Proc. Schl. Eng. Tokai Univ., Ser. E
49 (2024) 19-25
DOI: 10.18995/24343641. 49. 19

Analyses of the Membrane Topology and Physical Interactions of Yeast Dolichyl-Phosphate-Glucose Synthase Alg5p

by

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(Received on Mar. 2, 2024 and accepted on May 16, 2024)

Abstract

In the biosynthetic pathway of dolichol-linked oligosaccharide (DLO) in the eukaryotic cell, yeast dolichyl-phosphate-glucose synthase (DPGS), Alg5p, catalyzes glucosylation of dolichyl-phosphate (Dol-P) using UDP-glucose, yielding dolichyl-phosphate-glucose (DPG), which is in turn utilized by three luminal glucosyltransferases (Alg6p, Alg8p and Alg10p) as a substrate. In this study, we cloned yeast *ALG5* gene encoding DPGS into the vectors for the yeast split-ubiquitin system (YSUS) and explored its membrane topology and physical interaction in detail. Our results indicated that Alg5p possesses two transmembrane domains (TMDs), with both termini orientated toward the cytoplasmic side of the rough ER (rER) membrane and the protein physically interacts with Dpm1p, Sec59p, Cwh8p and Alg7p, which are involved in DLO assembly. In addition, we revealed that the *C*-terminal loop region (LR4) of Alg5p is essential for the interaction with the Alg7p.

***Keywords*:** Dolichyl-phosphate-glucose synthase, Membrane topology, Physical interaction

1. Introduction

The assembly of dolichol-linked oligosaccharides (DLOs) on the rough endoplasmic reticulum (rER) membrane is essential for the protein *N*-glycosylation in yeast[1-5]. As shown in Fig. 1, three glucose residues are added from dolichyl-phosphate-glucose (DPG) to DLOs by three glucosyltransferases (Alg6p, Alg8 and Alg10p) localized on the rER membrane for the completion of DLOs assembly. DPG synthase (DPGS) catalyzes the transfer of glucose from UDP-glucose to dolichyl-phosphate (Dol-P) and produces DPG[6], providing the glucosyltransferases with DPG (red arrow in Fig. 1). Yeast Alg5p protein is the firstly characterized DPGS. Its gene was firstly identified as *ALG5* gene by complementation assay of yeast *alg5* mutation[7], which causes accumulation of $Man_9GlcNAc_2$-PP- dolichol, a glucose-lacking intermediate DLO, at non-permissive temperature[8),9)]. Later on, a human cDNA encoding DPGS was isolated[10)]. Recently, it has been reported that a defect of human DPGS (hDPGS) gene causes atypical polycystic kidney disease[11)].

Despite the importance of properties of DPGS as described above, the membrane topology and the physical interactions of DPGS have not been characterized in detail. Therefore, in the previous study, we investigated and reported those of hDPGS[12)], by using the yeast split- ubiquitin system (YSUS)[13-17)], a yeast two-hybrid system that can analyze the membrane topology[18),19)] and detect the physical interaction between two membrane proteins[20-23)].

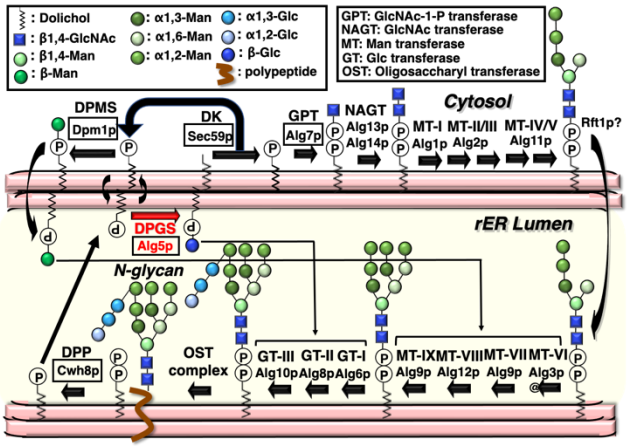

Fig. 1 Assembly of dolichol-linked oligosaccharides (DLOs) on the rough ER membrane in yeast. DPMS, DPGS and DK indicate dolichyl-phosphate-mannose synthase, dolichyl-phosphate-glucose synthase and dolichol kinase, respectively. The five dolichyl-phosphate (Dol-P)-related enzymes (Alg5p, Dpm1p, Sec59p, Alg7p and Cwh8p) are indicated with black frames.

Here we report those of yeast Alg5p using the YSUS. In

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Table I The PCR primers used in this study. Additional sequences containing a *Sfi* I- cleavage site are shown in lowercase letters.

Primer name	Nucleotide sequence	Purpose
AG5_fw	Ref. 24	Cloning of the full-length and *C*-terminally truncated *ALG5*
AG5_rv2	5'-gcctttggccgaggcggccCATTTCTTATTATCTCTATATATCCCTAA-3'	Cloning of the full-length *ALG5*
AG5HR1_rv	5'-gcctttggccgaggcggcCGAAAATAAATAGACCAATAAATACAGTG-3'	Cloning of the truncated *ALG5* from *N*-terminus to HR1
AG5LR2_rv	5'-gcctttggccgaggcggccgaGTTTCTTATCATTGATCTCTTTATGACG-3'	Cloning of the truncated *ALG5* from *N*-terminus to LR2
AG5HR2_rv2	Ref. 24	Cloning of the truncated *ALG5* from *N*-terminus to HR2
AG5LR3_rv	5'-gcctttggccgaggcggccGCCATCTTAGAGCCATCAACCTC-3'	Cloning of the truncated *ALG5* from *N*-terminus to LR3
AG5HR3_rv	5'-gcctttggccgaggcggccAATAAATAGGCCATTCTTATAATAACCAAG-3'	Cloning of the truncated *ALG5* from *N*-terminus to HR3

advance of this study, we have developed a novel set of control preys, especially for analysis of Alg5p by the YSUS[24]. Using these control preys and truncated Alg5 bait constructs that were newly prepared here, we successfully found out the novel properties of Alg5p regarding its membrane topology and physical interactions.

2. Experimental Methods

2.1 *Prediction of the membrane topology of Alg5p*

In order to predict the membrane topology of Alg5p protein, a WWW algorithms server, TOPCONS[25] (https://topcons.cbr.su.se) was used. On its WEB site, the amino acid sequence of Alg5p protein consisting of 400 residues was registered and surveyed regarding its potential transmembrane domains (TMDs) and membrane topology.

2.2 *Construction of recombinant plasmids for the YSUS*

The coding region of full-length *ALG5* gene has been already cloned into pBT-C vector, constructing as the pBT-C-Alg5 in our previous study[24]. Similar cloning into the pBT-N vector was performed via PCR using the AG5_fw and AG5_rv2 primers according to the standard method[26]. In addition, in order to create the bait constructs expressing the five types of truncated Alg5p (Fig. 3), each of the five reverse primers (AG5HR1_rv, AG5LR2_rv, AG5HR2_rv2, AG5LR3_rv and AG5HR3_rv), listed in Table 1, was combined with the AG5_fw forward primer and PCRs were carried out using the yeast genomic DNA as a template. Then amplified DNA fragments were cloned into the pBT-C vector according to the same procedure as previously described[24]. On the other hand, as for the prey constructs expressing Dol-P-related enzymes (Fig. 1), DNA fragments of their genes, which were amplified by PCR, were cloned into the pPR-N, pPR-C or pPR-STE vector in the same manner. The fabricated bait and prey constructs are explained with their expressing proteins in Fig. 3 and Fig.6, respectively.

2.3 *Assays for the membrane topology of Alg5p*

The bait constructs shown in Fig. 3 were used for co-transformation of *Saccharomyces cerevisiae* NMY51 strain, together with the positive or negative control prey construct, pDL2-Alg5 or pAI-Alg5[14], standardly prepared in the YSUS. The transformation of yeast cells was carried out by the standard method[27]. The co-transformants grown on the synthetic dextrose (SD) medium lacking leucine and tryptophan (SD-LW) were then subject to the growth examination on the SD medium lacking leucine, tryptophan and histidine (SD-LWH) and SD medium lacking leucine, tryptophan, histidine and adenine (SD-LWHA) according to the manual supplied by Dualsystems Biotech (www.dualsystems.com).

2.4 *Assays for the physical interaction of Alg5p*

Each of the bait construct of Alg5p and its derivatives (Fig. 3) was combined with each of the prey constructs of Dol-P-related enzymes (Fig. 6), then they were used for co-transformation of yeast NMY51 strain. After the co-transformation, the co-transformants grown on SD-LW medium were subject to growth examination with SD-LWH and SD-LWHA media, according to the same procedure as described above.

3. Results and Discussion

3.1 *The membrane topology of Alg5p*

Alg5p is a membrane protein with an apparent molecular size of 35 kDa[28], which is composed of 400 amino acid residues. First, we started from predicting the membrane topology of Alg5p protein with TOPCONS algorithms, a server freely available on WEB sites. As shown in Fig. 2, TOPCONS algorithm consequently predicted at most four loop regions (LR 1 to LR4) and three hydrophobic regions (HR 1 to HR3) corresponding to potential TMDs in Alg5p protein (Fig. 2). With respect to the orientation of *N*- and *C*-termini, the result of prediction was different among five programs (Fig. 2).

In order to ascertain the predictions, we applied the YSUS to our analysis of Alg5p. In our previous research, it has been revealed that Alg5p bait physically interacted with Alg5p prey[24]. As will be described in detail below, in this research, we concluded that the negative and positive control prey constructs (pDL2-Alg5 and pAI-Alg5, respectively) prepared in the YSUS were unsuitable for analysis of Alg5p.

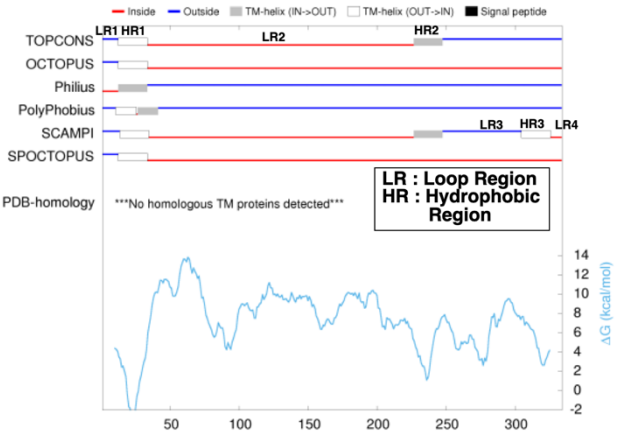

Fig. 2 Prediction of the membrane topology of Alg5p by TOPCONS algorithm server (http://topcons.cbr.su.se/). The three hydrophobic regions (HR1 to HR3) are indicated with boxes, as candidates of transmembrane domains (TMDs), and the four loop regions (LR1 to LR4) are indicated with red or blue lines, as cytoplasmic or luminal loops, respectively.

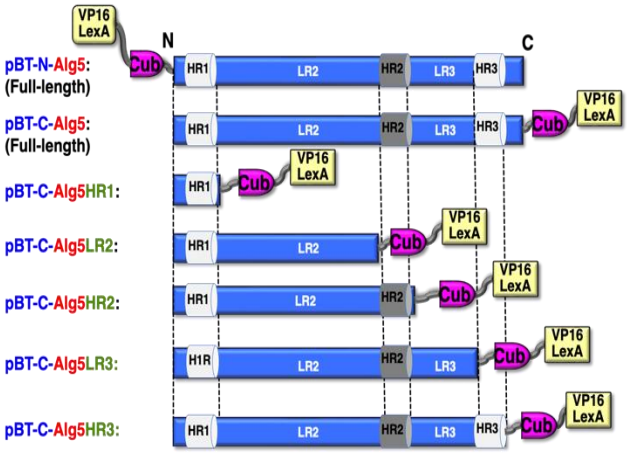

Fig. 3 The bait constructs used in this study and structure of Alg5p bait proteins expressed by them. The LRs and HRs are based on the prediction by TOPCONS,

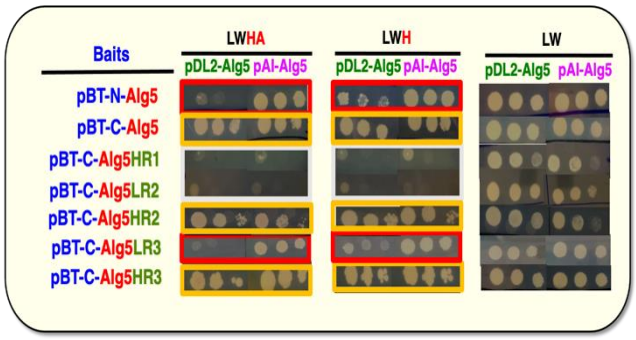

Fig. 4 Growth examination of co-transformants with Alg5p bait and control prey constructs. After the selection of colonies derived from the co-transformants on the SD-LW medium, their suspensions were diluted with sterilized water and adjusted to the OD_{600} values of 1.0, 0.1 and 0.01 (from left to right). These diluents were orderly spotted on the SD-LWH and SD-LWHA media for reporter detection, and SD-LW media for growth control, and then incubated at 30 ºC for 2-4 days.

To explain the reason, we firstly represent the results using them as controls in Fig. 4.

It has already been demonstrated that the *C*-terminus of Alg5p is located in the cytosol[14]. In our growth examination, co-transformants of the pBT-N-Alg5 bait with the pAI-Alg5 positive control prey have well grown on SD-LWH and SD-LWHA plates (right side within an upper red frame in Fig. 4), indicating that the Cub tag at the *N*-terminus of Alg5p bait spontaneously interacted with NubI (normal type of Nub) tag at the *C*-terminus of Alg5p prey, and consequently the *N*-terminus of Alg5p is also located in the cytosol. On the contrary, those with the pDL2-Alg5 exhibited little growth on both media (left side within an upper red frame in Fig. 4). As NubG (mutational type of Nub) tag at the *C*-terminus of Alg5p prey is not able to spontaneously interact with Cub tag without specific bait-to-prey interaction[29], this means that the *N*-terminus of Alg5p does not interact with the *C*-terminus of Alg5p prey. The same result was obtained using those of the pBT-C-Alg5LR3 bait (a lower red frame in Fig. 4), indicating that the *C*-terminus of Alg5p lacking HR3 and LR4 does not interact with the *C*-terminus of Alg5p prey.

Co-transformants of the pBT-C-Alg5/HR1 or pBT-C-Alg5/LR2 with pAI-Alg5 or pDL2-Alg5 did not display growth activities on SD-LWH and SD-LWHA media (Fig. 4), indicating that the *C*-termini of this two truncated Alg5p baits are not located in the cytosol, but in rER lumen.

On the other hand, growth activities of those of pBT-C-Alg5, pBT-C-Alg5/HR2 or pBT-C-Alg5/LR3 with pDL2-Alg5 were similar to those with pAI-Alg5 (compare the left side with the right side within orange frames in Fig. 4). These results indicate that *C*-termini of these truncated Alg5p baits indeed interact with *C*-terminus of Alg5 prey.

Taken together, our membrane topological analysis demonstrated that the *N*-terminal LR1, LR3, HR3 and *C*-terminal LR4 of Alg5p are located in the cytoplasmic side of the rER membrane and that the long LR2 of Alg5p is located in the luminal side of the rER membrane (Fig. 9).

Although the possibility that the HR3 of Alg5p is a membrane-embedded domain cannot be so far excluded, our model represented in Fig. 9 is in well concordance with the models previously proposed as for Alg5p[7] and human orthologous hDPGS[12].

As shown with two green frames in Fig. 9, Alg5p possesses a DxD motif, which might bind the co-factor Mg^{2+} ion, and a putative catalytic PxY motif. Both of them are located in the LR2. Therefore, this long loop region is estimated to be a catalytic domain of Alg5p. As it was demonstrated to be localized in the rER lumen in this study, transfer reaction of glucose from UDP-glucose to Dol-P should occur within the lumen. Although UDP-glucose is yielded in the cytosol, it is known that it can be partially transported into the rER lumen by the specific transporters[30),31]. This fact means that the donor UDP-glucose available for Alg5p might be limited.

On the other hand, the availability of the acceptor Dol-P for Alg5p seems to be not relatively limited. It is *de novo*

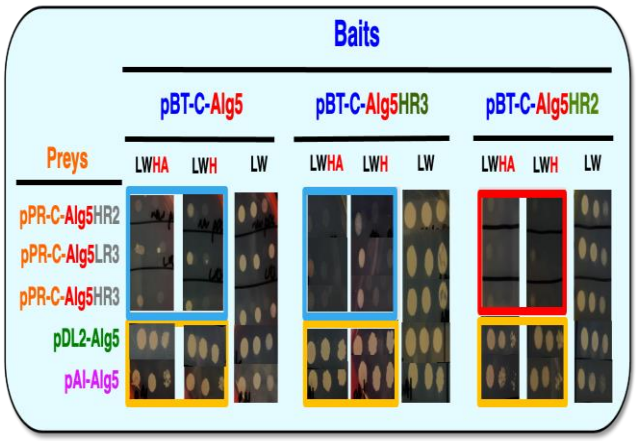

Fig. 5 Growth examination of co-transformants of full-length or truncated Alg5p bait with full-length or truncated Alg5p prey. The experiments were carried out according to the same procedure as described in the legend of Fig. 4.

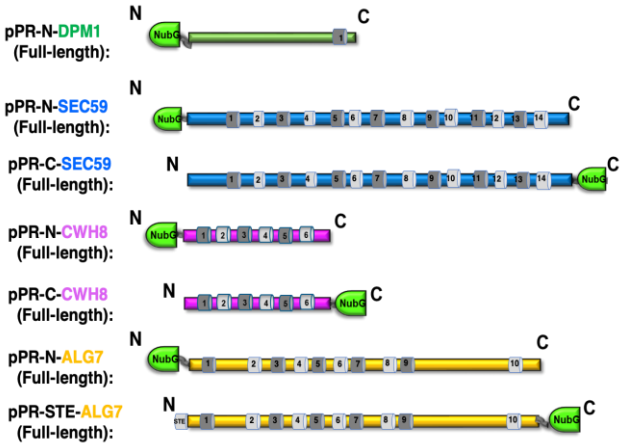

Fig. 6 The prey constructs for four kinds of enzymes (Dpm1p, Sec59p, Cwh8p and Alg7p) used in this study and structure of the prey proteins expressed by them. Boxes with numbers indicate hydrophobic regions (HRs) predicted by TOPCONS as well as Alg5p. A construct expressing *C*-terminally tagged Dpm1p was unavailable for the interaction analysis and so it was not made, because the Dpm1p has only one TMD with its *C*-terminus orientated towards the luminal side of the rER membrane.

biosynthesized by dolichol kinase (Sec59p) on the cytosolic side of the rER membrane and freely flip-flopped through the rER membrane (Fig. 1). Moreover, Dol-P is also reproduced by dolichol pyrophosphatase (Cwh8p) in the luminal side of the rER membrane (Fig. 1). As two Dol-P-utilizing enzymes, DPMS (Dpm1p) and GPT (Alg7p), orientate their catalytic domains toward cytoplasmic side (Fig. 1), Alg5p would not directly compete with these enzymes for Dol-P.

3.2 *The physical interaction of Alg5p with itself*

Alg5p has been utilized as a negative control prey in the YSUS on the assumption that it does not physically interact with a bait of any membrane protein[14]. However, our previous study and results shown in Fig. 4 (again shown with three orange frames in Fig. 5) clearly demonstrated that full-length Alg5p (expressed by the pBT-C-Alg5) and two truncated baits (expressed by the pBT-C-Alg5HR2 and pBT-C-Alg5HR3) physically interacts with full-length Alg5p prey expressed by the pDL2-Alg5 (which is substantially the same as pPR-C-Alg5). Therefore, in order to investigate this self-interaction of Alg5p in more detail, we used three novel types of the prey constructs (pRR-C-Alg5HR3, pPR-C-Alg5LR3 and pPR-C-Alg5HR2) expressing truncated Alg5p preys, instead of the pDL2-Alg5. Our results of growth examination of co-transformants are shown in Fig. 5. Compared with co-transformants of each of three baits with pDL2-Alg5 (upper row within three orange frames), those of pBT-C-Alg5 or pBT-C-Alg5HR3 with each of three truncated preys greatly decreased growth on SD-LWH and SD-LWHA media (two blue frames in Fig. 5), and those of pBT-C-Alg5HR2 displayed no growth on these media (a red frame in Fig. 5).

On the other hand, the fact that full-length and two truncated Alg5p baits really interact with full-length Alg5p prey derived from the pDL2-Alg5 means that the pDL2-Alg5 prepared in the YSUS does not serve as a negative control prey and consequently that it is not suitable for the analysis of Alg5p bait with other enzymes. In contrast, the truncated AG5HR2p prey expressed by the pPR-C-Alg5HR2 (designated

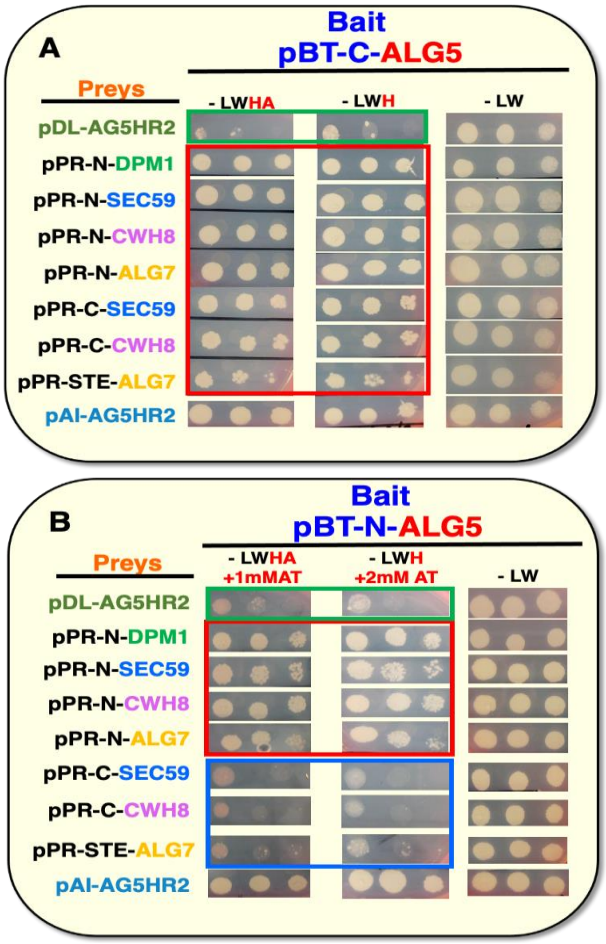

Fig. 7 Growth examination of co-transformants of full-length Alg5p baits with full-length preys of other enzymes exhibited in Fig. 6. The experiments were carried out according to the same procedure as described in the legend of Fig. 4.

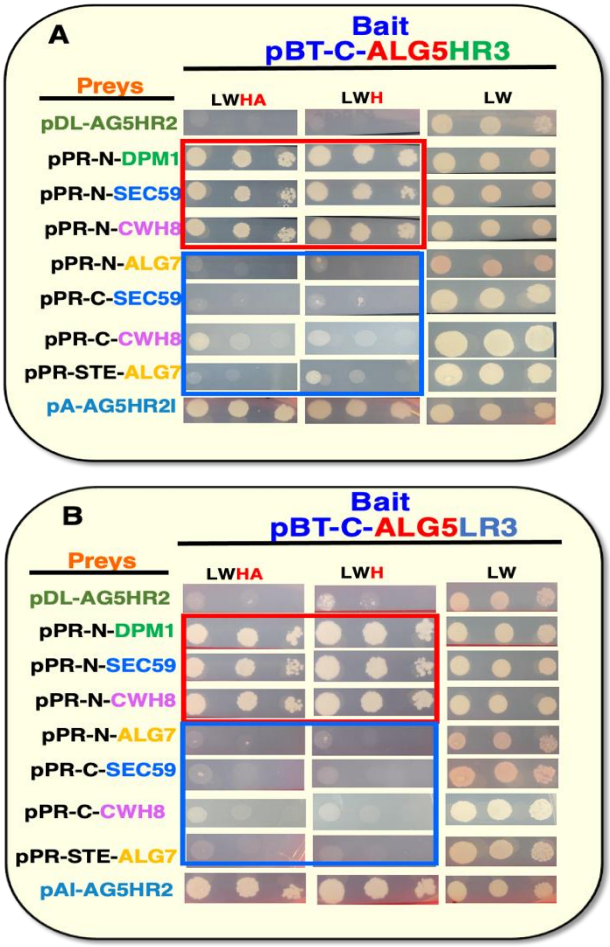

Fig. 8 Growth examination of co-transformants of the truncated Alg5p baits with the full-length preys of other enzymes exhibited in Fig. 6. The experiments were carried out according to the same procedure as described in the legend of Fig. 4.

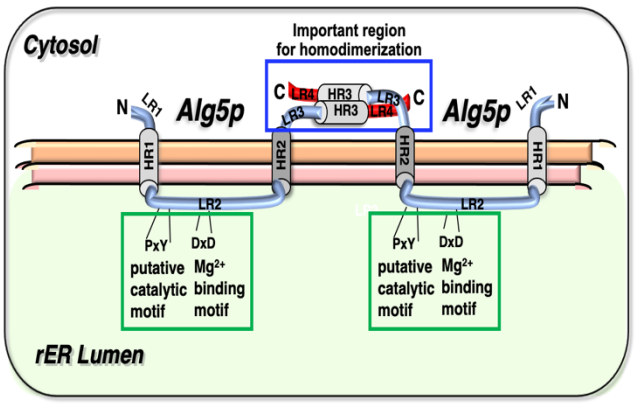

Fig. 9 The model of the membrane topology and physical interactions of Alg5p based on the results represented in Fig. 4, Fig. 5, Fig. 7 and Fig.8. A region critical for self-interaction of Alg5p is represented in a blue frame. The LR4, which is indicated in red color, is also essential for the interaction with Alg7p.

as pDL-AG5HR2 in the Ref. 24) hardly interacted with Alg5p baits as described above. In addition, it has been demonstrated that truncated AG5HR2p prey expressed by the pAI-AG5HR2 well interacted with full-length Alg5p bait[24]. Therefore, we decided to use the pDL-AG5HR2 and pAI-AG5HR2 as negative and positive for the analyses described in Section 3.3, respectively (Fig. 7 and Fig. 8).

3.3 *The physical interaction of Alg5p with other enzymes*

In our growth examination using four types of Dol-P-related enzymes as preys (shown in Fig. 6), all the co-transformants of pBT-C-Alg5 grew on SD-LWH and SD-LWHA media (a red frame in Fig. 7A), while those of pBT-N-Alg5 did not grow on the media, in case of combinations with the three *C*-terminally tagged preys (a blue frame in Fig. 7B). These results suggest that *C*-terminus of Alg5p bait would be accessible to all enzymes examined, but that *N*-terminus of Alg5p bait might have selectivity for orientation of accessing enzyme, probably due to conformational reasons.

Subsequently, we examined the physical interaction using two truncated Alg5p baits. As a result, co-transformants with the pPR-N-DPM1, pPR-N-SEC59 and pPR-N-CWH8 maintained the growth on SD-LWH and SD-LWHA media (red frames in Figs. 8A and 8B), while those with the pPR-N-ALG7, pPR-C-SEC59, pPR-C-CWH8 and pPR-STE-ALG7 abolished the growth on the media (blue frames in Figs. 8A and 8B). These observations indicated that the *C*-terminal LR4 of Alg5p was essential for the interaction between Alg5p bait and Alg7p prey and that a region from the *N*-terminus to the HR2 of Alg5p was enough for the interaction with other three enzymes examined. It is interesting that Alg7p only interacts with Alg5p via a different region. Alg7p serves as an initiator of DLO assembly, whilst Alg5p is necessary for the final three stages of DLO assembly. This fact raises the possibility that the interaction of Alg5p with Alg7p might specifically regulate the amount of DLOs yielded, in a feed-back manner.

4. Conclusion

Recently we briefly researched the membrane topology and physical interaction of Alg5p using the YSUS and demonstrated that its *C*-terminus is orientated towards cytoplasm and that it physically interacts with itself via a region from the LR3 to the *C*-terminus[24]. As we developed a novel set of control preys for the analysis of Alg5p in that study, we used it to proceed the research in more detail, combined with the novel truncated baits of Alg5p, in this study.

As for the membrane topology, we obtained a novel information that Alg5p has two TMDs, from the results shown in Fig. 4. Additionally, its *N*-terminus were found to be orientated towards cytoplasm by the analysis using the pBT-N-Alg5 bait construct (Fig.4).

Regarding the physical interaction with itself, we obtained an additional information that interaction between the two regions from the LR3 to the *C*-terminus of Alg5p is essential for homodimerization, from the results shown in Fig. 5.

We successfully detected the specific interactions of Alg5p with Dpm1p, Sec59p, Cwh8p and Alg7p, by using

pDL-AG5HR2 and pAI-AG5HR2 as control preys. We found out that both termini of Alg5p bait physically interacted with *N*-termini of all preys examined (Fig. 7). Moreover, we revealed that the *C*-terminal LR4 of Alg5p bait was critical for the interaction with Alg7p prey (compare Fig.8A with Fig. 7A).

Based on the results explained above, we propose a tentative model of the membrane topology and physical interaction of Alg5p, as shown in Fig. 9. In our previous study, we have demonstrated that hDPGS, a human ortholog of Alg5p, interacts with itself via only the LR3 and that it interacts with hGPT, a human ortholog of Alg7p, via the HR3[12]. Consequently, the model shown in Fig. 9 is slightly different from that for hDPGS, regarding the physical interactions, although they match each other well regarding the membrane topology. This study has demonstrated that Alg5p physically interacts with Dpm1p, Sec59p or Cwh8p via a region from the *N*-terminus to the LR3 of Alg5p. As a next step, a region critical for each interaction should be further refined by an analysis using novel truncated Alg5p baits.

The physical interaction between two membrane enzymes could play a role in positively or negatively altering their enzymatic activities. Here, we discovered the physical interactions of Alg5p with the four enzymes involved in DLO biosynthesis. In addition, our previous research detected the physical interaction of Alg5p with itself[24]. These facts raises the possibility that enzymatic activity of Alg5p might be regulated based on the status of homo- or hetero-oligomerization at protein level. In order to establish importance of Alg5p in enzymes participating in Dol-P metabolism, further executions of biochemical and stoichiometric analyses would be required.

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