A Specific Segment of the Transmembrane Domain of Wbp1p Is Essential for Its Incorporation into the Oligosaccharyl Transferase Complex†

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ABSTRACT: Wbp1p, a type I transmembrane protein, is an essential component of oligosaccharyl transferase (OT), which consists of nine different subunits in yeast. It has been proposed that three subunits, Wbp1p, Ost2p, and Swp1p, physically interact with each other, but the mechanism of these interactions is unknown. To explore the mode of interaction, we have focused on the single-transmembrane protein, Wbp1p, and made several deletions and mutations within the short cytosolic domain and the transmembrane domain. Our results show that the deletion of the cytosolic domain has no effect on cell growth, but mutation of all 17 amino acids in the transmembrane domain to 17 Leu residues or replacement of the transmembrane and cytosolic domains with the counterparts of Ost1p results in lethality. Immunoprecipitation experiments show that Wbp1p mutated in these two ways is not incorporated into the OT complex. This finding suggests that the transmembrane domain of Wbplp may mediate its association with the other subunits. A series of mutations of the transmembrane domain have revealed that block alterations in the half of the transmembrane domain facing the lumen of the endoplasmic reticulum (ER) impaired cell viability. Seven single-Lys mutants in the same domain were temperature sensitive for growth at 37 °C. In contrast, block mutations in the other half of the transmembrane domain facing the cytosol did not result in lethality and indicated that this portion of the transmembrane domain was not involved in stable incorporation of Wbp1p into the OT complex.

The key enzyme in the process of N-glycosylation of proteins, oligosaccharyl transferase (OT),[1] catalyzes the transfer of preassembled high-mannose oligosaccharide from a lipid-linked oligosaccharide donor (Dol-PP-$GlcNAc_2Man_9Glc_3$) onto asparagine acceptor sites on nascent polypeptide chains (*1*−*3*). In *Saccharomyces cerevisiae*, OT consists of nine subunits that contain a total of 31 predicted transmembrane segments. Studies demonstrating genetic and biochemical interactions among different OT subunits have led to the suggestion that the OT subunits can be grouped into three subcomplexes: Ost1p−Ost5p, Ost2p−Wbp1p−Swp1p, and Stt3p−Ost4p−Ost3p (*4*, *5*). To obtain a detailed picture of the OT complex, more information about the function and mode of interaction of these subunits is required.

Earlier, the yeast two-hybrid system was used to detect possible interactions between the lumenal segments of the yeast OT subunits, Ost1p, Swp1p, Wbp1p, and Stt3p (*6*). No interaction was observed among the lumenal segments of Ost1p, Swp1p, Wbp1p, and Stt3p in yeast OT, though an interaction between the lumenal segments of ribophorin I (homologue of yeast Ost1p) and OST48 (homologue of yeast Wbp1p) was observed in the mammalian OT using the same method (*7*). Interestingly, the only interaction that was observed in our yeast two-hybrid search was with Pkc1p (*6*). The significance of this interaction is unclear, because of the lack of evidence for the presence of this kinase in the lumen of the yeast ER. However, this possibility cannot be excluded. On the basis of these observations, we hypothesized that either the transmembrane domains or the cytoplasmic domains of yeast OT subunits, rather than the lumenal segments, must be involved in forming a functional enzyme complex.

In previous studies, it was reported that Ost4p interacted with Stt3p and Ost3p through its transmembrane domain and the region for interaction was localized in the half of the transmembrane domain facing the cytosol (*8*, *9*). Among the subunits of the Ost2p−Wbp1p−Swp1p subcomplex, Wbp1p and Swp1p have very short cytosolic domains and Ost2p has short lumenal and cytosolic domains. We hypothesized that perhaps the transmembrane domains are functionally important in the association of these three subunits. In the study presented here, we found that the cytosolic domain of Wbp1p, which is an essential subunit of OT, was not required for its function. Next, we examined the function of the transmembrane domain. We found that the transmembrane domain of Wbp1p functioned as a membrane anchor and that a segment of the transmembrane domain is essential for incorporation of the protein into the OT complex. Specifically, those amino acids in the half of the transmembrane segment of Wbp1p that is facing the ER lumen are essential for the incorporation of Wbplp into the OT complex, while the remaining residues in the other half of the

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[1] Abbreviations: OT, oligosaccharyl transferase; ER, endoplasmic reticulum; N-glycosylation, asparagine-linked glycosylation; HA, hemagglutinin; 5-FOA, 5-fluoroorotic acid.

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transmembrane domain exhibit no effect on its incorporation into the complex.

MATERIALS AND METHODS

Strains. The W303 diploid strain (MAT **a**/α *ade2 can1 his3 leu2 trp1 ura3)* was used to generate diploid strain L1 [MAT **a**/α *ade2 can1 his3 leu2 trp1 ura3 WBP1/*Δ*wbp1::his5*$^+$ (*S. pombe)*]. Strain L1 was transformed with pRS316-*WBP1* and sporulated. Haploid strain L2 [MAT **a** *ade2 can1 his3 leu2 trp1 ura3* Δ*wbp1::his5*$^+$ (*S. pombe*) pRS316-*WBP1*] was selected on −His−Ura plates. All the mutant strains were generated using L2 as the parental strain.

Plasmids. A 1.7 kb fragment was generated by PCR using QYY103 (MAT **a** *ade2 can1 his3 leu2 trp1 ura3 WBP1::HA-his5*) genomic DNA as a template and 5′-CCCTCGAG-GCGGAGTAAGATCTCTGGATG-3′ and 5′-CCGAGCTC-CTGCAGCCCGGGGGATCCAC-3′ as primers. The resulting PCR product was subcloned into pRS314 which was digested with *Xho* I and *Sac* I to generate the plasmid pRS314-*WBP1HA* with a triple HA epitope on the C-terminus of Wbp1p. The full length of *WBP1* was amplified from the W303 diploid strain genomic DNA, and digested with *Sac* I and *Hin*d III, and the fragment was ligated to pRS316 to generate pRS316-*WBP1*.

Spotting Assay for Growth. To determine the growth rate of yeast transformants carrying Wbp1p mutants, equal number of cells were collected after the strains were grown to early log phase in −Trp media at 25 °C. Seven microliters of 1:10 serial dilutions of the cells was spotted on −Trp plates and incubated at 25, 30, or 37 °C for 2 days.

OT Activity Analysis. The activity of OT in N-glycosylation was carried out as described previously (*10*) except that a different peptide substrate, [^{3}H]Ac-Asn-Bpa-Thr-Am, was used instead of [^{125}I]Bh-Asn-Lys(BzN_3)-Thr-NH_2. The activity of OT was expressed as the amount of labeled glycopeptide formed (in counts per minute) per unit of protein per unit of time.

PCR Mutagenesis for Block and Point Mutants. PCR mutagenesis was performed according to the manufacturer's protocol (Stratagene, La Jolla, CA). For all the block and point mutations mentioned in this paper, pRS314-*WBP1HA* was used as the PCR template. Mutated plasmids were sequenced, and those with the expected sequence were transformed into strain L2. The transformants were selected for Trp and Ura prototrophy and then further by the 5-FOA selection procedure.

Co-Immunoprecipitation under Mild Conditions. Yeast spheroplast lysates or crude microsomes were prepared from yeast strains L2 carrying the wild-type Wbp1p or the point or block mutations that had been tagged with HA (*11*). The spheroplast lysates or microsomes were suspended in 5% glycerol, 20 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, and 1 mM PMSF. The suspension was adjusted to contain 1.5% digitonin, 0.5 M NaCl, 20 mM Tris-HCl (pH 7.4), 3.5 mM $MgCl_2$, and 1 mM $MnCl_2$. The mixture was centrifuged for 20 min at 100000*g*, and the clarified supernatant was used for immunoprecipitation under the conditions described previously (*12*). Samples were resolved on SDS−PAGE, transferred to nitrocellulose membranes, and then probed by Western blot analysis using antibodies to various OT subunits.

Membrane Association of Wbp1p. To study the membrane association of wild-type Wbp1p and its mutants, 1 mg of the microsomal fraction was resuspended in 200 μL of 0.1 M Na_2CO_3 (pH 11). The suspension was incubated for 30 min on ice and then subjected to centrifugation at 100000*g* for 30 min at 4 °C. The resulting membrane pellet was rinsed once gently with distilled water and resuspended in 200 μL of 50 mM Tris-HCl (pH 7.4). Supernatant and pellet fractions were subjected to SDS−PAGE and analyzed by Western blot. The protein concentration was determined using the BCA protein assay reagent (Pierce, Rockford, IL) according to the manufacturer's instructions.

RESULTS

The Cytosolic Domain of Wbp1p Is Not Essential for Its Function. On the basis of hydropathy analysis, Wbp1p is a type I membrane protein with a long lumenal domain at its N-terminus, a transmembrane span containing 17 amino acid residues, and a short cytosolic tail containing only 16 amino acid residues at the C-terminus (*13*). The cytosolic domain contains a dilysine motif (KKXX) with two lysine residues located at positions −3 and −4 from the C-terminal end. Among the nine subunits of OT, Wbplp is the only protein in the complex containing a potential KKXX ER retention signal. This motif has been reported to serve as a retention signal in other type I ER transmembrane proteins (*14*). Previous studies showed that the C-terminus of Wbp1p retained protein chimeras in the ER membrane when it was fused to the C-terminus of the mammalian protein Tac antigen or yeast protein Suc2p (*15*, *16*). However, it was shown that the KKXX motif is not essential for the function of Wbp1p (*16*). We prepared a deletion construct Wbp1HAp-(Δ415−430), which lacked all 16 amino acid residues of the C-terminus (Figure 1). It was found that the mutant with the entire cytosolic domain deleted was found to support cell growth (data not shown) and exhibited no defect in the OT activity measured *in vitro* (Figure 2). This result clearly indicates that the OT complex is retained in the ER even without the whole cytosolic domain of Wbp1p, which confirms the previous results (*16*). It is obvious that neither the entire cytosolic domain nor the dilysine motif is necessary for the function of Wbplp, and that another mechanism for the retention of the yeast OT complex in the ER must exist.

It has been reported that the nine C-terminal cytosolic amino acid residues are dispensable for the function of Ost1p (*12*). Therefore, we made a *wbp1* and *ost1* chromosomally deleted strain containing plasmid copies of Wbp1HAp-(Δ415−430) and Ost1HAp(Δ468−476) with both of the cytosolic domains deleted. We found that deletion of the C-termini of both Wbp1p and Ost1p had no effect on cell growth (data not shown). This result clearly showed that no specific interaction was essential between the C-termini of Wbp1p and Ost1p. On the basis of this finding, it seems obvious that the transmembrane domain and/or the lumenal domains must play an important role in the formation of a functional complex between at least some of, if not all, the subunits of OT.

The Transmembrane Domain of Wbp1p Functions Not Only as a Membrane Anchor But Also in Incorporation of the Protein into the OT Complex. We prepared a truncated construct containing only the lumenal domain of

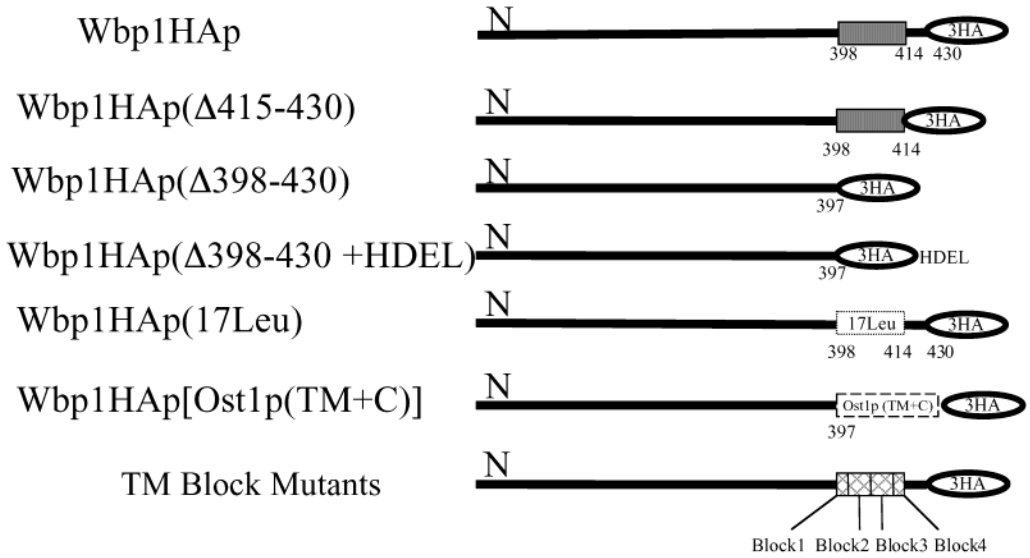

FIGURE 1: Diagram of mutants prepared in the transmembrane domain and C-terminus of Wbp1p. All the constructs contain a triple HA epitope at the C-terminus, except Wbp1HAp(Δ398−430+HDEL), in which the triple HA precedes the HDEL motif. The dark gray box near the C-terminus represents the wild-type hydrophobic transmembrane domain. The dotted box represents the 17 Leu residues. The dashed box represents the transmembrane and cytosolic domains of Ost1p. In Wbp1HAp(17Leu), the transmembrane domain of Wbp1p is replaced with 17 Leu residues. In Wbp1HAp[Ost1p(TM+C)], the transmembrane and cytosolic domains of Wbp1p are replaced with the counterparts of Ost1p. Four transmembrane domain block mutants (Block1−4) were prepared one at a time by changing four or five residues to a block of four or five leucines, respectively: block 1, 398−401LLLL; block 2, 402−406LLLLL; block 3, 407−410LLLL; and block 4, 411−414LLLL.

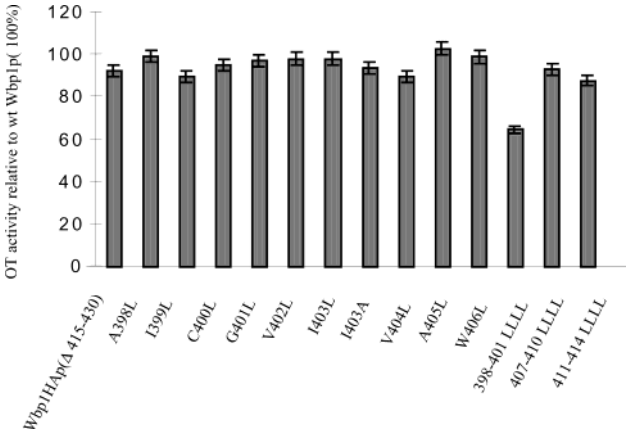

FIGURE 2: Measurement of the OT activity of lysates of the indicated mutant strains by using an *in vitro* assay of the N-glycosylation of a labeled [^{3}H]Ac-Asn-Bpa-Thr-Am peptide. Values shown are relative to 100% for the strain bearing wild-type Wbp1p; shown are the standard deviation and the average of three independent experiments.

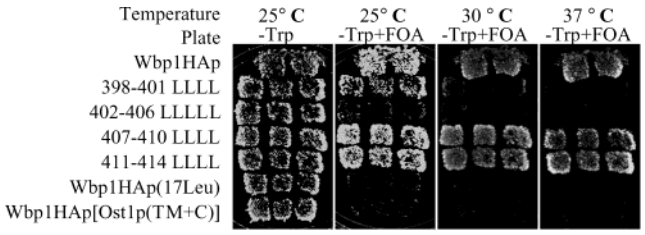

FIGURE 3: 5-FOA selection reveals that Wbp1p mutants Wbp1HAp-(17Leu), Wbp1HAp[Ost1p(TM+C)], and 402−406LLLLL are lethal and 398−401LLLL is temperature sensitive. Mutagenized plasmids were transformed into strain L2. The transformants were selected for Trp and Ura prototrophy, patched on a −Trp plate, and incubated at 25 °C for 1 day, and then the cells were replicated to three −Trp plates with 5-FOA and incubated at 25, 30, and 37 °C for 1 day, in succession.

Wbp1p, Wbp1HAp(Δ398−430), and a protein chimera in which four amino acid residues (HDEL) were fused to the C-terminus of the Wbp1p lumenal domain, Wbp1HAp-(Δ398−430+HDEL) (Figure 1). This HDEL tag might be expected to mediate the retention of the truncated protein in the ER. These plasmids were transformed into strain L2, and the plasmid shuffling procedure was performed as described above. It was found that neither of the mutated lumenal proteins could support cell growth (data not shown). These experiments, taken together with the results described above, suggested that the transmembrane domain of Wbp1p was likely to play an important role in the function of this protein.

To further study the role of the transmembrane domain of Wbp1p, we carried out a series of mutation studies. We prepared a construct, Wbp1HAp(17Leu), in which the 17 amino acids in the transmembrane region of Wbp1p were replaced with 17 leucine residues (Figure 1). In addition, we prepared another plasmid in which the transmembrane and cytosolic domains of Wbp1p were replaced with the counterparts of Ost1p, Wbp1HAp[Ost1p(TM+C)] (Figure 1). It was found that neither of the mutants yielded cells that were viable, even at 25 °C (Figure 3). These results suggested that at least a portion of the sequence of the transmembrane domain of Wbp1p is very important for its function and does not merely function to anchor it in the ER membrane.

However, it was important to determine that Wbp1HAp-(17Leu) and Wbp1HAp[Ost1p(TM+C)] proteins were expressed. The control and mutated plasmids were transformed into strain L2 containing a plasmid copy of the wild-type *WBP1* gene to support growth. Microsomes were prepared, and Western blot analysis was performed with anti-HA antibody. Western blot analysis showed all these proteins were expressed, although the amount of Wbp1HAp[Ost1p-(TM+C)] was significantly smaller than the amount of wild-type control and Wbp1HAp(17Leu) (data not shown).

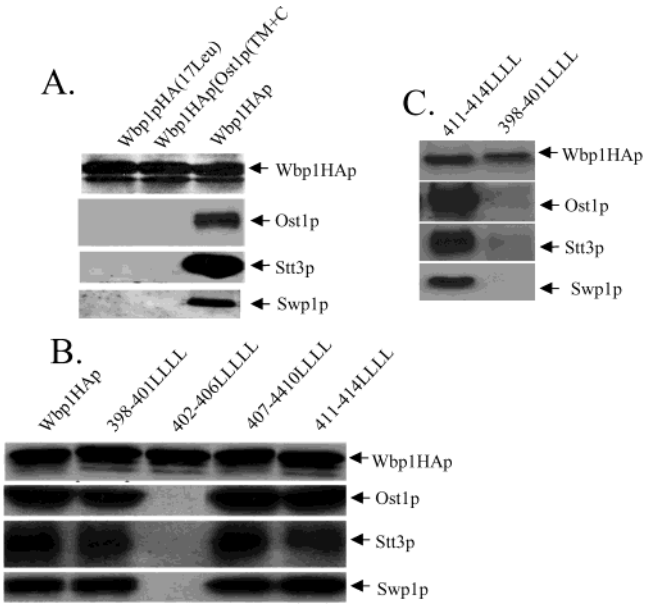

FIGURE 4: Association of Wbp1p mutants with the OT complex. (A) Wbp1HAp(17Leu) and Wbp1HAp[Ost1p(TM+C)] and (B) block mutant 402−406LLLLL are not integrated into the OT complex at 25 °C, whereas block mutants 398−401LLLL, 407−410LLLL, and 411−414LLLL are. (C) At 37 °C, block 4 mutant 411−414LLLL is integrated into the OT complex, but not block 1 mutant 398−401LLLL. Microsomes and spheroplast lysates were prepared from each mutant and the wild-type strains. Immunoprecipitation under mild detergent conditions was carried out using mouse anti-HA antibody as described in Materials and Methods. Rabbit anti-HA, anti-Ost1p, anti-Swp1p, and anti-Stt3p antibodies were used for Western blot analysis.

These mutants were further studied with respect to their ability to be incorporated into the OT complex. It was reported that eight OT subunits could be co-immunoprecipitated by using an anti-HA antibody against Ost3HAp after mild detergent solubilization (*17*). This method was utilized in this study to analyze if the mutant proteins were in the OT complex. Since the expression levels of the mutant proteins were not the same, the protein amounts were normalized to be the same before the co-immunoprecipitation. The monoclonal anti-HA antibody was utilized for co-immunoprecipitation against Wbp1HAp. Western blot analysis was carried out using antibodies that recognize subunits Stt3p, Ost1p, and Swp1p to check for the association of these subunits with Wbp1p. The results showed that wild-type Wbp1p was co-immunoprecipitated with Stt3p, Ost1p, and Swp1p. However, neither the Wbp1HAp(17Leu) protein nor the Wbp1HAp[Ost1p(TM+C)] protein was co-immunoprecipitated with Stt3p, Ost1p, or Swp1p (Figure 4A). This analysis confirms that unlike wild-type Wbp1p, these two Wbp1p mutants are not incorporated into the OT complex.

It was reported that wild-type Wbp1p is a type I ER transmembrane protein (*13*). The mutations mentioned above that caused growth defects and the disruption of Wbp1p from the OT complex were located in the transmembrane domain. Therefore, it was possible that the mutations might not just prevent incorporation into the OT complex but could also actually prevent entry of the mutant proteins into the ER membrane. Consequently, the membrane association of Wbp1p mutants was checked. Microsome membrane fractions were extracted with Na_2CO_3 (pH 11) and then fractionated into a membrane pellet and a soluble supernatant by centrifugation at 100000*g*. Western blot analysis was carried out, and Wbp1p mutants, Wbp1HAp(17Leu) and Wbp1HAp-[Ost1p(TM+C)], were found to be localized to the membrane pellet. As a control, unsoluble ER lumenal protein, Pdi1p, was shown to be present in the supernatant fraction (Figure 5). Thus, it is clear that Wbp1p mutants, Wbp1HAp(17Leu) and Wbp1HAp[Ost1p(TM+C)], were incorporated into the ER membrane, but did not become associated with the other OT subunits. This result indicates that it is likely that at least some of the amino acid residues of the transmembrane domain of Wbp1p function in stable incorporation of this protein into the OT complex.

The Half of the Transmembrane Domain Facing the ER Lumen Is Functionally Important for Integration in the OT Complex. To identify which region in the transmembrane domain of Wbp1p associates with the other OT subunits, block mutations in the transmembrane domain were prepared. The transmembrane domain was divided into four blocks, and all of the amino acids in each block were mutated to polyleucine: block 1 (398−401LLLL), block 2 (402−406LLLLL), block 3 (407−410LLLL), and block 4 (411−414LLLL) (Figure 1). Subsequent analysis of the ability of these mutants to grow revealed cells with block 3 or 4 mutations grew well at all temperatures that were tested. However, cells with the block 1 mutation grew only at 25 °C, but not at 30 or 37 °C (Figures 3 and 6A). The cells with block 2 mutations exhibited a lethal phenotype at 25 °C. Thus, only one-half of the transmembrane domain was affected by the polyleucine mutations, suggesting that the amino acid residues in this region are important. On the basis of the topology of Wbp1p (*13*), blocks 1 and 2 are facing the ER lumen.

It was found that the four Wbp1p block mutants were expressed and associated with the ER membrane (Figure 5). Western blot analysis revealed that the level of block mutant expression varied: the amount of block 1 (398−401LLLL) and block 2 (402−406LLLLL) mutants was significantly smaller than that of the wild type or block 3 or block 4 mutant (Figure 5). Next, co-immunoprecipitation was carried out as described above, and it was found that block 1, block 3, and block 4 Wbp1p mutants could be co-immunoprecipitated with Stt3p, Ost1p, and Swp1p at 25 °C and therefore were incorporated into the OT complex. On the other hand, the block 2 mutant protein was not co-immunoprecipitated with Stt3p, Ost1p, and Swp1p and was not incorporated into the OT complex (Figure 4B). Furthermore, at 37 °C, the block 4 mutant (411−414LLLL) protein was found in the complex, but the block 1 mutant (398−401LLLL) was not (Figure 4C). Next, we examine the OT activity of the mutants. The block 1 mutant exhibited a significant decrease in the OT activity, but the block 3 and block 4 mutants exhibited normal enzyme activity (Figure 2). These results confirm the hypothesis that approximately one-half of the transmembrane domain of Wbp1p facing the ER lumen is responsible for stable incorporation of the subunit into the OT complex.

Effect of Point and Block Mutations in the Half of the Transmembrane Domain Facing the ER Lumen. To determine if there was a specific region for certain amino acids in blocks 1 and 2, we mutated each of the nine amino acid residues to Ala and/or Leu in blocks 1 and 2. It was found that the single-amino acid, Ala, and Leu mutants exhibited

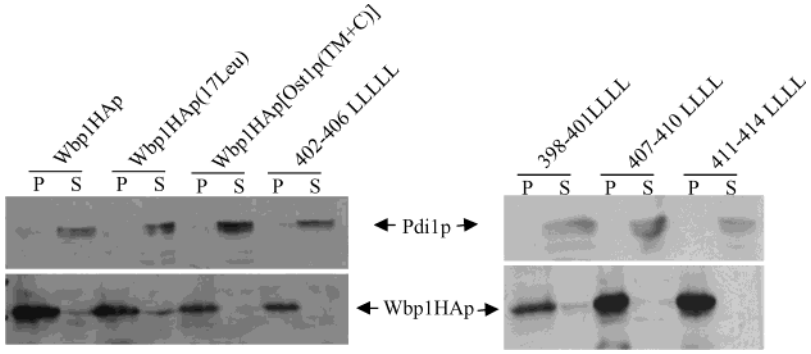

FIGURE 5: Membrane association of Wbp1p. Microsomes from the wild type and mutants of Wbp1p were treated with 0.1 M Na_2CO_3 (pH 11) and separated into a supernatant (S) and membrane pellet (P) by centrifugation at 100000*g*. These fractions were analyzed by Western blotting using polyclonal anti-Pdi1p and monoclonal anti-HA antibodies.

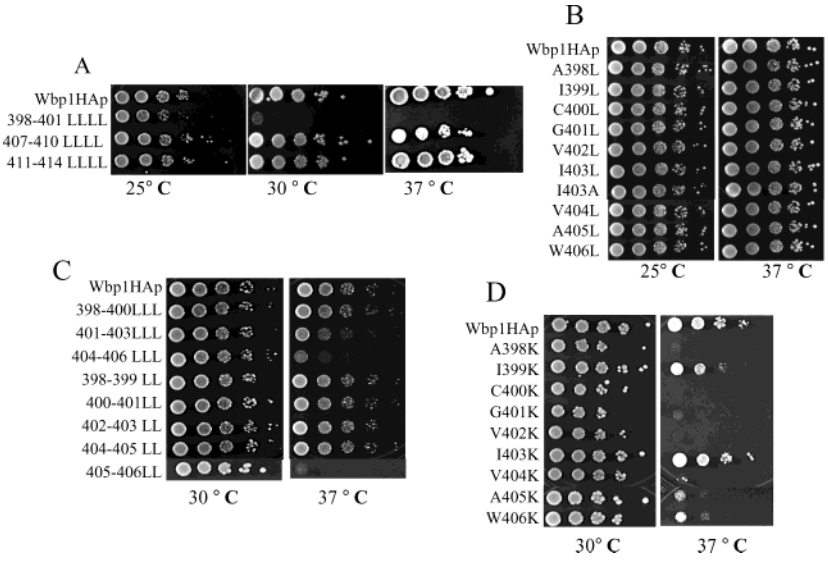

FIGURE 6: Spotting growth assay of Wbp1p mutant strains. Comparison of the growth phenotype of strains carrying Wbp1p mutated at the indicated amino acid positions. Cultures of wild-type control and mutant strains were diluted serially and spotted on −Trp plates. After 2 days, the growth of colonies at different temperatures was compared: (A) block mutation with four Leu replacements, (B) single-site mutations with uncharged amino acids (Leu or Ala), (C) block mutations with two or three Leu replacements, and (D) single-site mutations with charged amino acid Lys.

neither growth defects (Figure 6B) nor impaired *in vitro* glycosylation activity (Figure 2). These results suggest that the growth defects of the block 1 and block 2 mutants were probably caused by an additive effect of the nine amino acids. Block mutations containing two amino acid residues exhibited no growth defect at 37 °C except for 405−406LL. Block mutations of three amino acid residues (401−403LLL and 404−406LLL) exhibited temperature sensitivity at 37 °C, whereas block mutant 398−400LLL grew well even at 37 °C (Figure 6C).

Next, we proceeded to make single-amino acid changes likely to disrupt hydrophobic interactions. We did this by changing residues in block 1 and 2 regions to Lys, one at a time. The results showed that all point mutations except I399K and I403K exhibited a serious growth defect at 37 °C (Figure 6D).

DISCUSSION

Oligosaccharyl transferase (OT) is a membrane-associated enzyme complex that catalyzes the N-glycosylation of proteins. Yeast OT is more complicated than the other members of the general class of glycosyl transferases that have been studied, and contains nine different subunits residing in the ER. Conceivably, several subunits of the yeast OT complex may not participate directly in oligosaccharide transfer, but may instead serve other functions. This view has been supported by the observation that several subunits, Ost3p−Ost6p, are not essential for the viability of yeast, yet loss of these proteins reduces the level of oligosaccharide transfer to nascent glycoproteins (*18*−*21*). Possible roles for OT subunits not directly engaged in catalysis could include mediating interactions within the complex or with the components of the translocation apparatus. They also might function in regulating the lipid-linked oligosaccharide assembly pathway or glycoprotein folding. Our objective is to understand the function of each of these transmembrane protein subunits and how they interact with each other in the ER.

Each of the nine subunits of yeast OT contains at least one transmembrane domain, and the largest and most conserved OT subunit among all organisms that were studied, Stt3p, contains 10−12 transmembrane domains (*4*). Furthermore, two OT subunits, Ost2p and Ost4p, are almost entirely located within the ER membrane (*4*). All of these facts imply

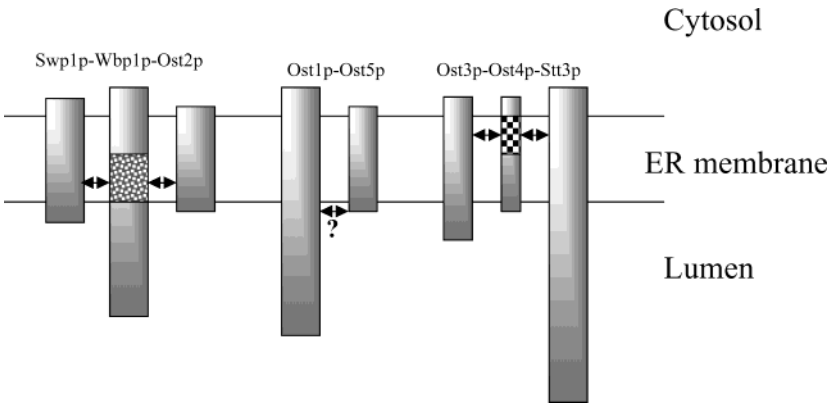

FIGURE 7: Model for the interactions in the three subcomplexes of the OT complex. As discussed, the speculation is based on the results of Knauer *et al*. (*4*), Spirig *et al*. (*5*), Kim *et al*. (*8, 9*), Yan *et al*. (*12*), and Yan *et al*. (*23*). The masses of the subunits are not to scale.

that the transmembrane domains have important functions. Among the nine subunits, Ost1p, Ost4p, and Wbp1p contain a single transmembrane domain, and each belongs to one of the three subcomplexes. In previous studies, the importance of the transmembrane domains of Ost1p and Ost4p was examined and it was found that the transmembrane domains behaved differently (*8*, *9*, *12*). The transmembrane domain of Ost1p is probably only involved in anchoring the protein in lipid bilayer, and it has no specific role in interaction with the transmembrane domains of the other OT subunits (*12*). In contrast, Ost4p, the mini-membrane protein, mediates the interaction with Stt3p and Ost3p through its transmembrane domain (*8*, *9*).

In this study, we investigated the function of the transmembrane domain of another single-transmembrane protein in OT, Wbp1p. Interestingly, we found that the Wbp1p mutants were not incorporated into the OT complex when the transmembrane domain was replaced either with 17 Leu residues or with the transmembrane domain of Ost1p. This finding indicated that the transmembrane domain of Wbp1p does not merely serve as an ER membrane anchor, but also may be necessary for association with other OT subunits. It was reported that Ost2p, Wbp1p, and Swp1p form a subcomplex (*4*). In this complex, Ost2p has short cytosolic and lumenal domains and three hydrophobic segments to form two or three predicted transmembrane domains (*22*). Several temperature sensitive mutations were found to be located in the second and third hydrophobic segments, and it was suggested that both the second and third hydrophobic segments of the Ost2p protein might interact with the membrane-spanning segments of the other OT subunits (*22*). Recent studies showed that Swp1p and Ost2p could be cross-linked to Wbp1p, but they could not be cross-linked to each other, using a membrane-permeable thio-cleavable cross-linking reagent, dithiobis(succinimidyl propionate) (DSP) (*23*). These results suggest that in this subcomplex, Wbp1p functions as a bridge between Ost2p and Swp1p (*23*). It was also reported that no interaction was detected between the lumenal segments of Wbp1p and Swp1p using the two-hybrid system (*6*). Our results, taken together with these observations, indicate that Wbp1p interacts with Swp1p and Ost2p through its transmembrane domain. Further block mutations showed that only half of the transmembrane domain of Wbp1p is essential for the interaction. The half-transmembrane domain (residues 398−414) responsible for the defect in incorporation of Wbp1p faces the ER lumen. In this respect, it is different from the case of Ost4p, in which the half-transmembrane domain essential for interaction is that closest to the cytosolic face of the membrane (*9*).

Since the three single-transmembrane proteins belong to different subcomplexes, it is not surprising to find different modes of interaction in the OT complex. These findings, taken together with the earlier results (*4*, *5*, *8*, *9*, *12*) shed light on some of the interactions among the subunits of the OT complex. A model for the interactions in the OT complex is shown in Figure 7. We believe that in the Stt3p−Ost4p−Ost3p subcomplex, Ost4p interacts with Stt3p and Ost3p through the half of its membrane segment that faces the cytosol (*8*, *9*). In the case of the Ost2p−Wbp1p−Swp1p subcomplex, Wbp1p mediates the interaction between Ost2p and Swp1p through the half of the transmembrane domain facing the lumen of the ER. For the Ost1p−Ost5p complex, it was found that the specific transmembrane domain of Ost1p was not important for interaction and the C-terminal cytosolic domain was unnecessary for its function (*12*). It is possible that the interaction region may be located in the lumenal domain of Ost1p. This hypothesis is supported by the observation that certain mutations in the lumenal domain were found to prevent Ost1p from being incorporated into the OT complex (*12*). But more direct evidence is required to test this hypothesis.

Recently, it was reported that Ost1p could cross-link with all the other eight subunits (*23*) and suggested that Ost1p might be in the center of the complex and close to all eight other subunits. Therefore, in our hypothesized model, the Ost1p−Ost5p subcomplex is in the center in the complex with the other two subcomplexes (Swp1p−Wbp1p−Ost2p and Stt3p−Ost4p−Ost3p) surrounding it (Figure 7). But we still do not know how the three subcomplexes combine to constitute the OT complex.

Membrane proteins of the ER may be localized to this organelle by mechanisms that involve retention, retrieval, or both (*24*). For lumenal ER proteins containing a KDEL or HDEL motif, and for type I transmembrane proteins carrying the dilysine motif (KKXX), specific retention mechanisms have been identified. However, most ER membrane proteins do not contain easily identifiable retrieval or retention motifs, and ER localization information has been found in cytoplasmic, transmembrane, and lumenal domains of different proteins (*24*). In yeast, Wbp1p is the only subunit in the OT complex that contains an ER retention signal with a typical cytosolic dilysine motif. If this dilysine motif is

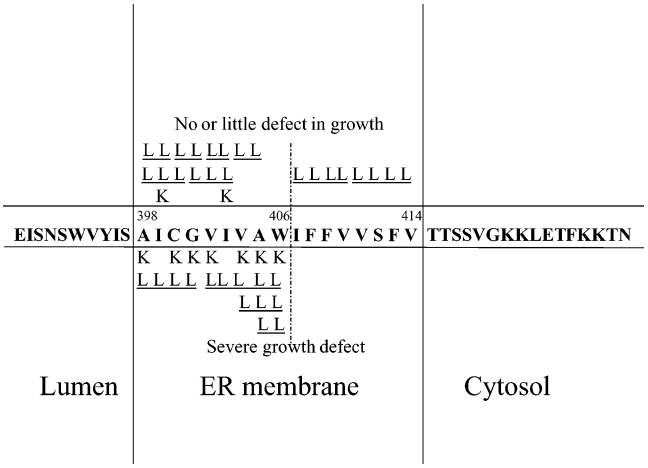

FIGURE 8: Summary of growth effects on strains bearing block and single mutations at the transmembrane domain of Wbp1p. Each block mutation is underlined.

the retention signal for the whole OT complex, it would be essential. But previous work showed that the KKXX motif was not essential for Wbp1p's function (*16*), and our mutagenesis study confirmed this finding. Thus, there must be another mechanism for retaining all of the proteins of the OT complex in the ER. A similar result was observed in the mammalian OT complex, in which the ER localization domains are reported to be in the three type I transmembrane proteins ribophorin I (homologue of yeast Ost1p), ribophorin II (homologue of yeast Swp1p), and Ost48p (homologue of yeast Wbp1p). Yet when the KKXX motif of Ost48p was mutated to the SSXX motif, the mutated Ost48p was still retained in the ER, when this mutant was coexpressed with ribophorin I or II (*24*). These results suggested that the retention signal of the yeast OT complex may be located in other OT subunits rather than Wbp1p.

A summary of the growth phenotype of the mutants carrying the mutations on the transmembrane domain of Wbp1p is presented in Figure 8. Though we observed that the block of four or five polyleucine Wbp1p mutations on the half of the transmembrane domain facing the ER lumen had a serious growth defect, the single-Leu and/or -Ala mutations on the same region showed no growth phenotypes. We speculate that growth defect phenotypes 398−401LLLL and 402−406LLLLL are probably caused by an additive effect of the four or five amino acids. It was found that mutation of 405−406LL showed a growth defect at 37 °C. Two other 3-polyleucine mutations, 401−403LLL and 404−406LLL were found to be temperature sensitive at 37 °C. These results were helpful in the identification of amino acids 405 and 406 which are essential for cell growth at 37 °C. Since previous studies showed that introduction of ionizable residues might disrupt tight hydrophobic packing of helices in the membrane (*9*), we introduced single Lys residues in the sensitive half of the transmembrane segment of Wbp1p, and found that except for I399K and I403K, the other seven single-Lys mutants exhibited serious growth defects at 37 °C. The reason the two mutations of Ile to Lys did not result in significant growth defects is unknown. Overall, these results unambiguously confirmed the hypothesis that the segment of the transmembrane domain facing the lumen of the ER mediates interaction with other subunits of the OT complex.

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