C* is 120 Å.

subunits have their larger hydrophilic segments on the luminal side of the ER membrane. The two dashed white lines in Fig. 4 *A–C* represent the potential bilayer site. OT was estimated to be ≈ 120 Å in height. Excluding the 40 Å embedded on the lipid bilayer, there would be ≈ 40 Å in either side of the membrane. This structural feature is consistent with the knowledge that the active site of the OT complex is located ≈ 30 – 40 Å away from the membrane in the lumen (39).

The physiological role of the dimeric nature of the OT complex is currently unclear. However, we note that the actively translocating translocon was recently determined to form a dimer on ribosomes (40). Because OT is known to physically associate with and to function in collaboration with the translocon (41), we speculate that a dimeric configuration of the OT complex may be required for effective association with the translocon dimer and efficient N-glycosylation of newly synthesized polypeptides. Furthermore, a dimeric OT might facilitate the proposed allosteric regulation mechanism of the yeast complex (27).

Materials and Methods

Standard yeast media were used. T4 DNA ligase and shrimp alkaline phosphatase were obtained from Roche Diagnostics. Restriction enzymes and primers were obtained from Invitrogen. Anti-HA mAb HA.II was obtained from Covance (Richmond, CA). Anti-Myc and anti-HA polyclonal antisera were obtained from Santa Cruz Biotechnology. Horseradish peroxidase (HRP)-labeled anti-rabbit IgG raised in goats was obtained from Chemicon International (Temecula, CA). Protein G-agarose beads were purchased from Invitrogen. Triple Master DNA polymerase was obtained from Eppendorf-Brinkman, and Pfu polymerase was obtained from Stratagene. Nitrocellulose membrane was obtained from Schleicher & Schuell. Anti-FLAG (M2) affinity gel, affinity-purified monoclonal anti-FLAG antibody, and 3XFLAG peptide were obtained from Sigma. Zymolyase 20T was obtained from Seikagaku Kogyo (Tokyo). Highly purified digitonin was obtained from Calbiochem. Antisera to the N-terminal 16-aa residues of Ost2p was raised by Genemed Synthesis (San Francisco) after coupling the peptide to keyhole limpet hemocyanin. Protein estimation was carried

Multireference alignment and classification were further carried out in IMAGIC-V several times by using class averages from previous iterations as references until stable class averages were generated. A starting model was calculated in EMAN from selected final class averages based on the cross-common line technique. Selection of class averages was based on the structural features and their corresponding variance maps. The model was refined in a reprojection matching procedure with a total of 4,900 negatively stained particles to produce a refined map. The resolution of the resultant map (25 Å) was estimated by Fourier shell correlation of two maps calculated separately from two halves of the data set. The map was low-pass filtered to the

estimated resolution and rendered into surface views with PYMOL.

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