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Construction of a Novel Set of Control Preys for the Yeast Split-Ubiquitin System

by

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Abstract

In order to investigate the physical interaction between two membrane proteins, we usually use the yeast split-ubiquitin system (YSUS). In the YSUS, two control prey constructs, pDL2-Alg5 and pAI-Alg5, which were developed based on the yeast *ALG5* gene, have been prepared as negative and positive controls for detection of protein interaction, respectively. These control prey constructs are available only when yeast Alg5p protein does not physically interact with any other protein. However, our previous study demonstrated that human orthologous protein, hDPGS, interacted with itself. This fact suggests the possibility that yeast Alg5p would also interact with itself. If so, both control preys might not be suitable for analysis of Alg5p. Therefore, we tried to develop a novel negative control prey that does not interact with Alg5p at all. For this purpose, we focused on our recent observation that hDPGS exhibited no interaction with truncated hDPGS, which contains only a region from the *N*-terminus to the second hydrophobic region (HR2). In order to replace a portion of the full-length Alg5p with that of truncated Alg5p (Alg5HR2p), which corresponds to truncated hDPGS, a coding region of Alg5p from the *N*-terminus to HR2 was amplified by PCR, and ligated with pPR3-C vector to transform *Escherichia coli* for molecular cloning. This manipulation yielded the negative control prey construct (pDL2-AG5HR2), which would express Alg5HR2p-NubG fusion protein in yeast cells. In addition, to create the positive control prey construct, combinatorial PCR with primers for introduction of point mutation was carried out using pDL2-AG5HR2 as a template. After PCR, the resultant fragment was ligated with pPR3-C vector to transform *E. coli*, yielding the positive control prey construct (pAI-AG5HR2), which would express Alg5HR2p-NubI fusion protein in yeast cells. Using these constructs as controls, physical interaction of Alg5p was analyzed using the YSUS. As a result, it was revealed that Alg5p did not interact with Alg5HR2p-NubG, but did interact with Alg5HR2p-NubI. This observation indicates that the novel prey constructs work well as negative and positive controls.

Keywords: Split-ubiquitin system, Control prey, Physical interaction

1. Introduction

The study of protein-to-protein interaction has become important more and more, because elucidation of the relationship among various proteins is one of key points for understanding a number of biological phenomena. As a quite useful tool for this purpose, the yeast two-hybrid system was devised, which can detect physical interaction between two proteins *in vivo*. However, it has been demonstrated not to be suitable for analysis of membrane proteins, as it forces to artificially localize two proteins of interest into the nucleus of the yeast cell.

To overcome this disadvantage, the yeast split-ubiquitin system (YSUS) was later developed for analysis of physical interaction between two membrane proteins[1-4]. This system can detect protein-to-protein interaction naturally occurring on the rough endoplasmic reticulum (rER) membrane. Using the YSUS, various physical interactions among membrane proteins, including enzymes and subunits of an enzymatic complex residing on the rER membrane, have been revealed[5–8].

We also have used the YSUS, and investigated physical interactions among human glycosyltransferases and related proteins that play a role in biosynthesis of dolichol-linked oligosaccharide (DLO) on the rER membrane[9-12]. Recently, we analyzed physical interactions of human dolichol-P-glucose synthase (hDPGS)[13]. From a result of the growth examination using co-transformants with hDPGS bait and hDPGSl prey, we noticed that hDPGS physically interacted with itself to form homo-dimer[13]. As yeast Alg5p is orthologous to hDPGS, they share similar primary structure and conformation with each other[14]. Hence, it is possible that Alg5p might interact with Alg5p-NubG, which is produced by the negative control prey pDL2-Alg5 prepared in the YSUS. As the negative and positive control preys in the YSUS are presupposed to exhibit no physical interaction of Alg5p with

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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	5'-tgtaatggccattacggccATGAGAGCGTTGAGATTCCTGATTG-3'	Cloning of *ALG5HR2*
AG5HR2_rv	5'-gcctttggccgaggcggccgaTGTGTCTTTGATAGACCTGATGCCG-3'	Cloning of *ALG5HR2*
NubI_fw	5'-CTTTGACCGGTAAAACCATAACATTGGAAGTTGAATC-3'	Mutagenesis from *NubG* to *NubI*
NubI_rv	5'-GATTCAACTTCCAATGTTATGGTTTTACCGGTCAAAG-3'	Mutagenesis from *NubG* to *NubI*
NUB_rv	5'-TTACATGACTCGAGGTCGACATCC-3'	Cloning of *ALG5HR2-NubI*
dl2xn_seqfw	5'-GTCGAAAATTCAAGACAAGG-3'	Cloning of *ALG5HR2-NubI*

any membrane protein, these observations suggest that the conventional set of these control preys could not be suitable for analysis of DPGS derived from any organism.

Therefore we attempted to construct the novel set of control preys in order to entirely exclude the possibility that yeast Alg5p interacts with any protein, including itself. For this purpose, we focused on the observation that hDPGS did not interact with truncated hDPGS which has a region from the *N*-terminus to the second hydrophobic region (HR2)[13]. Hence, our strategy for creating the novel preys was to exchange full-length *ALG5* portion (1002 bp) in pDL2-Alg5 and pAI-Alg5 for truncated AG5HR2 portion (741 bp) that encodes a region from the *N*-terminus to HR2 of Alg5p.

2. Experimental Methods

2.1 *Construction of the negative control prey (pDL2-AG5HR2)*

In order to produce the DNA fragment which encodes a region from the *N*-terminus to HR2 of yeast Alg5p, PCR with AG5_fw and AG5HR2_rv primers (Table 1) was performed using yeast genomic DNA as template by KOD ver.2 DNA polymerase (TOYOBO) [condition: 94 ^{0}C 2 min, (98 ^{0}C 10 sec, 60 ^{0}C 30 sec, 68 ^{0}C 1 min) by 36 times, 68 ^{0}C 2 min]. Amplified 741bp fragment and prey vector pPR3-C, which contains NubG, were digested with *Sfi* I restriction enzyme (TOYOBO) at 50 ^{0}C overnight, purified and ligated with each other by DNA ligase (TOYOBO) at 16 ^{0}C for 1 hour. Transformation of *Escherichia coli* JM109 with ligation mix by a method developed by Inoue *et al*.[15] yielded several colonies on ampicillin-containing LB plates. Plasmids were prepared from cultures inoculated from colonies, digested with *Bam* HI and *Eco* RI restriction enzymes (TOYOBO) at 37 ^{0}C for 2 hours, and subjected to analysis by agarose gel electrophoresis[16]. The plasmids which yielded a 741bp fragment were selected as candidates of pDL2-AG5HR2 and subjected to DNA sequencing analysis.

2.2 *Construction of the positive control prey (pAI-AG5HR2)*

Using pDL2-AG5HR2 as template, combinatorial PCR[17] was conducted in order to convert *ALG5HR2*-NubG portion to *ALG5HR2*-NubI portion as follows. PCR with dl2xn_seqfw and NubI_rv primers was carried out under the following condition: 94 ^{0}C 2 min, (98 ^{0}C 10 sec, 60 ^{0}C 30 sec, 68 ^{0}C 1 min) by 36 times, 68 ^{0}C 2 min. This yielded a head part of *ALG5HR2*-NubI portion (841 bp). On the other hand, PCR with NubI_fw and NUB_rv primers was carried out under the following condition: 94 ^{0}C 2 min, (98 ^{0}C 10 sec, 60 ^{0}C 30 sec, 68 ^{0}C 30 sec) by 36 times, 68 ^{0}C 1 min, to produce a tail part of *ALG5HR2*-NubI portion (101 bp). Both parts were mixed, heated at 100 ^{0}C for 5 min and cooled down to room temperature so that the two fragments hybridized with each other. Using the mixture as template, the second round PCR was performed with dl2xn_seqfw and NUB_rv primers under the same condition as amplification of the head part, to produce the full-length fragment of *ALG5HR2*-NubI portion (942 bp). Amplified 942 bp fragment and pPR3-C were digested with *Bam* HI and *Sal* I restriction enzymes (TOYOBO) at 37 ^{0}C for 3 hours, purified and ligated with each other by DNA ligase (TOYOBO) at 16 ^{0}C for 1 hour. Transformation and selection of desired recombinant plasmid pAI-AG5HR2 were performed by a standard cloning method[16] described above.

2.3 *Validation of pDL2-AG5HR2 and pAI-AG5HR2*

The bait construct, pBT-C-Alg5 was used for co-transformation of the *Saccharomyces cerevisiae* NMY51 strain, together with a conventional set of control preys (pDL2-Alg5 and pAI-Alg5) or the novel set of control preys (pDL2-AG5HR2 and pAI-AG5HR2). The transformation of the yeast NMY51 cells was carried out by the standard method[18]. The co-transformants obtained on the synthetic dextrose (SD) medium lacking leucine and tryptophan (SD-LW) were then subjected 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).

3. Results and Discussion

3.1 Molecular *cloning of pDL2-AG5HR2 and pAI-AG5HR2*

The YSUS takes advantage of the property of the yeast ubiquitin protein, in which *C*-terminal half portion (Cub) spontaneously associates with wild type *N*-terminal half portion (NubI) to form split-ubiquitin (Fig. 1A). To detect the physical interaction between two membrane proteins, NubG which is no longer able to spontaneously associates with Cub was devised by point mutagenesis of NubI (Fig. 1B). Despite abolishment of spontaneous association with Cub, NubG still can form split-ubiquitin only when it locates near Cub (Fig. 1C). This proximity between NubG and Cub occurs when the Nub-fused membrane protein physically interacts with the Cub-fused membrane protein (Fig. 1C). Therefore, the "negative" control prey protein fused with NubG must not interact with any bait protein fused with Cub (Fig. 1B). In contrast, the "positive" control prey protein fused with NubI should always form split-ubiquitin together with any bait protein fused with Cub (Fig. 1A). As mentioned above, we

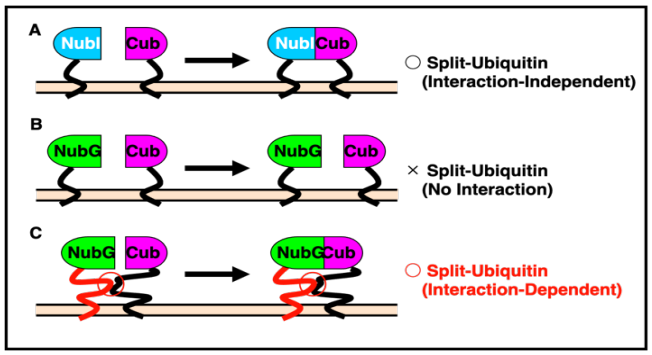

Fig. 1 The principal of the yeast split-ubiquitin sysyem (YSUS). A. bait protein fused with Cub vs. Alg5p-NubI expressed by pAI-Alg5. B. bait protein fused with Cub vs. Alg5p-NubG expressed by pDL2-Alg5. C. bait protein fused with Cub vs. prey protein fused with NubG.

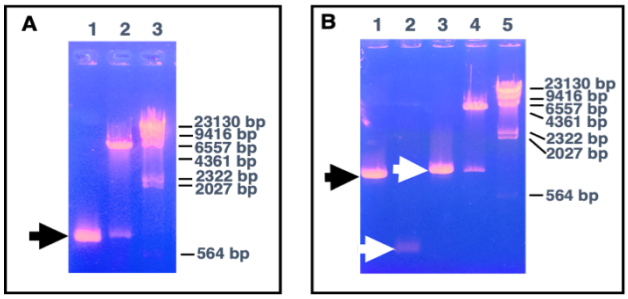

Fig. 2 A. Electrophoresis of PCR product described in section **2.1** (lane 1), pDL2-AG5HR2 after digestion with *Bam* HI and *Eco* RI (lane 2) and λ/*Hin*d III size marker (lane 3). B. Electrophoresis of combinatorial PCR products described in section **2.2** (head part; lane 1, tail part; lane 2, entire; lane 3), pAL-AG5HR2 after digestion with *Bam* HI and *Sal* I (lane 4) and λ/*Hin*d III size marker (lane 5).

suspected that Alg5p fused with NubG expressed from pDL2-Alg5 construct, might interact with Alg5p fused with Cub. In order to exclude this possibility, we had an idea that a region from the *N*-terminus to HR2 of Alg5p (Alg5HR2p) should be used rather than full-length Alg5p as the negative control prey. This idea was based on our observations that full-length hDPGS interacted with itself, and that a region from the *N*-terminus to HR2 of hDPGS (hDPGSHR2) no longer interacted with full-length hDPGS [13].

At the same time, full-length Alg5p portion expressed from positive control prey pAI-Alg5 also should be replaced with the same Alg5HR2p for comparison. Hence, we constructed both pDL2-AG5HR2 and pAI-AG5HR2 as the novel set of control preys.

Firstly, pDL-AGHR2 was produced by PCR cloning. The 741 bp fragment containing *AG5HR2*-coding region was successfully amplified by PCR (a black arrow of lane 1 in Fig. 2A) and cloned into pPR3-C vector via *Sfi* I sites (lane 2 in Fig. 2A).

Subsequently, pAI-AG5HR2 was constructed by introducing G13I substitutional mutation into *Nub* gene to convert *NubG* to *Nub*I. For this purpose, combinatorial PCR using pDL2-AG5HR2 containing *AG5HR2-NubG*-coding region as template was carried out. PCR with dl2xn_seqfw and NubI_rv primers generated 810 bp fragment containing head part of *AG5HR2-NubI* (a black arrow of lane 1 in Fig. 2B),

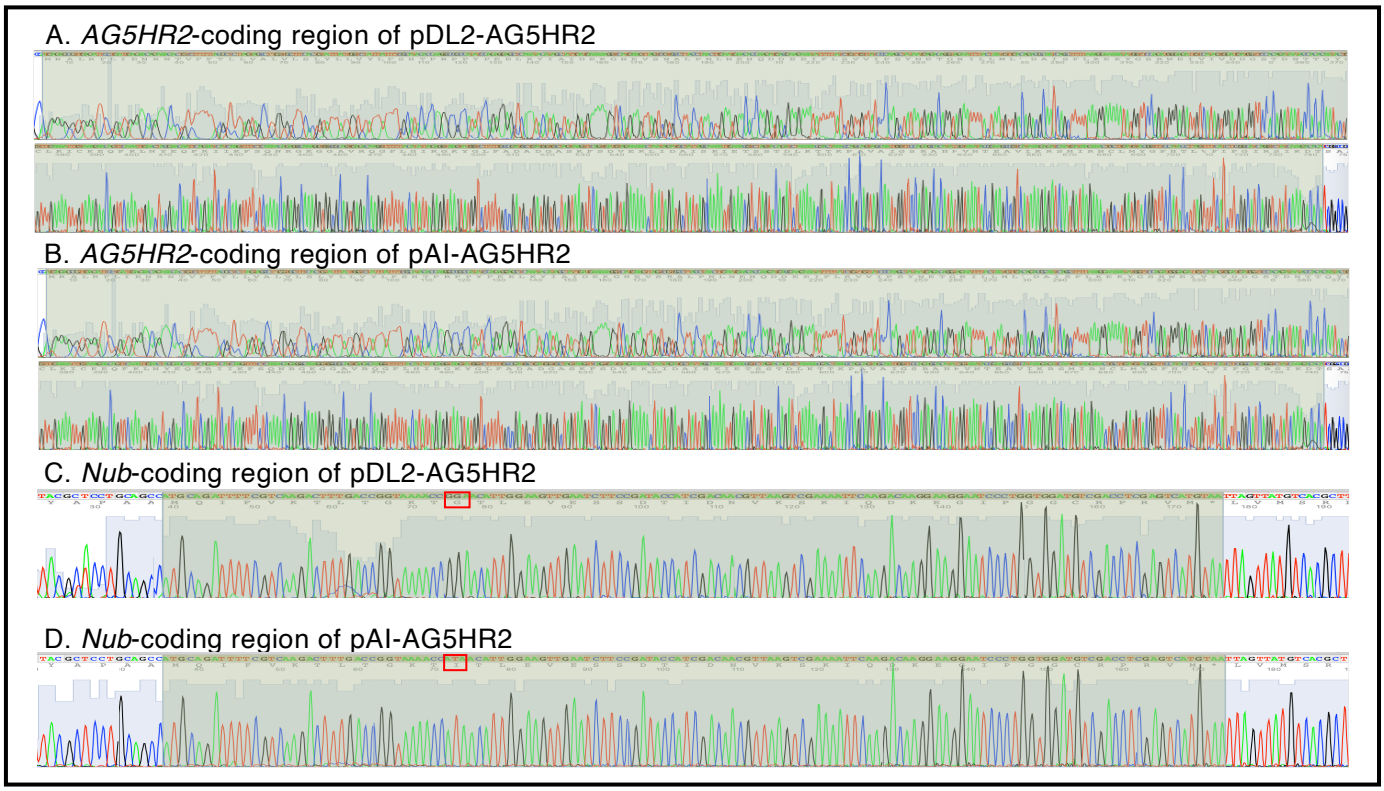

Fig. 3 Sequencing analysis. Nucleotide sequences of pDL2-AG5HR2 and pAI-AG5HR2. Sequencing reaction for analysis of *AG5HR2* or *Nub* portion was primed with dl2xn_seqfw (A and B) or Nub_fw primer (C and D), respectively. The dl2xn_seqfw or Nub_fw primer aneals against sequences upstream from *AG5HR2*- or *Nub*- initiation codon, respectively. Each coding region is highlighted in yellow color.

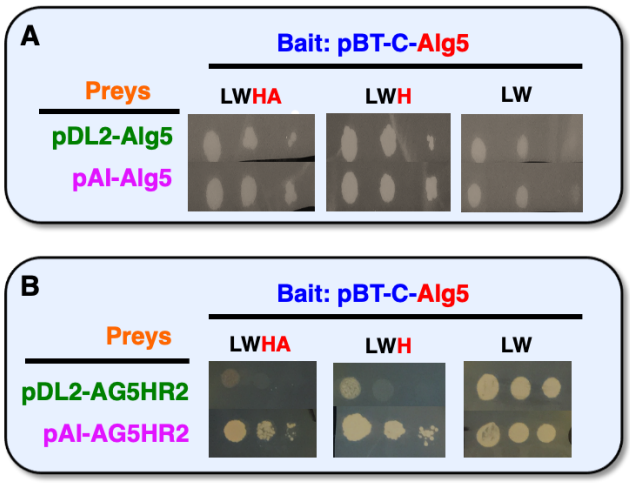

Fig. 4 Growth examination of co-transformant with pBT3-C-Alg5 (A and B). In A, the conventional control preys (pDL2-Alg5 and pAI-Alg5) were used, while the novel control preys (pDL2-AG5HR2 and pAI-AG5HR2) were used in B.

while that with NubI_fw and NUB_rv primers generated 110 bp fragment containing tail part of *AG5HR2-NubI* (a white arrow of lane 2 in Fig. 2B). Hybridization of both fragments and subsequent PCR with dl2xn_seqfw and NUB_rv primers produced 910 bp fragment including entire *AG5HR2-NubI*-coding region (a white arrow of lane 3 in Fig. 2B). The resulting fragment was cloned into pPR3-C vector via *Bam* HI and *Sal* I sites. (lane 4 in Fig. 2B). Both prey constructs obtained were then subjected to nucleotide sequencing and respectively confirmed as proper recombinant clones (Fig. 3A). Hence, nucleotide sequences of *AG5HR2*-coding portion derived from pDL2-AG5HR2 and pAI-AG5HR2 perfectly accorded (Fig. 3A and Fig. 3B), and those of *Nub*-coding portion matched with each other except only the thirteenth codon (indicated by red boxes in Fig. 3C and Fig. 3D).

3.2 The *novel control prey set properly works on the analysis of yeast Alg5p*

In order to test the nature of the set of prey constructs newly prepared above, they were individually co-introduced into yeast NMY51 cells together with bait construct pBT3-C-Alg5 and co-transformants were subjected to growth examination on SD-LWH and SD-LWHA media.

When the conventional set of prey constructs were used in such co-transformation and growth examination, co-transformants with pDL2-Alg5 grew on both selective media, as well as those with pAI-Alg5 (Fig. A). This result obviously indicated that Alg5p-NubG from pDL2-Alg5 physically interacts with Alg5p-Cub from pBT3-C-Alg5, via a portion of Alg5p. The possibility that Alg5p might form a homodimer has been already reported by Drake *et al.*[19] and therefore our result strongly supports this possibility.

In contrast, co-transformants with pDL2-AGHR2 barely grew on both selective media, although those with pAI-AG5HR2 normally grew on both media (Fig. 4B). These observations indicated that *C*-terminus of Alg5HR2p must be indeed oriented towards cytoplasmic side, and that Alg5HR2p itself should no longer physically interact with full-length Alg5p.

Taken together, in case of analyses of the physical interaction of yeast Alg5p, pDL2-AG5HR2 and pAI-AG5HR2 constructs successfully functioned as negative and positive controls, respectively.

4. Conclusion

Since we started using the YSUS twenty years ago, our question as to whether the Alg5p portion expressed by control preys might physically interact with any membrane protein, particularly itself, has remained unsolved. In order to clarify this issue, we developed the novel set of control preys (pDL-AGHR2 and pAI-AG5HR2) and examined their properties as experimental controls. In conclusion, pDL2-AG5HR2 construct expressed Alg5HR2p-NubG, which did not interact with an Alg5p portion of Alg5p-Cub bait protein, while pAI-AG5HR2 construct expressed Alg5HR2p-NubI, which did interact with a Cub portion of Alg5p-Cub bait protein via its NubI portion. These results show that the novel set of control preys were more suitable than conventional set of those for analysis of physical interaction regarding at least Alg5p. Although we presently cannot exclude the possibility that the Alg5HR2p portion might interact with unknown protein, our further research will prove availability of these preys.

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Analysis of the Stability of Human Mannose-Phosphate-Dolichol Utilization Defect 1 Protein Using the Yeast Split-Ubiquitin System

by

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Abstract

In the process of biosynthesis of dolichol-linked oligosaccharide (DLO), human mannose-phosphate-dolichol utilization 1 (MPDU1) protein, which is localized on the rough endoplasmic reticulum (rER) membrane, plays an essential role in the initiation of the latter stages. Nevertheless, its precise function remains to be clarified. In this study, we analyzed the stability of human MPDU1 (hMPDU1) protein by using constructs based on the pBT-STE vector of the yeast split-ubiquitin system (YSUS). The results demonstrated that full-length hMPDU1 bait protein with STE sequence improved the stability compared with that without STE sequence, but was still unstable. Moreover, several truncated hMPDU1 bait proteins were more unstable than the full-length hMPDU1 bait protein. Our finding demonstrating that these truncated hMPDU1 bait proteins exhibited different stabilities suggests that the hMPDU1 protein possesses several potential protease-sensitive sites.

Keywords: Split-ubiquitin system, MPDU1 protein, Protein stability

1. Introduction

Human mannose-P-dolichol utilization defect 1 (hMPDU1) protein, which is localized on the rough endoplasmic reticulum (rER) membrane, plays an essential role in the latter half stages of dolichol-linked oligosaccharide (DLO) assembly (Ref.1 and Fig. 1). When this protein is faulty or damaged, four steps of mannosylation dependent on the mannose-phosphate-dolichol (MPD) by the three mannosyltransferases (hAlg3, hAlg9, and hAlg12) do not proceed, even though MPD is normally biosynthesized, leading to severe defect of protein *N*-glycosylation known as congenital disorder of glycosylation (CDG) type If[2,3]. Hence, the protein name MPDU1 is derived from this phenomenon. Although it has been estimated that MPDU1 protein might be involved in the flipping of MPD (Fig. 1), the precise assignment remains to be clarified.

Therefore, we are analyzing the physical interactions of hMPDU1 protein with itself and other proteins which are involved in the biosynthetic pathway of DLO, using the yeast split-ubiquitin system (YSUS)[4-8]. The YSUS is a kind of yeast two-hybrid system for investigating whether two proteins (bait and prey) expressed in the rER membrane physically interact with each other. For this purpose, the bait and prey proteins are tagged with Cub-LexA/VP16 and NubG, respectively. Expression of reporter *HIS3* and *ADE2* genes, which respectively encode imidazole-glycerol-phosphate dehydratase and phospho-ribosyl amino imidazole carboxylase, is designed to occur only when bait interacts with prey. Using the YSUS, physical interactions of various membrane proteins have been demonstrated[9-12]. We also have revealed the physical interactions of several glycosyltransferases which

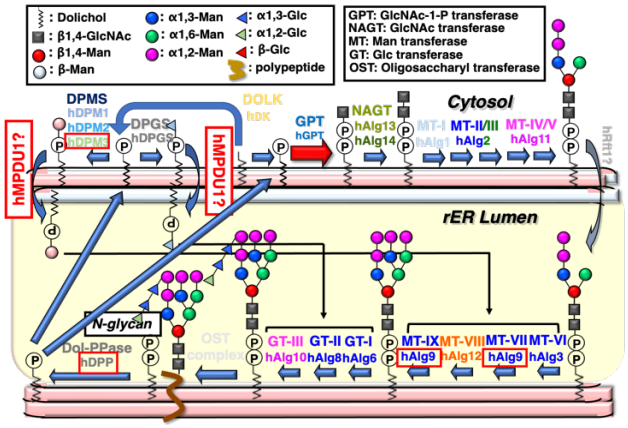

Fig. 1 Assembly of dolichol-linked oligosaccharides (DLOs) and site of action regarding the human mannose- phosphate-dolichol utilization defect 1 (hMPDU1) protein. DPMS, DK and DPP indicate dolichol-phosphate mannose synthase, dolichol kinase and dolichol pyrophosphatase, respectively.

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*3 Associate Professor, Department of Bioengineering

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
MU1BP_fw	5'-tgtaatggccattacggccATGGCGGCCGAGGCGGACGGACCG-3'	Cloning of full-length and truncated *hMPDU1*
MU1BP_rv	5'-gcctttggccgaggcggcCTGCGCCTTTTTCTGCTTGTGGGGAGG-3'	Cloning of full-length *hMPDU1*
MU1HR2BP_rv	5'-gcctttggccgaggcggccGGGAAGTTGTTAGTGATGCTGTAGA-3'	Cloning of truncated *hMPDU1/LR2*
MU1HR3BP_rv	5'-gcctttggccgaggcggcCACAGTCTGTCCTCTGTAGTGCAT-3'	Cloning of truncated *hMPDU1/LR3*
MU1HR4BP_rv	5'-gcctttggccgaggcggcCAAGGGCGTCAGAGGTGAGAGAA-3'	Cloning of truncated *hMPDU1/LR4*
MU1HR6BP_rv	5'-gcctttggccgaggcggcCCCAGCCATCAGGGGATCTCCG-3'	Cloning of truncated *hMPDU1/LR6*

reside on the rER membrane and are involved in DLO assembly using this system[13-17].

During previous investigations with the hMPDU1 bait construct (pBT-C-hMPDU1) prepared using the pBT-C vector of the YSUS, we noticed that the full-length hMPDU1 bait protein was a quite unstable in yeast cells, as its degradation frequently caused a high degree of activation of *HIS3* and *ADE2* reporter genes, which was independent of the protein-to-protein interaction to be detected. Such self-degradation of the hMPDU1 bait protein and subsequent activation of reporter genes will consequently affect and interfere with the results of analysis of its physical interaction using the YSUS. In order to more precisely assess the physical interaction of hMPDU1 protein in the YSUS, we developed new constructs which were prepared using the pBT-STE vector of the YSUS and attempted to validate its stability using the YSUS.

2. Experimental Methods

2.1 *Prediction of the membrane topology of hMPDU1 protein*

In order to predict the membrane topology of the hMPDU1 protein, a WWW algorithms server, Phobius (https://phobius.sbc.su.se/cgi-bin/predict.pl) was used. On its WEB site, the amino acid sequence of the hMPDU1 protein consisting of 247 residues was registered and surveyed for its potential transmembrane domain (TMD) and membrane topology.

2.2 *Preparation of the bait plasmid constructs*

There are seven hydrophobic regions (HR 1 to 7) and eight loop regions (LR 1 to 7) in the hMPDU1 protein. Using these regions as a guide, PCR with forward and reverse primers listed in Table 1 was carried out using KOD ver.2 DNA polymerase (TOYOBO CO., LTD.) under the following conditions: 94 ^{0}C 2 min, (98 ^{0}C 10 sec, 60 ^{0}C 30 sec, 68 ^{0}C 1 min) for 36 times, 68 ^{0}C 2 min. Amplified fragments and bait vector pBT-STE, which contains coding regions of the yeast STE sequence (for stabilization of expressed protein) and Cub-LexA/VP16 sequence, were digested with *Sfi* I restriction enzyme (TOYOBO) at 50 ^{0}C overnight, purified, and ligated with each other by DNA ligase (TOYOBO) at 16 ^{0}C for 1 hour. Transformation of *Escherichia coli* JM109 with a ligation mix by the method developed by Inoue *et al*.[18] yielded several colonies on ampicillin-containing LB plates. Plasmids were prepared from cultures inoculated from colonies, digested with *Xba* I and *Hin*d III restriction enzymes (TOYOBO) at 37 ^{0}C for 2 hours, and subject to analysis by agarose gel electrophoresis[19]. The plasmids which yielded desired fragments were selected as candidates and subjected to DNA sequencing analysis.

2.3 *Validation of the stability for full-length and truncated hMPDU1 proteins*

Using the bait construct pBT-C-hMPDU1, which was previously prepared, and the constructs prepared as described above, the transformation of the *Saccharomyces cerevisiae* NMY51 strain was conducted by the standard method[20]. The transformants obtained on the synthetic dextrose (SD) medium lacking leucine (SD-L) were then subjected to the growth examination on the SD medium lacking leucine and histidine (SD-LH) and SD medium lacking leucine, histidine and adenine (SD-LHA) according to the manual supplied by Dualsystems Biotech (www.dualsystems.com). Additionally, SD-LWH and SD-LHA media containing 3-amino triazole (3-AT) were used.

3. Results and Discussion

The YSUS is an excellent system for detecting the physical interaction between two membrane proteins *in vivo*. However, degradation of bait membrane protein triggers the release of a portion of LexA/VP16 from bait residing on the rER membrane, leading to the activation of *HIS3* and *ADE2* genes. This activation which does not depend on the interaction with prey is called "self-activation". To address the self-activation of the hMPDU1 bait protein, we tried to assess its stability in yeast cells.

Firstly, we analyzed the membrane topology of the hMPDU1 protein. By predicting its membrane topology with the Phobius algorithm, a server freely available on the WEB site (https://phobius.sbc.su.se/cgi-bin/predict.pl), it was revealed that it possesses seven hydrophobic regions (HRs 1 to 7), which act as potential transmembrane domains (Fig. 2). Moreover, Phobius predicted that the *C*-terminus

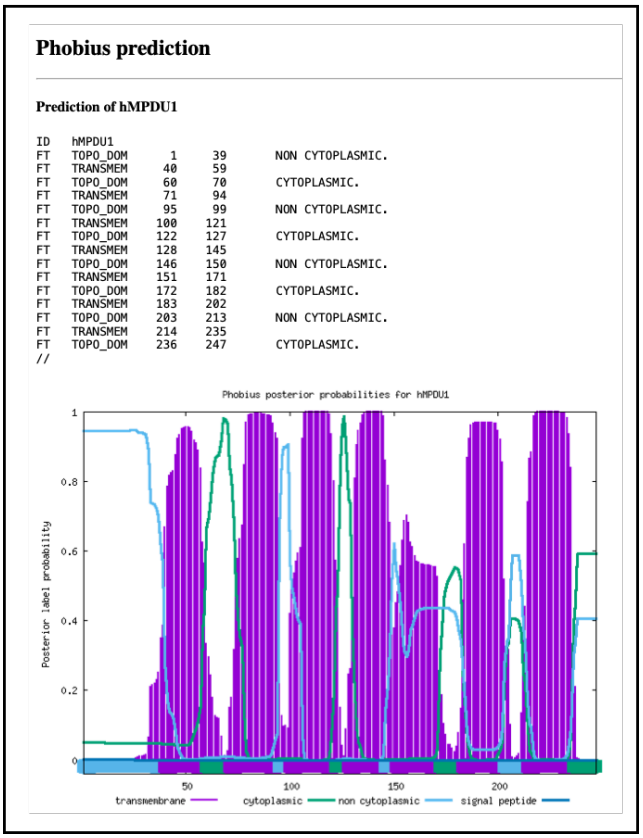

Fig. 2 Prediction of the membrane topology of hMPDU1 protein by Phobius algorithm server (https://phobius.sbc.su.se/cgi-bin/predict.pl). The seven hydrophobic regions (HRs 1 to 7) were detected in hMPDU1 protein as potential transmembrane domains (TMDs).

of the hMPDU1 protein is oriented towards the cytoplasmic side of the rER membrane (Fig. 2).

Based on these predictions, we prepared a series of bait constructs that would be able to express full-length (pBT-STE-hMPDU1) and four truncated hMPDU1 bait proteins (pBT-STE-hMPDU1/LR6, pBT-STE-hMPDU1/LR4, pBT-STE-hMPDU1/LR3 and pBT-STE-hMPDU1/LR2), which were N- and *C*- terminally tagged with STE and Cub-LexA/VP16, respectively, in yeast cells (Fig. 3). From our preliminary experiment, it has been already demonstrated that the *C*-terminal Cub-LexA/VP16 portions of the hMPDU1 proteins expressed by these bait constructs were located in the cytosol as shown in Fig. 3 (data not shown).

In the growth examination of resultant transformants obtained on SD-L plates, transformants with pBT-C-hMPDU1 remarkably grew on SD-LH and SD-LHA plates, but those with pBT-STE-hMPDU1 showed less growth on both plates (Fig. 4A). These observations indicate that the full-length hMPDU1 bait protein with STE sequence at *N*-terminus improves the stability compared with that without the STE sequence. On the other hand, transformants expressing the four truncated hMPDU1 bait proteins exhibited a better growth pattern on SD-LH and SD-LHA media than those expressing full-length hMPDU1

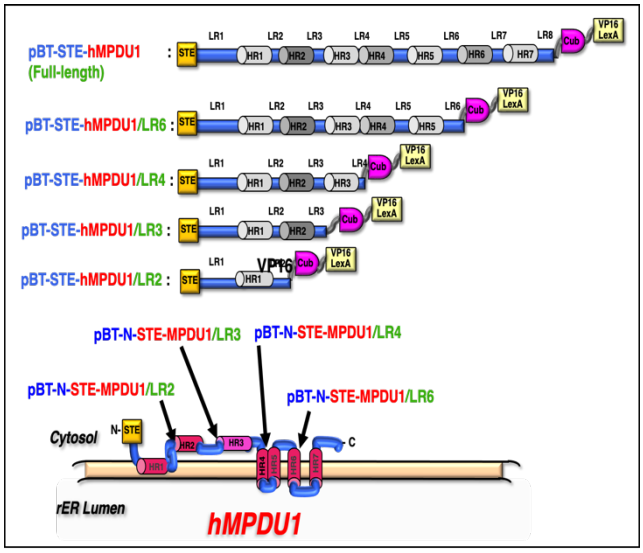

Fig. 3 Bait plasmid constructs used in this study. LR and HR mean loop region and hydrophobic region, respectively. The lower figure shows a model for the membrane topology of the hMPDU1 protein based on our preliminary experiment (unpublished data). Each arrow indicates the *C*-terminal portion of the truncated hMPDU1 bait protein expressed by each construct.

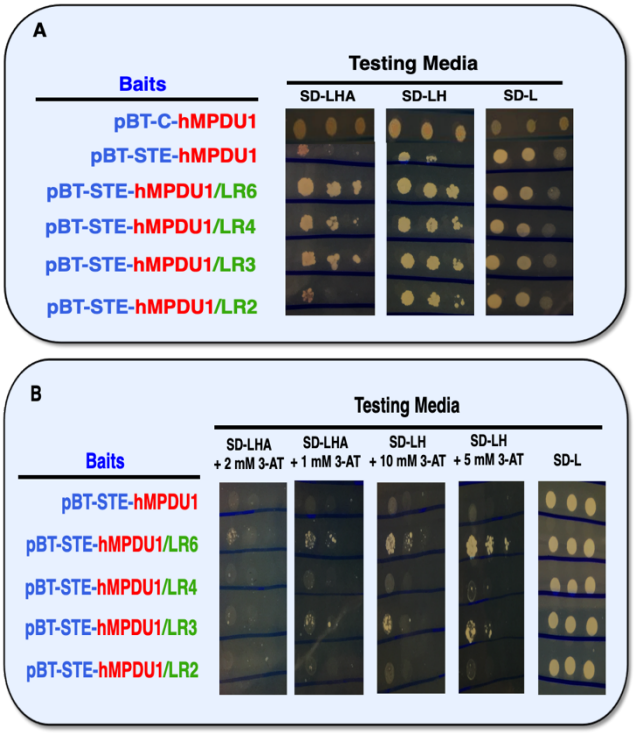

Fig. 4 Growth examination of transformants with pBT3-STE-hMPDU1, pBT3-STE-hMPDU1/LR6, pBT3-STE-hMPDU1/LR4, pBT-STE-hMPDU1/LR3 and pBT3-STE-hMPDU1/LR2. After the selection of colonies derived from the transformants on the SD-L medium, their suspensions were diluted with sterilized water and adjusted to the OD_{600} values of 1.0, 0.1, and 0.01 (from left to right). These diluents were orderly spotted on the SD-LH and SD-LHA media (panel A) or 3-AT-containing SD-LH and SD-LHA media (panel B) for reporter detection, and SD-L media for growth control, and then incubated at 30 ºC for 2~5 days.

bait protein (Fig. 4A), indicating that the added STE sequence has no effect on the stability. Particularly, it appeared that transformants with pBT-STE-hMPDU1/LR6 strongly grew on both selective media (Fig. 4A), suggesting that a hidden protease-sensitive site contained in hMPDU1/LR6 bait protein had become exposed.

Next, in order to assess the stability of the truncated hMPDU1 bait proteins in greater detail, we conducted a growth examination of transformants on the 3-AT-containing media, instead of SD-LH and SD-LHA media. The reagent 3-AT works as a competitive inhibitor of the *HIS3* gene product. Therefore, its addition to media allows growth arrest of transformants that have some level of *HIS3* gene activation on histidine-lacking media, such as SD-LH and SD-LHA, in proportion to its concentration ranging from 1 mM to 80 mM. As a result, transformants with pBT-STE-hMPDU1/LR6 and pBT-STE-hMPDU1/LR3 were able to grow on SD-LH medium containing 5 mM and 10 mM 3-AT, and SD-LHA medium containing 1 mM 3-AT, while the other transformants were not able to grow (Fig. 4B). These observations suggest that at least two cryptic protease-sensitive sites would exist within a region from HR2 to LR6 (Fig. 3). One of them might be located within a region from HR4 to LR6 (Fig. 3) because removal of this region seems to restore the stability of hMPDU1 protein (compare the growth of transformants expressing hMPDU1/LR6 bait protein with the growth of those expressing hMPDU1/LR4 bait protein in Fig. 4B). In the full-length hMPDU1 bait protein, this protease-sensitive site might be masked by a region from HR6 to the *C*-terminus. In addition, it is possible that there is another protease-sensitive site within a region from HR2 to LR3 (Fig. 3), which might be protected by a region from HR3 to LR4, because the hMPDU1/LR2 bait protein appeared to be more stable than the hMPDU1/LR3 bait protein (Fig. 4B). Although we do not investigate whether these protease-sensitive sites are really effective in human cells, our data raises the possibility that they might be critical for control of the activity of the hMPDU1 protein.

4. Conclusion

In this study, we concluded that hMPDU1 protein tends to be generally degraded in yeast cells, judging from the results of the growth examination of yeast transformants expressing the hMPDU1 bait protein on SD-LH and SD-LHA media. When the physical interactions of hMPDU1 bait protein with prey proteins of other enzymes involved in DLO assembly need to be precisely accessed by growth examination of co-transformant, self-activation of reporter genes due to degradation of MPDU1 bait protein should be carefully considered, because such self-activation cannot be differentiated from true reporter activation due to the physical interactions between hMPDU1 bait protein and certain prey enzymes, and consequently would interfere with precise observations.

As shown in Fig. 4, we were able to obtain data demonstrating that full length hMPDU1 bait protein bearing STE sequence at *N*-terminus improved the stability. This observation will be very useful in the analysis of physical interaction of the full-length hMPDU1 protein, because the amount of 3-AT used on growth examinations of co-transformants expressing the full-length hMPDU1 bait protein and various prey enzymes can be greatly regulated. On the other hand, it became clarified that the full-length hMPDU1 bait protein with STE sequence was still unstable. In addition, it was revealed that the truncated hMPDU1 bait proteins with STE sequence were also unstable, and their levels of the stability varied from one another. This means that it is necessary to individually optimize the concentration of 3-AT to be used according to these hMPDU1 bait proteins. Although more detailed analysis is needed for the optimization of 3-AT concentration on growth examinations, information concerning the stability of the hMPDU1 bait protein obtained in this study will be very useful for further analysis of physical interaction of this protein.

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Erratum: Characterization of the Membrane Topology and Physical Interaction of Human Dolichol-Phosphate-Glucose Synthase

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According to an offer from corresponding author, the following corrections were conducted in this article.

Page 2, **2.4** ***Assays for the physical interaction of hDPGS***, line 5-8, for :
“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.”

read :

“After the co-transformation, the co-transformants grown on SD-LW medium were subject to growth examination with SD-LWH containing 5mM 3-aminotriazole (3-AT) and SD-LWHA media, according to the same procedure as described above.”

Page 3, right row, line 3-4, for :
“On the contrary, those with the pDL-Alg5 negative control prey exhibited no growth on both media (Fig. 3A),”

read :

“On the contrary, those with the pDL-Alg5 negative control prey hardly exhibited growth on both media (Fig. 3A),”

Page 4, **3.2** ***The physical interaction of hDPGS***, line 11-13, for :
“Co-transformants with pBT-C-hDPGS/pPR-N-hDPGS were able to grow on LHW and LWHA media.”

read :

“Co-transformants with pBT-C-hDPGS/pPR-N-hDPGS were able to grow on LHW containing 5mM 3-AT and LWHA media.”

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