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Mutational Analyses of the Human Mannosyltransferase I

by

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Abstract

In eukaryotes, protein *N*-glycosylation is one of the most important co-translational modifications for the structure and function of many proteins. *N*-glycans used for protein *N*-glycosylation are derived from dolichol-linked oligosaccharides (DLOs), which are assembled by several glycosyltransferases of the dolichol pathway localized on the rough endoplasmic reticulum (rER) membrane. Among them, mannosyltransferase I (MT-I) involved in the early assembly of DLO has proven to be essential for multiple cellular functions. In this study, we developed the budding yeast system in which the activity of mutagenized human mannosyltransferase I (hMT-I) could be assessed, using the temperature-sensitive *alg1* mutant. Mutational analysis using this system and randomly mutagenized *HMT-1* genes was successful in identifying several detrimental missense mutations in both catalytic and noncatalytic regions of hMT-I protein. In addition, mutational analysis using this system and site-directedly mutagenized *HMT-1* genes demonstrated the importance of the three motifs that were evolutionarily conserved among all eukaryotic MT-I enzymes. The present data obtained from our analyses will be useful for genetic diagnosis of congenital disorders of glycosylation type I (CDG-I) due to *HMT-I* gene defect.

Keywords: Mannosyltransferase, Dolichol-linked oligosaccharide, *N*-glycosylation, Mutagenesis, Genetic diagnosis

1. Introduction

In eukaryote, the structure and function of most glycoprotein greatly depend on *N*-glycans, which are transferred into specific asparagine residues of nascent polypeptides within rough endoplasmic reticulum (rER)[1]. Dolichol-linked oligosaccharides (DLOs) are biosynthesized on the rER membrane as precursors of *N*-glycans. For completion of assembly from dolichol phosphate to full-sized DLO, eleven glycosyltransferases and other related enzymes are required (Fig.1), which are also localized on the rER membrane[2)-5)]. The normal biosynthesis of DLO has been demonstrated to be essential for cell viability. For example, the *Saccharomyces cerevisiae alg* mutants, each of which has defect in any glycosyltransferase (or its gene) involved in DLO assembly, displays severe cell growth arrest or lethal phenotype[6)]. Of them, the yeast *alg1* mutant has a nonsense mutation within the *ALG1* gene coding the yeast mannosyltransferase I (yMT-I) which catalyzes transfer of the first mannose from GDP-mannose to chitobiosyl-pyrophosphoryl dolichol ($GlcNAc_2$-PP-dolichol) (Fig.1), and represents temperature-sensitive growth phenotype[7,8)].

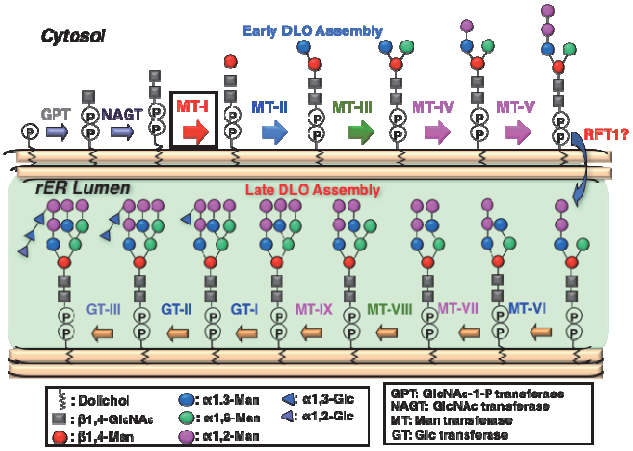

Fig. 1 Biosynthesis of dolichol-linked oligosaccharide (DLO) and site of action as for mannosyltransferase I (MT-I).

Previously, we were successful in cloning the *HMT-1* gene, which codes the functional human MT-I (hMT-I), by complementing this temperature-sensitive mutation of the *alg1* mutant[9)]. In other words, instead of the mutated *alg1* gene, the normal *HMT-1* gene has been demonstrated to be functional in the *alg1* cells under the non-permissive temperature in our previous study.

The defect of any enzyme involved in the DLO assembly is estimated to cause underglycosylation of almost N-linked glycoproteins leading to the malformations of multiple organs

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with various lesions. In particular, neuro-muscular system is severely damaged, including microcephaly, hypotonia, seizures, psychomotor retardation, cerebral and cerebellar atrophy and fluctuating hormone levels[10)-16)]. The inherited syndrome which represents such symptoms is known as congenital disorder of glycosylation type I (CDG-I).

Although the *HMT-1* gene was cloned as human ortholog of the yeast *ALG1* gene in 2000[9)], later, it has been demonstrated to be one of genes responsible for CDG-I. The CDG-I which arises from the defect of the *HMT-1* gene is called CDG-I type k (CDG-Ik) or CDG-ALG1. Since 2004, seven deleterious missense mutations have been identified in coding region of the *HMT-1* genes derived from patients with CDG-Ik[10)-16)]. Among them, S150R and G145D mutations are notably intriguing, because Ser^{150} and Gly^{145} of hMT-I are neither conserved nor located within a conserved domain among the species. This fact prompt us to investigate other novel potential mutational sites within the *HMT-1* gene, which could affect enzymatic function of hMT-I.

In this study, we constructed recombinant plasmids including various artificially mutangenized *HMT-1* gene, and examined effect of each mutation by introduction and expression in the *alg1* mutant cells under the non-permissive temperature.

2. Experimental Method

2.1 *Expression system of the yeast alg1 mutant cells*

The experimental expression system of mutagenized *HMT-1* gene is shown in Fig. 2. The PRY56 strain, which has temperature-sensitive mutation in the yeast *ALG1* gene. was constructed by Dr. P.W. Robbins[6)]. In these mutant cells, also called *alg1* cells, the mutated yeast MT-I is able to catalyze the native transfer reaction under permissive temperature (26 °C) and they normally grow on medium. However, under non-permissive temperature (36 °C), the enzyme is unable to catalyze the reaction, so that they lead to lethality. Introduction of normal human *HMT-1* gene into them has been already demonstrated to overcome the lethality under the non-permissive temperature (Fig. 3A and Fig.3B). Hence, by preparing various mutagenized *HMT-1* genes, introducing and expressing them in PRY56 cells, and then observing growth viability of the transformants at 36 °C, detrimental effect of each mutation would be easily accessed (Fig. 2).

2.2 *Random mutagenesis of the HMT-1 gene*

In order to prepare various mutagenized *HMT-1* genes, we used error-prone PCR technique[17), 18)]. This technique is able to control the average number of substitutional mutation which would be introduced by adjusting concentration of

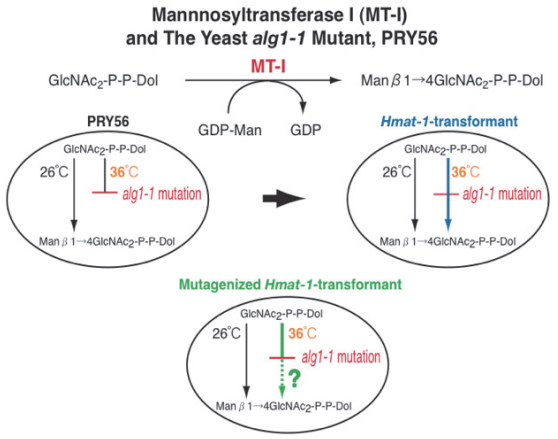

Fig. 2 Overview of experimental expression system of mutagenized *HMT-1* gene based on the yeast *alg1* cells..

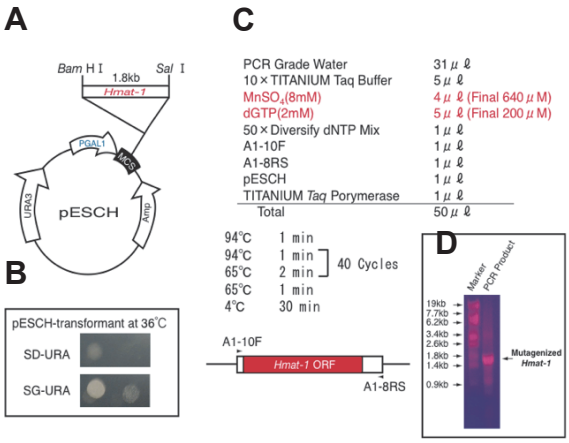

Fig. 3 Preparation of randomly mutagenized *HMT-1* genes by error-prone PCR. The original HMT-1-recombinant plasmid (pESCH) is shown in panel A. Growth phenotype of transformant with pESCH is represented in panel B. The PCR condition is shown in panel C, and the result of agarose gel electrophoresis is represented in panel D.

dGTP and Mg^{2+} ion. Under the reaction condition represented by Fig. 3C, approximately 10 substitutional mutations were estimated to be artificially introduced into 1.6 kb coding region of *HMT-1* (Fig, 3D). The informations of PCR primers, A1-10F and A1-8RS, used in the error-prone PCR, are shown in Table 1.

2.3 *Preparation of randomly mutagenized HMT-1-recombinant plasmids and screening procedure*

As shown in Fig. 4A and Fig. 4B, randomly mutagenized *HMT-1* fragments were subsequently cloned into pESC-URA plasmid vector based on PCR, and then mutagenized *HMT-1*-recombinant plasmids (mutagenized *HMT-1* gene library) were prepared via transformation of *Escherichia coli* (*E. coli*) JM109 strain. Transformation of the yeast PRY56 cells with this library allowed cloning of mutagenized *HMT-1* genes, and growth examination of each transformant with mutagenized

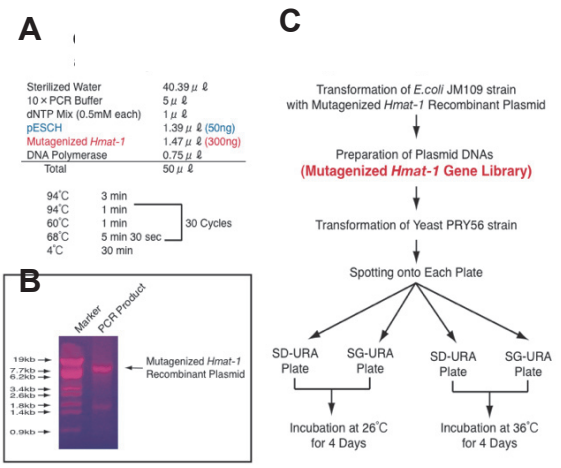

Fig. 4 Procedure for cloning and selection of non-functional mutagenized *HMT-1* genes. PCR condition is shown in panel A, and the result of agarose gel electrophoresis is shown in panel B. Screening procedure is shown in panel C.

HMT-1 under four growth conditions allowed selection of non-functional *HMT-1* (Fig. 4C). Expression of recombinant *HMT-1* is controlled under the *GAL1* promoter (P_{GAL1}) on pESC-URA plasmids (Fig. 3A), and repressed on SD-URA medium containing dextrose as sole carbon resource (Fig. 3B). In contrast, on SG-URA medium containing galactose as sole carbon resource, transformants can express the recombinant *HMT-1* (Fig. 3B). If the mutagenized *HMT-1* is functional, the transformants could grow on SG-URA media owing to the *HMT-1*, but not on SD-URA medium, at non-permissive temperature (36 °C). On the other hands, if mutation introduced into *HMT*-1 is harmful, the transformants could not grow on both SG-URA and SD-URA media at 36 °C. Therefore, such screening system using the yeast *alg1* cells under four culture conditions (Fig. 4C) enabled us to readily detect the dysfunctional *HMT-1* gene.

2.4 *Preparation of site-directedly mutagenized HMT-1-recombinant plasmids*

When we cloned the *HMT-1* and predicted amino acid sequence of hMT-I protein previously, we noticed that the three motifs (motif-I, II and III) highly conserved among various eukaryotes exist in this enzyme (Fig. 5B)[9]. Interestingly, as shown in Fig.5A, all these motifs are not located within the catalytic domain which is commonly well-conserved among the species. Such the functions of motif-I, II and III are not so far elucidated. Hence, in order to investigate their significances, site-directed mutagenesis was performed using combinatorial PCR[19] (Fig.6). The PCR primers, which were used for substitutional mutations of one amino acid to alanine within these motifs, are shown in Table 1. The mutagenized *HMT-1* genes were ligated into pESC-URA vector, and conventional cloning via transformation of *E. coli*

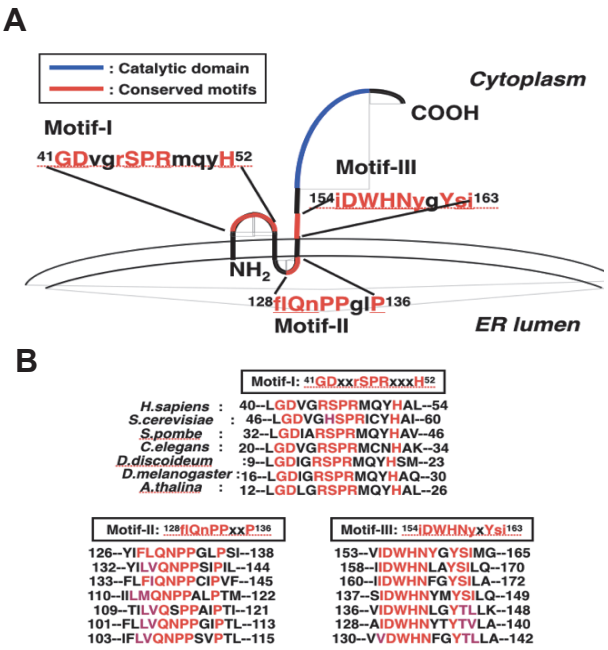

Fig. 5 Conserved motifs of hMT-I enzyme. Location of the three motifs is shown in panel A. Partial alignment of amino acid sequences of MT-I proteins derived from various organisms is shown in panel B. In panels A and B, the amino acid residues highly conserved among the seven species are indicated in red uppercase letters, and those semi-conserved are indicated in red lowercase letters.

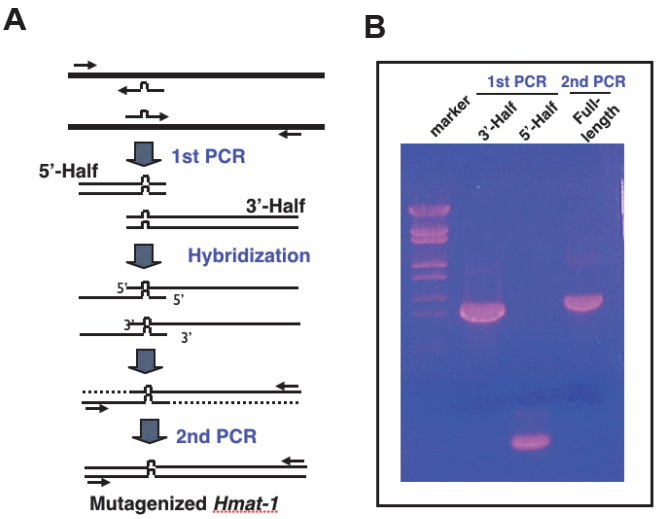

Fig. 6 Site-directed mutagenesis of the *HMT-1* by combinatorial PCR. Its scheme is shown in panel A, and the result of agarose electrophoresis is represented in panel B.

JM109 strain was carried out[20]. Recombinant plasmids containing only one missense mutation causing substitution to alanine residue in any conserved motif of *HMT-1* was used for transformation of the yeast PRY56 (*alg1*) cells. As illustrated in Fig.4C, the transformants obtained on SD-URA medium were then subject to growth examination.

3. Results and Discussion

Via screening of 600 mutagenized *HMT-1* clones for

Table I The PCR primers used in this study. Additional sequences or mutational sites are shown in lowercase letters.

Primer name	Nucleotide sequence	Purpose
A1-10F	5'-gtacggatccGGCGGGCCAGCCAAGATGGC-3'	Error-prone PCR
A1-8RS	5'-gtacgtcgacCAACGTGGACACACTCAGTT-3'	Error-prone PCR
G41A-F	5'-GCGGTGGTGCTGGcCGACGTGGGCCGCAG-3	Combinatorial PCR targeted to motif-I
G41A-R	5'-CTGCGGCCCACGTCGgCCAGCACCACCGC-3'	Combinatorial PCR targeted to motif-I
D42A-F	5'-GTGGTGCTGGGCGcCGTGGGCCGCAGCCC-3'	Combinatorial PCR targeted to motif-I
D42A-R	5'-GGGCTGCGGCCCACGgCGCCCAGCACCAC-3'	Combinatorial PCR targeted to motif-I
R45A-F	5'-GGGCGACGTGGGCgcCAGCCCCCGTATGC-3'	Combinatorial PCR targeted to motif-I
R45A-R	5'-GCATACGGGGGCTGgcGCCCACGTCGCCC-3'	Combinatorial PCR targeted to motif-I
S46A-F	5'-GCGACGTGGGCCGCgcCCCCCGTATGCAG-3'	Combinatorial PCR targeted to motif-I
S46A-R	5'-CTGCATACGGGGGgcGCGGCCCACGTCGC-3'	Combinatorial PCR targeted to motif-I
P47A-F	5'-GACGTGGGCCGCAGCgCCCGTATGCAGTAC-3'	Combinatorial PCR targeted to motif-I
P47A-R	5'-GTACTGCATACGGGcGCTGCGGCCCACGTC-3'	Combinatorial PCR targeted to motif-I
R48A-F	5'-GGGCCGCAGCCCCgcTATGCAGTACCACGC-3'	Combinatorial PCR targeted to motif-I
R48A-R	5'-GCGTGGTACTGCATAgcGGGGCTGCGGCCC-3'	Combinatorial PCR targeted to motif-I
H52A-F	5'-CCCGTATGCAGTACgcCGCGCTGTCGTTGG-3'	Combinatorial PCR targeted to motif-I
H52A-R	5'-CCAACGACAGCGCGgcGTACTGCATACGGG-3'	Combinatorial PCR targeted to motif-I
F128A-F	5'-CAGGTGCCTATATCgcTCTCCAGAACCCCC-3'	Combinatorial PCR targeted to motif-II
F128A-R	5'-GGGGGTTCTGGAGAgcGATATAGGCACCTG-3'	Combinatorial PCR targeted to motif-II
L129A-F	5'-GTGCCTATATCTTTgcCCAGAACCCCCCAG-3'	Combinatorial PCR targeted to motif-II
L129A-R	5'-CTGGGGGGTTCTGGgcAAAGATATAGGCAC-3'	Combinatorial PCR targeted to motif-II
Q130A-F	5'-CCTATATCTTTCTCgcGAACCCCCCAGGTC-3'	Combinatorial PCR targeted to motif-II
Q130A-R	5'-GACCTGGGGGGTTCgcGAGAAAGATATAGG-3'	Combinatorial PCR targeted to motif-II
N131A-F	5'-ATATCTTTCTCCAGgcCCCCCCAGGTCTGC-3'	Combinatorial PCR targeted to motif-II
N131A-R	5'-AGACCTGGGGGGgcCTGGAGAAAGATATAG-3'	Combinatorial PCR targeted to motif-II
P132A-F	5'-CTTTCTCCAGAACgCCCCAGGTCTGCCTAG-3'	Combinatorial PCR targeted to motif-II
P132A-R	5'-CTAGGCAGACCTGGGGcGTTCTGGAGAAAG-3'	Combinatorial PCR targeted to motif-II
P133A-F	5'-CTTTCTCCAGAACCCCgCAGGTCTGCCTAG-3'	Combinatorial PCR targeted to motif-II
P133A-R	5'-CTAGGCAGACCTGcGGGGTTCTGGAGAAAG-3'	Combinatorial PCR targeted to motif-II
P136A-F	5'-CCCCCCAGGTCTGgCTAGCATTGCTGTCTG-3'	Combinatorial PCR targeted to motif-II
P136A-R	5'-CAGACAGCAATGCTAGcCAGACCTGGGGGG-3'	Combinatorial PCR targeted to motif-II
I154A-F	5'-GAAGCAAGCTCGTCgcTGACTGGCACAAC-3'	Combinatorial PCR targeted to motif-III
I154A-R	5'-GTTGTGCCAGTCAgcGACGAGCTTGCTTC-3'	Combinatorial PCR targeted to motif-III
D155A-F	5'-CAAGCTCGTCATTGcCTGGCACAACTATGG-3'	Combinatorial PCR targeted to motif-III
D155A-R	5'-CCATAGTTGTGCCAGgCAATGACGAGCTTG-3'	Combinatorial PCR targeted to motif-III
W156A-F	5'-GCTCGTCATTGACgcGCACAACTATGGC-3'	Combinatorial PCR targeted to motif-III
W156A-R	5'-GCCATAGTTGTGCgcGTCAATGACGAGC-3'	Combinatorial PCR targeted to motif-III
H157A-F	5'-CGTCATTGACTGGgcCAACTATGGCTACTC-3'	Combinatorial PCR targeted to motif-III
H157A-R	5'-GAGTAGCCATAGTTGgcCCAGTCAATGACG-3'	Combinatorial PCR targeted to motif-III
N158A-F	5'-GTCATTGACTGGCACgcCTATGGCTACTCC-3'	Combinatorial PCR targeted to motif-III
N158A-R	5'-GGAGTAGCCATAGgcGTGCCAGTCAATGAC-3'	Combinatorial PCR targeted to motif-III
Y159A-F	5'-ATTGACTGGCACAACgcTGGCTACTCCATC-3'	Combinatorial PCR targeted to motif-III
Y159A-R	5'-ATGGAGTAGCCAgcGTTGTGCCAGTCAATG-3'	Combinatorial PCR targeted to motif-III
Y161A-F	5'-GGCACAACTATGGCgcCTCCATCATGGGTC-3'	Combinatorial PCR targeted to motif-III
Y161A-R	5'-GACCCATGATGGAGgcGCCATAGTTGTGCC-3'	Combinatorial PCR targeted to motif-III
S162A-F	5'-CAACTATGGCTACgCCATCATGGGTCTGG-3'	Combinatorial PCR targeted to motif-III
S162A-R	5'-CCAGACCCATGATGGcGTAGCCATAGTTG-3'	Combinatorial PCR targeted to motif-III
I163A-F	5'-CTATGGCTACTCCgcCATGGGTCTGGTGC-3'	Combinatorial PCR targeted to motif-III
I163A-R	5'-GCACCAGACCCATGgcGGAGTAGCCATAG-3'	Combinatorial PCR targeted to motif-III

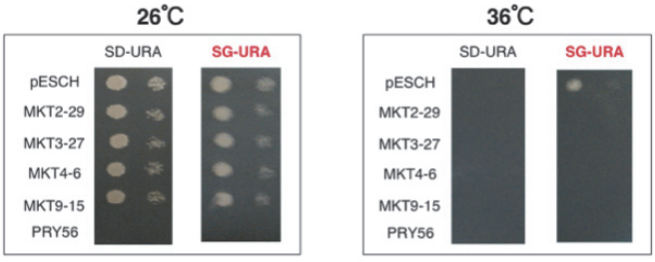

Fig. 7 Growth phenotype of the four transformants with non-functional mutagenized *HMT-1* genes. The top "pESCH" shows transformants with normal *HMT-1* as positive control.

Table 2 Identification of number of nucleotide substitutions and predicted amino acid substitutions in the four randomly mutagenized *HMT-1* genes.

Mutant	Nucleotide Substitution	Amino Acid Substitution
MKT2-29	13	8
MKT3-27	12	6
MKT4-6	3	1
MKT9-15	8	6

dysfunctionality, four clones, MKT2-29, MKT3-27, MKT4-6 and MKT9-15, which could not complement the yeast *alg1*

mutation at 36 °C, were obtained (Fig. 7, right panel). Analysis of nucleotide sequence of them revealed their numbers of mutation, indicating that error-prone PCR was well controlled. According to the sequencing data, mapping of mutational sites in the four MKT mutant version of hMT-I protein was subsequently conducted (Fig. 8). Interestingly, all four mutants had L387S missense mutation. Considering that the MKT4-6 mutant had no other mutation, Leu^{387} residue located in the catalytic domain is important for enzymatic activity of hMT-I. As three other MKT mutants has several mutations in addition to L387S, we perform re-correction of this mutation (i.e. S387L conversion) of them, by combinatorial PCR, in order to address whether other significant mutations would exist. The resulting MKT2-29L, MKT3-27L and MKT9-15L mutants were introduced into the *alg1* cells, respectively, and growth examination was carried out. As seen in Fig. 9, transformants with MKT3-27L and MKT9-15L successfully recovered growth ability on SG-URA media at 36 °C. These observations indicate that all mutations except L387S in both mutants are harmless. On the other hand, the transformant with KMT2-29L was not able to grow on SG-URA media at 36 °C (Fig. 9). The result strongly suggests that any of seven remaining mutations contained in KMT2-29L might significantly affect MT-I function. Among these seven mutations, Y161C is noteworthy, because Tyr^{161} was located in motif-III and well-conserved (Fig. 5). On the other hand, other six mutations were in catalytic domain (Fig. 8), and the inspection of effect by these mutations is in progress.

Next, we explored the significance of the three conserved motifs, which are not localized within the catalytic domain in hMT-I. The 21 residues were selected from conserved or non-conserved residues in each motif, and site-directed mutagenesis, in which only one residue would be substituted into alanine residue, was conducted by combinatorial PCR. The results of growth examination of transformants with the *HMT-1* mutagenized in motif-I, II and III were shown in Fig. 10, Fig, 11 and Fig. 12, respectively.

In the motif-I, substitution of conserved residues, Gly^{41}, Asp^{42}, Ser^{46}, Arg^{46} or His^{52} to Ala led to lethality on SG-URA media at 36 °C, suggesting that these residues play a role in hMT-I function (Fig.10). In contrast, semi-conserved Arg^{45} residue and conserved Pro^{47} residue could not be anticipated to be critical, because their substitutions appeared to have no effect on hMT-I activity (Fig.10).

With respect to motif-II, substitution of Phe^{128}, Leu^{129}, Asn^{131}, Gln^{130} or Pro^{136} to Ala led to lethality on SG-URA media at 36 °C, suggesting that these residues might play a role in hMT-I function (Fig.11).

Finally, substitution of conserved or semi-conserved residues, except Tyr^{161}, to alanine in the motif-III all caused lethality on SG-URA media at 36 °C (Fig. 12). Unexpectedly, Y161A mutation was harmless to hMT-I function, because Tyr^{161} residue is perfectly conserved among seven species (Fig.5B), and so Y161C mutation was considered as a

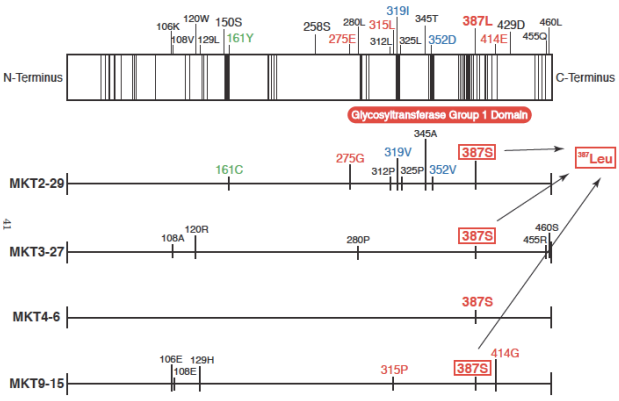

Fig. 8 Mapping of mutational sites of the four MKT mutant versions of hMT-I. Corresponding amino acid residues in the original hMT-I proteins are shown in top as reference.

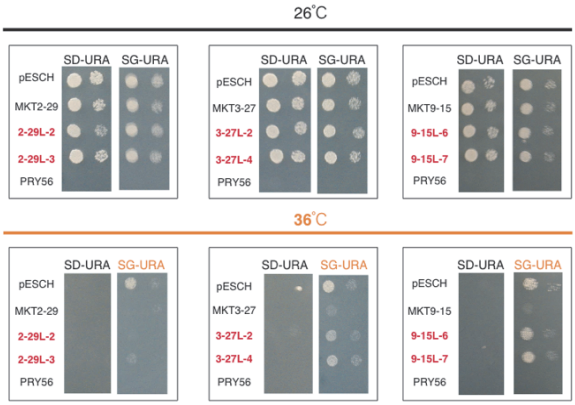

Fig. 9 Growth phenotype of the three transformants with MKT in which only L387 mutation was re-corrected. The top "pESCH" shows transformants with normal *HMT-1* as positive control. The second shows each of original MKT-transformants. The bottom shows parental *alg1* mutant cells (PRY56) as negative control.

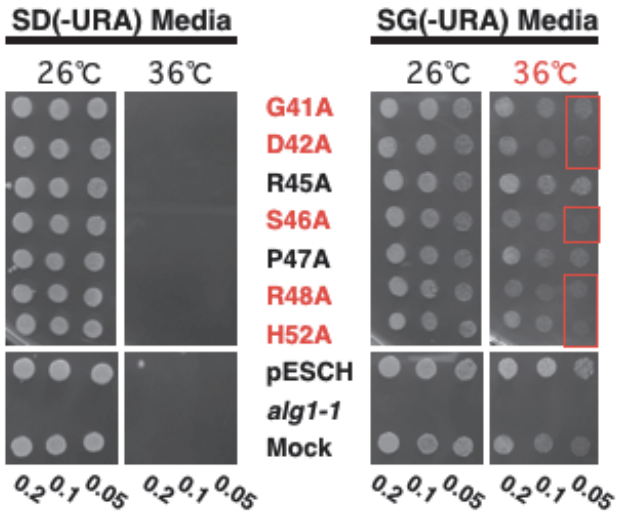

Fig. 10 Growth phenotype of transformants with *HMT-1* site-directedly mutagenized in motif-I. The bottom is transformant with pESC-URA vector plasmid as negative control. Diluted suspensions of transformants or parental *alg1-1* cells with OD_{600} of 0.2, 0.1 and 0.05 were used in the experiments.

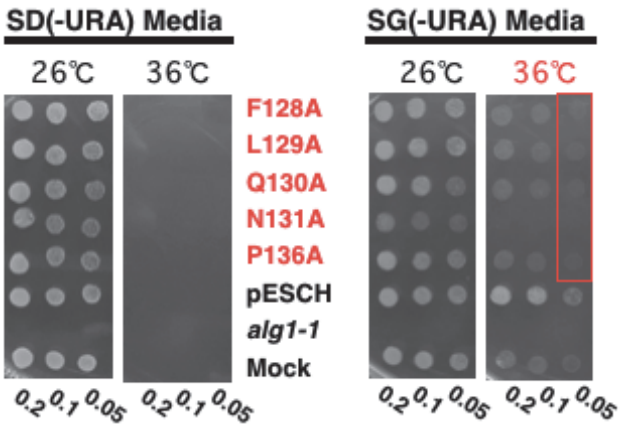

Fig. 11 Growth phenotype of transformants with *HMT-1* site-directedly mutagenized in motif-II. The bottom is transformant with pESC-URA vector plasmid as negative control. Diluted suspensions of transformants or parental *alg1-1* cells with OD_{600} of 0.2, 0.1 and 0.05 were used in the experiments.

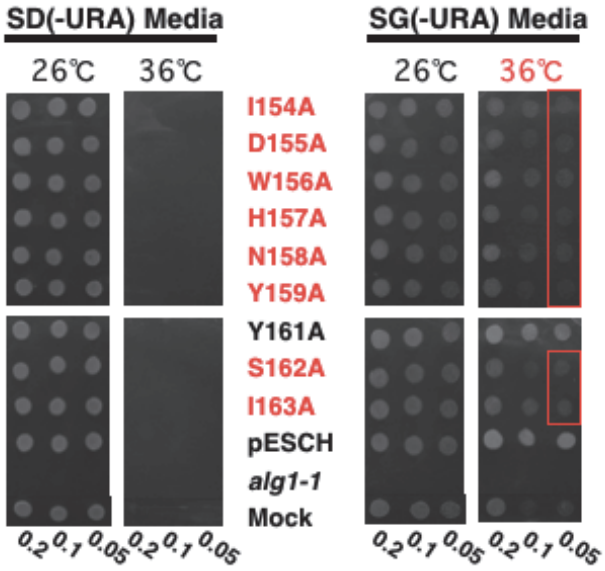

Fig. 12 Growth phenotype of transformants with *HMT-1* site-directedly mutagenized in motif-III. The bottom is transformant with pESC-URA vector plasmid as negative control. Diluted suspensions of transformants or parental *alg1-1* cells with OD_{600} of 0.2, 0.1 and 0.05 were used in the experiments.

candidate for dysfunction of MKT2-29L (Fig.8). Although the possibility that the substituted Cys^{161} might have deleterious effect on hMT-I activity remain to be investigated, Tyr^{161} residue itself seems to be not so crucial.

As these residues are not localized within the catalytic domain of hMT-I, determination of their precise functions would be difficult. As predicted function, protein stability, allosteric effect, recognition of donor or acceptor substrates, association with other enzyme or regulatory protein are considered. In fact, it has been demonstrated that the yeast ALG1 protein forms enzymatic complex with ALG2 or ALG11 mannosyltransferases[21]. We also analyze the physical interaction of enzymes involved in DLO biosynthesis, including hMT-I, and detected the interaction of hMT-I with other human mannosyltransferases[22]. Therefore, the mutation characterized in this study should be further investigated for significance of protein interactions.

5. Conclusion

In this study, we search amino residues in hMT-I which might profoundly participate in MT-I function. Random mutational analysis revealed that Leu^{387} in its catalytic domain is critical and that other important residue(s) might exist.

On the other hand, site-directed mutational analysis revealed Gly^{41}, Asp^{42}, Ser^{46}, Arg^{46} and His^{52} in the motif-I, Phe^{128}, Leu^{129}, Asn^{131}, Gln^{130} and Pro^{136} in the motif-II, and Ile^{154}, Asp^{155}, Trp^{156}, His^{157}, Asn^{158}, Tyr^{159}, Ser^{162} and Ile^{163} in the motif-III should be essential for the function of hMT-I.

In CDG-Ik patients, seven mutations which cause S258L, E342P, S150R, M377V, G145D, C396* or R276W alternations in hMT-I have been reported so far[10)-16)]. In addition to these mutations, the novel mutations identified in this study are expected to be useful for genetic diagnosis of *HMT-1* in the case of clinic judgement or calculation of risk for CDG-Ik.

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