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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

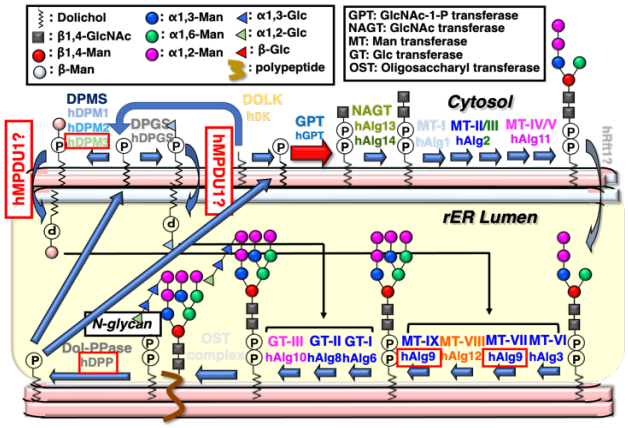

Fig. 1 Assembly of dolichol-linked oligosaccharides (DLOs) and site of action regarding the human mannose- phosphate-dolichol utilization defect 1 (hMPDU1) protein. DPMS, DK and DPP indicate dolichol-phosphate mannose synthase, dolichol kinase and dolichol pyrophosphatase, respectively.

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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

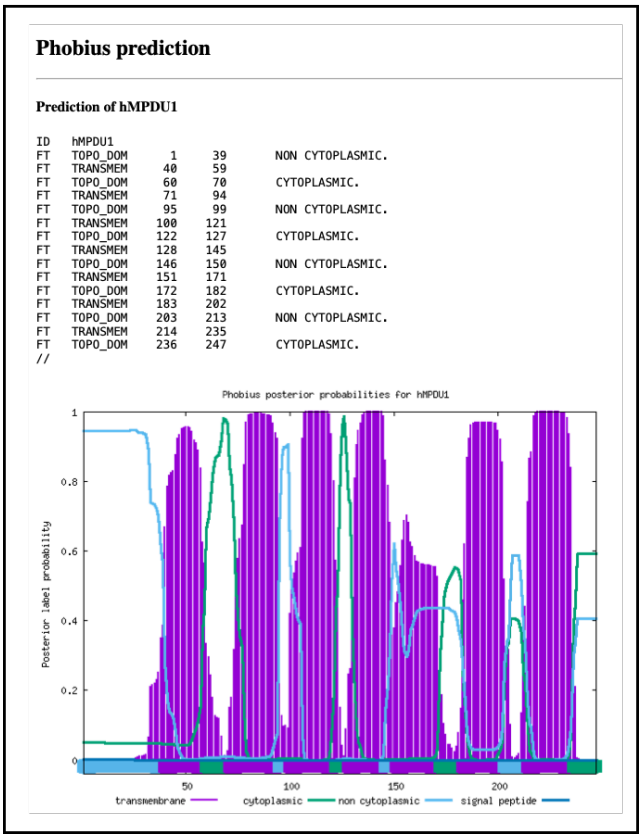

Fig. 2 Prediction of the membrane topology of hMPDU1 protein by Phobius algorithm server (https://phobius.sbc.su.se/cgi-bin/predict.pl). The seven hydrophobic regions (HRs 1 to 7) were detected in hMPDU1 protein as potential transmembrane domains (TMDs).

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

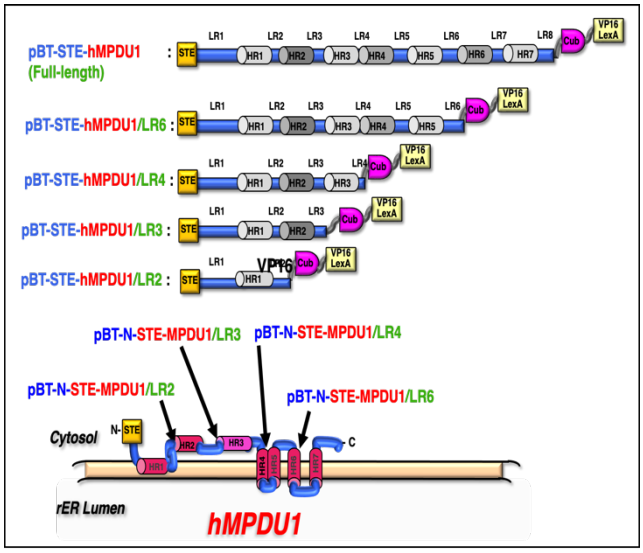

Fig. 3 Bait plasmid constructs used in this study. LR and HR mean loop region and hydrophobic region, respectively. The lower figure shows a model for the membrane topology of the hMPDU1 protein based on our preliminary experiment (unpublished data). Each arrow indicates the *C*-terminal portion of the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

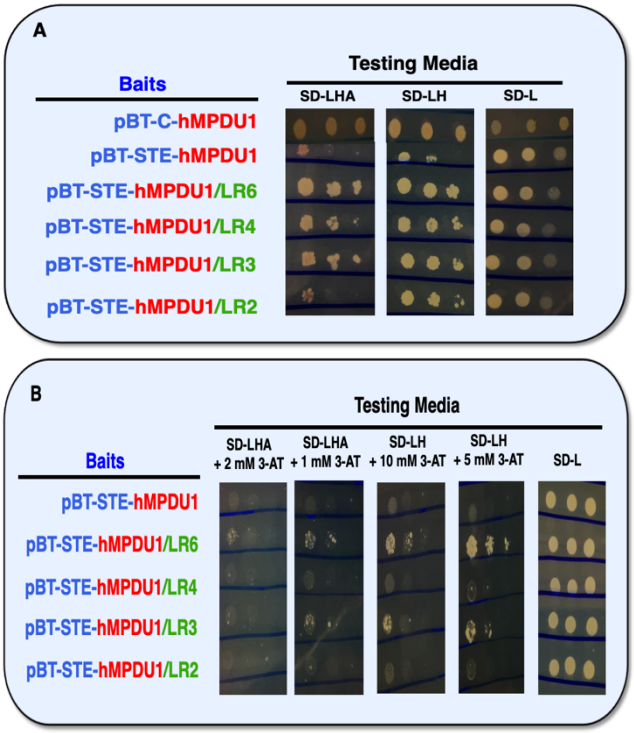

Fig. 4 Growth examination of transformants with pBT3-STE-hMPDU1, pBT3-STE-hMPDU1/LR6, pBT3-STE-hMPDU1/LR4, pBT-STE-hMPDU1/LR3 and pBT3-STE-hMPDU1/LR2. After the selection of colonies derived from the transformants on the SD-L medium, their suspensions were diluted with sterilized water and adjusted to 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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