厚生労働科学研究費補助金

障害者対策総合研究事業（神経・筋疾患分野）

中枢神経症状を伴う筋疾患α-ジストログリカノパチーの分子病態と治療法開発に関する研究

平成２３年度　総括研究報告書

研究代表者　萬谷　博

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研究代表者 萬谷　博、 地方独立行政法人東京都健康長寿医療センター、主任研究員

研究要旨　　遺伝性の神経疾患や筋疾患は進行性で極めて難治性であり、その代表的疾患として筋ジストロフィーがある。筋ジストロフィーは一般的に単一遺伝子の異常によるもので、これまでに多くの原因遺伝子が発見され、発症メカニズムの解明や治療法開発に期待が寄せられている。我々はこれまでに、先天性筋ジストロフィーに分類される muscle-eye-brain 病（MEB）と Walker-Warburg syndrome（WWS）の原因遺伝子産物 POMGnT1 と POMT1、POMT2 が *O*-マンノース型糖鎖を合成する糖転移酵素あることを示し、MEB と WWS が *O*-マンノース型糖鎖不全を起因とする疾患であることを明らかにしている。これらの知見は *O*-マンノース型糖鎖が神経や筋組織の正常な発生や機能に重要な役割を果たしていること示している。本年度は、昨年度末に *O*-マンノース型糖鎖がリン酸化された新奇構造が報告されたことから、新奇構造の生合成機構について検討した。また、POMT1 と POMT2 の複合体形成機構について、酵母ホモログである ScPmt1 で報告されている複合体形成機構との比較解析を行った。

A. 研究目的

福山型先天性筋ジストロフィー症（FCMD）、Muscle-eye-brain 病（MEB）、Walker-Warburg 症候群（WWS）は中枢神経系の障害を伴う先天性筋ジストロフィー症である。これらの疾患はジストロフィン糖蛋白質複合体の構成分子であるα-ジストログリカンの *O*-マンノース型糖鎖不全を起因とし、α-ジストログリカノパチーと総称される。本研究では、α-ジストログリカノパチーの原因遺伝子産物及び *O*-マンノース型糖鎖の機能を解明することで、病態解明から診断・治療法への応用を目指している。

我々はこれまでに、MEB と WWS の原因遺伝子産物 Protein *O*-linked mannose β1,2-*N*-acetyl glucosaminyltransferase 1（POMGnT1）と protein *O*-mannosyltransferase 1（POMT1）及び POMT2 が *O*-マンノース型糖鎖（Siaα2-3Galβ1-4 GlcNAcβ1-2Man-Thr/Ser）の生合成に関わる糖転移酵素であることを明らかにしている。

哺乳類の *O*-マンノース型糖鎖はα-ジストログリカンなどの限られた蛋白質でしか検出されないことから、特定のアミノ酸配列に特異的な修飾である可能性がある。*O*-マンノース型糖鎖合成の開始酵素は protein *O*-mannosyl transfease（POMT）であり、Man 修飾の特異性は主に POMT に依存すると考えられるが、POMGnT1 は GlcNAc 以降の構造の部位特異性に関与する可能性がある。また最近、マンノースの 6 位がリン酸化され、リン酸ジエステルを介して糖鎖が付加する可能性が報告された。また、リン酸ジエステルがα-ジストログリカンとラミニンの結合に関与することが示唆されている。そこで、POMGnT1 の基質特異性および生合成過程におけるマンノースのリン酸化と POMGnT1 の関係について検討した。

また、ヒト *O*-マンノース転移酵素 POMT1 と

POMT2 は酵素活性の発現に POMT1-POMT2 複合体の形成が必要である。しかし、各サブユニットの役割や複合体形成のメカニズムはよく分かっていない。出芽酵母（*S. cerevisiae*）の POMT ホモログである ScPmt1 と ScPmt2 は複合体を形成する。ScPmt1 は 7 回膜貫通型タンパク質であり小胞体内腔側に位置する loop 5 が活性中心と予想されているが、loop 1 に位置する Arg64、Glu78、Arg138 も酵素活性に重要であり、特に Arg138 は複合体形成に必須であることが報告されている。我々はこれまでにヒト POMT の二次構造を解析し、ScPmt1 と同様の膜配向性をとることを明らかにした。また、ScPmt1 の Arg64、Glu78、Arg138 は POMT1（Arg30, Glu44, Arg105）と POMT2（Arg72, Glu86, Arg145）ですべて保存されていた。そこで、これらのアミノ酸の重要性について検討した。

B. 研究方法

①POMGnT1 の基質特異性とマンノースリン酸化の解析

α-ジストログリカンのアミノ酸配列を基に Man-*O*-Thr を含むペプチドを有機化学的に合成し、アミノ酸組成や残基数の影響を反応速度論的に解析した。また、マンノースの 6 位をリン酸化したマンノース-6-リン酸化ペプチド（Man6P-pept）を合成し POMGnT1 の基質となるか調べた。

②酵母 ScPmt1 の重要アミノ酸のヒト POMT1, 2 における重要性の解析

site-directed mutagenesis 法によりヒト POMT1 の Arg30、Glu44、Arg105 および POMT2 の Arg72, Glu86, Arg145 をそれぞれ Ala に置換した変異体（R30A、E44A、R105A、R72A、E86A、R145A）を HEK293T 細胞に共発現させ、酵素活性と複合体形成への影響を調べた。ウェスタンブロットにより各タンパク質の発現量を定量し、発現量当たりの酵素活性を測定した。複合体形成は免疫沈降法により確認した。

（倫理面への配慮）

実験動物の使用に関しては、「動物愛護管理法」および「動物実験に関する指針」に基づいて動物愛護に十分配慮するとともに、所内実験動物委員会の承認を受けている。放射性同位元素の使用関しては「放射線障害防止法関連法令」および「所内規定」に基づき安全を確保する。組換え DNA 実験に関しては、「遺伝子組換え生物等の使用等の規制による生物の多様性の確保に関する法律」に基づくとともに所内組換え DNA 実験安全委員会の承認を受けている。

C. 研究結果

①POMGnT1 の基質特異性とマンノースリン酸化の解析

アミノ酸残基数 3～8 ではペプチドの伸長に伴って *Km* 値は減少、*Vmax* 値は増加した。また、合成ペプチドのアミノ酸組成は *Km* 値、*Vmax* 値に影響した。また、POMGnT1 は Man6P-pept に GlcNAc を転移することができなかった。

②酵母 ScPmt1 の重要アミノ酸のヒト POMT1, 2 における重要性の解析

変異型 POMT1（R30A、E44A、R105A）と

野生型 POMT2 との共発現では酵素活性は著しく減少し、正常型（100%）に対してそれぞれ 25%（R30A）、20%（E44A）、5%（R105A）であった。一方、野生型 POMT1 と変異型 POMT2（R72A、E86A、R145A）との共発現では酵素活性の減少は認められなかった。免疫沈降実験では、すべての組み合わせにおいて共沈が観察され、いずれの変異体も複合体を形成することが確認された。

D. 考察

①POMGnT1 の基質特異性とマンノースリン酸化の解析

POMGnT1 はアミノ酸 1 つからなるマンノシルスレオニン（Man-*O*-Thr）に GlcNAc を転移することができず、基質として認識するためには少なくともマンノースに 3 残基以上のアミノ酸が結合している必要がある。さらにアミノ酸の長さや配列により GlcNAc への転移効率が変化したことから、POMGnT1 がアミノ酸配列をある程度認識していると考えられる。また、POMGnT1 が Man6P-pept に GlcNAc を転移できなかったことから、マンノースのリン酸化は POMGnT1 による GlcNAcβ1-2Man 形成の後に起こる必要がある。このことから、GlcNAcβ1-2Man 形成がマンノース 6 位のリン酸化により制御されている可能性が考えられる。さらに、マンノースの 6 位には GnT-IX (VB)により GlcNAc が転移され、分岐構造 GlcNAcβ1-2(GlcNAcβ1-6)Man が形成されることも報告されていることから、マンノースの 6 位では GlcNAc 化とリン酸化が競合する可能性が考えられる。こうした生合成の制御機構が *O*-マンノース型糖鎖の機能調節に関与しているのかもしれない。

②酵母 ScPmt1 の重要アミノ酸のヒト POMT1, 2 における重要性の解析

変異型 POMT1 では酵素活性が減少したことから、POMT1 の loop1 も ScPmt1 と同様に酵素活性に必要であることが示された。一方、変異型 POMT2 では酵素活性への影響が見られなかったことから、POMT1 と POMT2 では loop1 の働きがことあることが明らかとなった。この結果は、POMT1 と POMT2 が機能的に異なる可能性を示すものである。また、複合体の形成に関しては POMT1、POMT2 ともに変異の影響を受けなかったことから、複合体形成のメカニズムは酵母とヒトでは異なることが示唆された。

E. 結論

O-マンノース型糖鎖の生合成では、マンノースの 6 位リン酸化は、POMGnT1 による GlcNAcβ1-2Man 形成の後に起こることが明らかとなった。ヒト POMT1 の loop1 も酵母 ScPmt1 と同様に酵素活性の発現に重要であることが明らかとなった。しかしながら、複合体形成のメカニズムはヒトと酵母では異なると考えられた。POMT1 と POMT2 の働きが異なる可能性を示した。

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研究成果の刊行に関する一覧表

書籍

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研究成果の刊行物・別刷

PEPTIDE SCIENCE 2011 : K.Sakaguchi (Ed.)
The Japanese Peptide Society (2012)

Synthesis of Glycopeptide Containing 6-*O*-Phosphorylated Mannose for an α-Dystroglycan/Laminin Interaction Study

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α-Dysrtoglycan (α-DG) is one of the best known O-mannosylated proteins in mammals, and O-mannosyl glycan of α-DG play an important role in its binding to laminin. Recently, new glycan containing phosphorylated mannose residue was found. In order to study α-DG/laminin interaction, 6-O-phosphorylated mannosyl peptide was synthesized.

Keywords: 6-*O*-phosphorylated mannose, α-dystroglycan, laminin, POMGnT1

Introduction

Dystroglycan (DG) is a membrane protein and is a component of the dystrophin-glycoprotein-complex in skeletal muscle. DG is composed of an extracellular α-DG subunit and a transmembrane β-DG subunit, and α-DG binds to laminin. Some muscular dystrophies, such as muscle-eye-brain disease (MEB) and Fukuyama congenital muscular dystrophy (FCMD), are caused by deletion of α-DG/laminin interaction [1]. It was found that *O*-mannosyl tetrasaccharide (NeuAcα2-3Galβ1-4GlcNAcβ1-2Man) exists in brain and muscle α-DG, and this mannosyl saccharide was required for laminin binding [2]. In the previous study, we found protein *O*-linked mannose β1,2-N-acetylglucosaminyltransferase 1 (POMGnT1) which catalyzes the transfer of GlcNAc to *O*-mannose of glycoproteins. Additionally, we have shown that mutations in the *POMGnT1* gene cause MEB.

Recently, laminin-binding activity of trisaccharide containing 6-*O*-phosphorylated mannose residue (GalNAcβ1-3GlcNAcβ1-4Man-6-P) was reported, and it was found that this phosphorylation occurs independently from the mannose-6-phosphate synthetic pathway that is required for lysosomal protein modification [3]. In order to study α-DG/laminin interaction, glycopeptide containing 6-*O*-phosphorylated mannose was synthesized. Additionally, to obtain information about the step of phosphorylation in *O*-mannosyl glycan biosynthesis, we examined whether or not 6-*O*-phosphorylated mannosylpeptide worked as an acceptor substrate of POMGnT1.

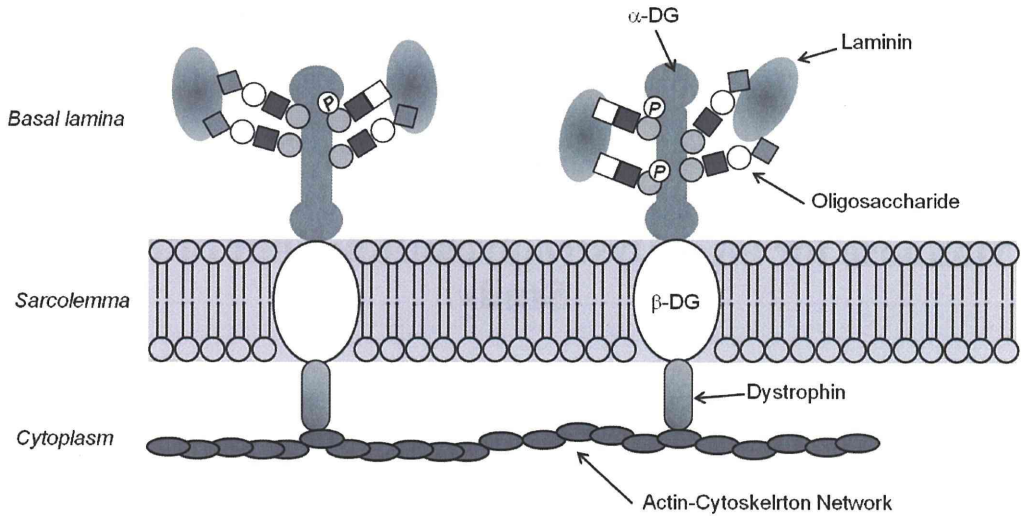

Fig.1 Schematic model of the dystrophin-glycoprotein complex as a transsarcolemmal linker between the subsarcolemmal cytoskeleton and extracellular matrix.

Results and Discussion

The synthetic route of building block Fmoc-Thr[αMan{2,3,4-tri-*O*-benzyl-6-P(OCH_2CCl_3)$_2$}]-OPfp **5α** is shown in Fig. 2. In this study, odourless thiol, 2-methyl-5-tert-butylthiophenol (MbpSH) [4], was used to provide thioglycoside donor. The thioglycoside **2** was prepared from the mannose peracetate **1** and Mbp-SH in presence of the BF_3 diethyl ether complex. After removal of O-Ac groups, the trityl group was used for the protection of the C-6 hydroxyl and the benzyl (Bn) ethers for the C-2 and C-3, C-4 hydroxyls.

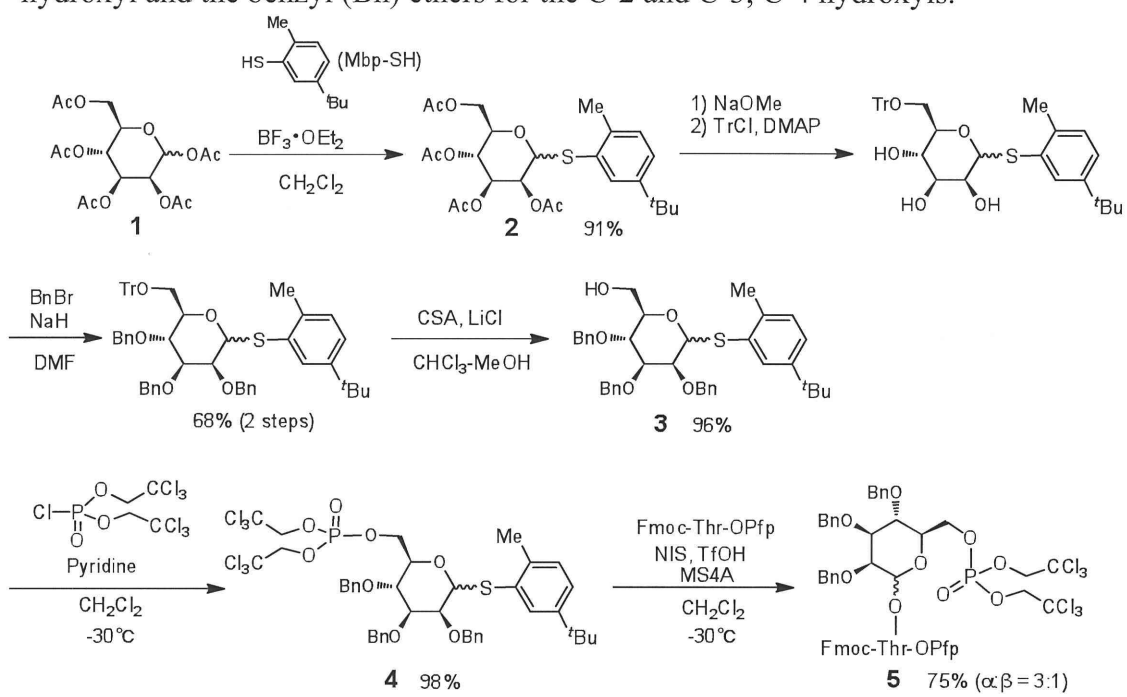

Fig.2 Synthesis of 6-O-phosphorylated mannosyl threonine derivative.

The cleavage of C-6 trityl group using 10-camphorsulfonic acid (CSA) and lithium chloride gave compound **3**. Reaction of compound **3** with bis (2,2,2-trichloroethyl) phosphorochloridate [5] and pyridine in CH_2Cl_2 yielded the thioglycoside **4** in almost quantitative yield (98%). The thioglycoside **4** was coupled with Fmoc-threonine Pfp ester using NIS-TfOH to give mannosyl threonine **5** as the anomeric mixture (75% α:β = 3:1). After silica-gel column purification, α-glycoside **5α** was isolated and used as the building block of glycopeptides synthesis

Next, phospho-mannosyl peptide was prepared by solid-phase manner (Fmoc chemistry). Amino acid sequence of mannosyl peptide was selected same sequence as our previous studies [6, 7]. Starting from Fmoc-Pro-CLEAR-amide resin, peptide synthesis was carried out with a five-hold excess of amino acids and using PyBOP as the activator system. The introduction of mannosyl threonine residue was performed using one equivalent of Pfp ester **5α** by a double coupling method. Finally, N-terminal amino group was capped with acetyl group. The resulting glycopeptides resin was treated with 95% aqueous TFA to give the crude product of protected phospho-mannosyl peptide. Removal of trichloroethyl groups of phosphoryl moiety and benzyl ethers of mannose moiety was accomplished by hydrogenolysis over palladium hydroxide on carbon in MeOH for 2 hours. After HPLC purification, the target phospho-mannnosyl peptide **6** was obtained in 3.9% overall yield from the starting resin.

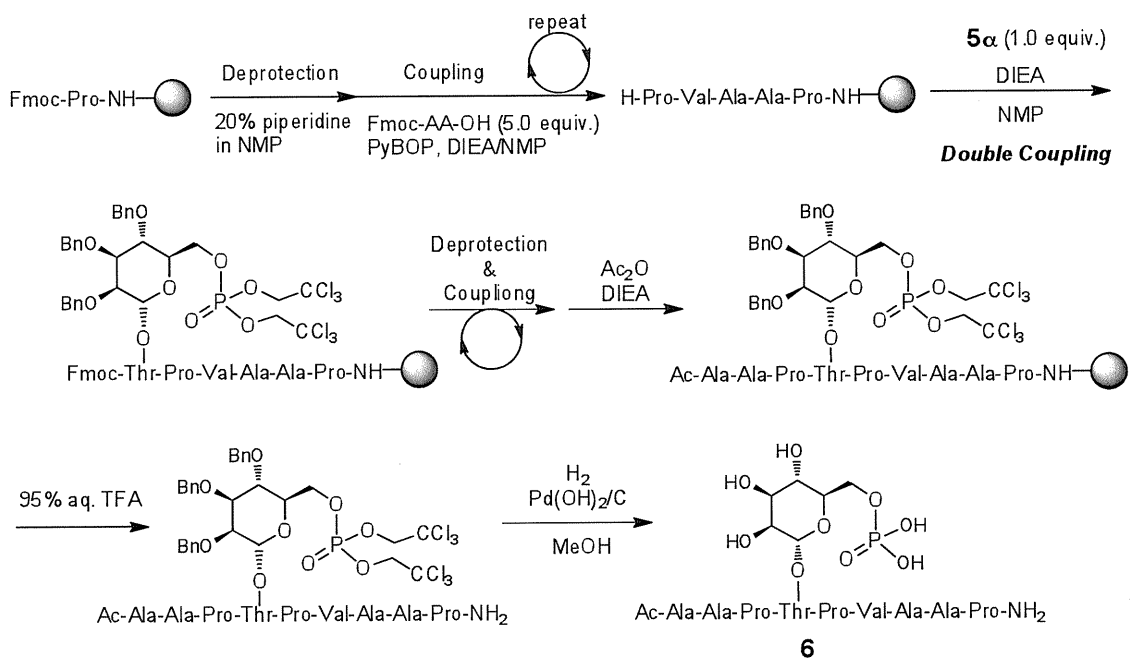

Fig.3 Synthesis of phospho-mannnosyl peptide.

The POMGnT1 activity was obtained from the amount of [^{3}H]GlcNAc transferred from UDP-GlcNAc to a substrate [2]. The reaction product was purified with a reverse-phased HPLC and radioactivity was measured. The mannosyl peptide (Man-peptide, Ac-AAPT(Man)PVAAP-NH_2) and the 6-*O*-phosphorylated mannosyl peptide **6** were used as substrates. The soluble form of human POMGnT1 used as an enzyme source was described previously [7]. The mannosyl peptide was worked as an acceptor for POMGnT1 (*Km* and *Vmax* values are 3.6 mM and 0.6 μmol/min/mg, respectively), whereas the POMGnT1 activity against the phospho-mannosyl peptide **6**

was not detected. This result demonstrated that the POMGnT1 cannot recognize the 6-*O*-phosphorylated mannose as an acceptor. Thus, if the phosphorylation of mannose occurred earlier than POMGnT1 reaction, the formation of GlcNAcβ1-2Man linkage should be inhibited. Taken together, it is possible that the phosphorylation of mannose may occur after forming GlcNAcβ1-2Man linkage. Not only 6-*O*-phosphorylated mannose structure, Galβ1-4GlcNAcβ1-2Man(6-P) [3], but also the 2,6-substituted *O*-mannose structure, GlcNAcβ1-2(GlcNAcβ1-6)Man, have been identified in α-DG [8]. The GlcNAcβ1-6Man linkage was formed by GnT-IX (GnT-VB). Because the transfer of GlcNAc to C6-position of *O*-mannose by GnT-IX (GnT-VB) requires also the GlcNAcβ1-2Man structure [9, 10], the phosphorylation and GlcNAcylation of C6-position of mannose may be competitive mutually.

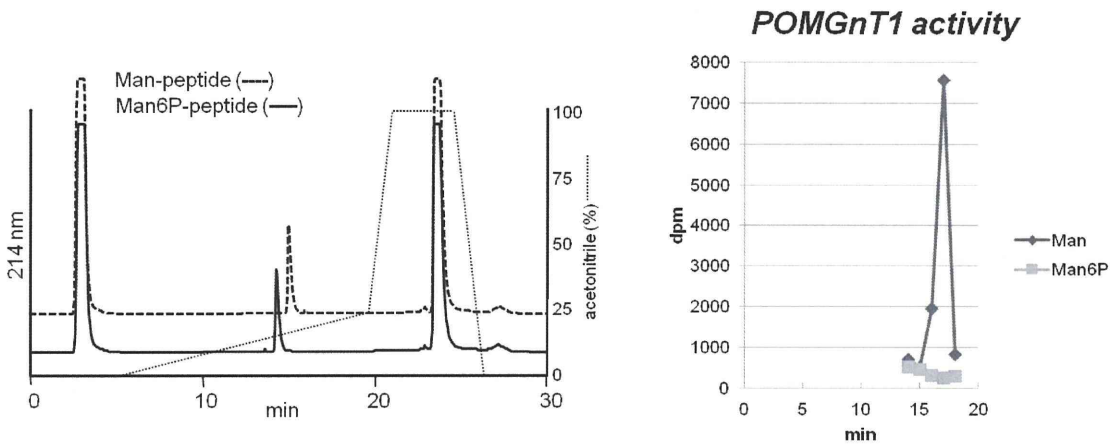

Fig.4 POMGnT1 activity against the phospho-mannosyl peptide **6** *and mannosyl peptide. The radioactivity of each fraction (Fr.=1ml/min, count=0.5ml) was measured using a liquid scintillation counter.*

In conclusion, synthesis of 6-*O*-phosphorylated mannosyl peptide was achieved. It was found that 6-*O*-phosphorylated mannosyl peptide does not worked as an acceptor substrate of POMGnT1. Future studies are necessary to clarify the phosphorylation mechanism of *O*-mannosyl glycan.

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Effects of length and amino acid sequence of *O*-mannosyl peptides on substrate specificity of protein *O*-linked mannose β1,2-*N*-acetylglucosaminyltransferase 1 (POMGnT1)

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ABSTRACT

Protein *O*-linked mannose β1,2-*N*-acetylglucosaminyltransferase 1 (POMGnT1) catalyzes the transfer of GlcNAc to *O*-mannose of glycoproteins. Mutations in the *POMGnT1* gene cause muscle–eye–brain disease (MEB). POMGnT1 is a typical type II membrane protein, which is localized in the Golgi apparatus. However, details of the catalytic and reaction mechanism of POMGnT1 are not understood. To develop a better understanding of POMGnT1, we examined the substrate specificity of POMGnT1 using a series of synthetic *O*-mannosyl peptides based on the human α-dystroglycan (α-DG) sequence as substrates. *O*-Mannosyl peptides consisting of three to 20 amino acids are recognized as substrates. Enzyme kinetics improved with increasing peptide length up to a length of 8 amino acids but the kinetics of peptides longer than 8 amino acids were similar to those of octapeptides. Our results also show that the amino acid sequence affects POMGnT1 activity. These data suggest that both length and amino acid sequence of mannosyl peptides are determinants of POMGnT1 substrate specificity.

1. Introduction

Protein *O*-linked mannose β1,2-*N*-acetylglucosaminyltransferase 1 (POMGnT1) catalyzes the transfer of GlcNAc to *O*-mannose of glycoproteins. POMGnT1 is responsible for muscle–eye–brain disease (MEB), which is an autosomal recessive disorder characterized by congenital muscular dystrophy, ocular abnormality, and brain malformation. The human *POMGnT1* gene was cloned from a cDNA sequence homologous to human *GnT-I* (UDP-GlcNAc: α-3-D-mannoside β1,2-*N*-acetylglucosaminyltransferase I). Although the overall amino acid sequence identity of POMGnT1 and GnT-I was only 23.2%, the predicted catalytic domains of both are highly homologous [1]. Such high conservation of the catalytic domain suggests that the reaction mechanism of POMGnT1 is partly similar to that of GnT-I. POMGnT1 and GnT-I are typical type II membrane proteins that are localized in the Golgi apparatus, and both enzymes form GlcNAcβ1–2Man linkages. A difference is that POMGnT1 catalyzes the GlcNAcβ1–2Man linkage in *O*-mannosyl glycans but not in *N*-glycans, while GnT-I catalyzes the same linkage in *N*-glycans but not in *O*-mannosyl glycans. The reason for these specificities is unclear.

Mammalian POMGnT1 is known to recognize synthetic mannosyl-peptide and benzyl-α-D-mannose as an acceptor [1,2], but not free mannose or *p*-nitrophenyl-α-mannose [1]. Thus, in order for POMGnT1 to recognize mannose, the mannose must be linked to certain other moieties.

It is known that two distinct *O*-fucosyl glycan-processing pathways exist: one is specific for epidermal growth factor-like (EGF) repeats and the other is specific for thrombospondin type 1 repeats (TSRs) [3,4]. *O*-Fucose-specific β1,3-*N*-acetylglucosaminyltransferase is able to modify *O*-fucose on an EGF repeat but not on a TSR, while *O*-fucose-specific β1,3-glucosyltransferase is able to modify *O*-fucose on a TSR but not on an EGF repeat. These results suggest that enzymes responsible for elongation of glycan following *O*-fucose are different and that each elongation enzyme strictly recognizes a particular amino acid sequences or conformation. These unique specificities of the two elongation enzymes prompted us to examine whether adding GlcNAc to *O*-mannose by POMGnT1 depends on the amino acid sequence near the *O*-mannosylated site. To answer this question, we analyzed the substrate specificity of POMGnT1 using a series of synthetic *O*-mannosyl peptides.

2. Materials and methods

2.1. Materials

O-Mannosyl peptides were synthesized in a solid-phase manner using 9-fluorenylmethyloxycarbonyl (Fmoc) chemistry [5]. The

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structures of each mannosyl peptide were identified by ^{1}H NMR, amino acid analysis (6 M HCl, 110 °C, 24 h) and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS).

2.2. Preparation of recombinant baculovirus and expression of soluble His-POMGnT1

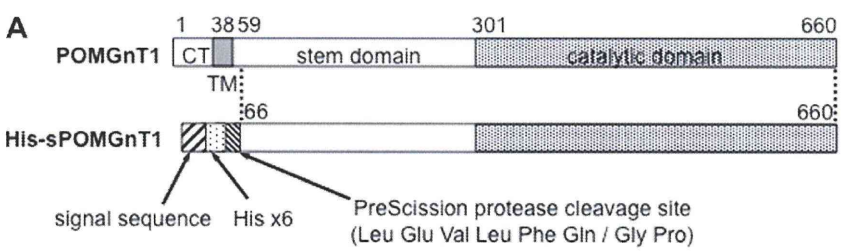
The soluble form of human POMGnT1 used as an enzyme source was prepared using the baculovirus (*Autographa californica nucleopolyhedro virus*) expression vector system (Nosan Corporation, Kanagawa, Japan; Protein Sciences Corporation, Meridien, CT). The soluble POMGnT1 (Ser66-Thr660) fused His-tag and PreScission protease cleavage site (Leu-Glu-Val-Leu-Phe-Gln-Gly-Pro) was secreted from *expres*SF+ Insect cells (Protein Sciences Corp.) by using the Ig kappa chain secretion signal (Fig. 1A). The transfer vector for generating recombinant baculovirus was constructed by ligating two PCR products. The primer pairs and template plasmid DNAs were: fragment 1 encoding the secretion signal, His-tag epitope and protease cleavage site, 5′-GATCTAGAATGGAGACAGACACAC-3′, 5′-CTGGTACCGGGCCCCTGGAACAGAACTTCCAGATGATGATGATGA TGATGAG-3′ and *HX-sPOMGnT1/pcDNA3.1 Zeo*(+) [1]; fragment 2 encoding Ser66-Thr660 of POMGnT1, 5′-CCCGGTACCAGTGAAGCC AATGAAGACCC-3′, 5′-TAAGATCTTCATGTCTGTTCTGGGGCTCC-3′ and the *POMGnT1/pcDNA3.1 Zeo*(+) [1]. The *pcDNA3.1 Zeo*(+) was purchased from Invitrogen (Carlsbad, CA). Two fragments were ligated at an *Acc65* I site and introduced into the *Xba* I site and *Bgl* II sites of *pPSC8* (Protein Sciences Corp.). The recombinant baculovirus was produced by baculovirus expression vector system using transfer vector and *expres*SF+ cells according to the manufacturer's instructions (Protein Sciences Corp.). The single clones of the recombinant baculovirus were isolated and expanded, and the high titer (>10^8 pfu/ml) virus stock was prepared. Isolation of clones and determination of virus titers were performed by plaque assay. The *expres*SF+ cells were maintained in Sf900-II medium (Invitrogen) supplemented with 50 μg/ml Kanamycin at 27 °C. A 10-l culture of *expres*SF+ cells was infected with 10 ml of virus stock and incubated for 72 h to express soluble His-POMGnT1 protein.

2.3. Purification of recombinant human POMGnT1

The recovered 10-l culture medium was concentrated 100-fold by 50 kDa cut-off VIVAFLOW, ultrafiltration membrane (Sartorius AG, Goettingen, Germany). The collected medium (approximately 100 ml) was first subjected to Ni^{2+}-chelating Sepharose (HisTrap HP 5 ml, GE Healthcare UK Ltd., Buckinghamshire, England). After washing with 100 ml of 20 mM sodium phosphate buffer (pH 7.0) containing 300 mM NaCl and 20 mM imidazole, recombinant His-POMGnT1 was eluted from the column with 25 ml of 20 mM sodium phosphate buffer (pH 7.0) containing 300 mM NaCl and 500 mM imidazole. After dialysis against 20 mM sodium phosphate buffer (pH 7.0) containing 300 mM NaCl, the eluted enzyme was incubated with 250 μl of PreScission protease (GE Healthcare) and 250 μl of 100 mM dithiothreitol at 4 °C for 4 h to eliminate His-tag from His-POMGnT1. After PreScission protease digestion, recombinant POMGnT1 was subjected to the tandem columns combination of HisTrap column and Glutathione Sepharose column (GSTrap HP, GE Healthcare). The recombinant POMGnT1 was recovered in unbound fraction from the tandem columns by using 20 mM sodium phosphate (pH 7.0) containing 300 mM NaCl for washing buffer. The purity of recombinant POMGnT1 of each step was confirmed by SDS–PAGE.

2.4. Assay for POMGnT1 activity

POMGnT1 activity was obtained from the amount of [^{3}H] GlcNAc transferred to a substrate [1]. The reaction buffer containing 140 mM MES buffer (pH 7.0), 2 mM UDP-[^{3}H] GlcNAc



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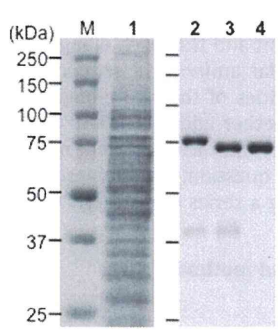

C

Purification of recombinant human POMGnT1	
step	Specific activity (nmol/h/μg)
1. Culture medium	0.01
2. Ni^{2+}-affinity column	2.5
3. PreScission protease digested	2.6
4. Purified after the tandem columns	3.0

Fig. 1. (A) Schematic representation of human POMGnT1 and recombinant soluble POMGnT1 used in this study. POMGnT1 is a typical type II membrane protein, which is composed of, in order from the N-terminus, a cytoplasmic tail (CT), transmembrane domain (TM), stem domain, and catalytic domain. To prepare soluble enzyme, amino acid residues 1–65 were replaced by the sequence of the signal sequence, His tag, and PreScission protease cleavage site. The numbers above the boxes indicate the amino acid residue numbers of human POMGnT1. (B), (C) Electrophoretic patterns and POMGnT1 activity of recombinant human POMGnT1 at each purification step. Specific activity was determined using mannosyl nonapeptide. Lane 1, culture medium; lane 2, Ni^{2+}-affinity column absorbed protein; lane 3, PreScission protease-digested protein; lane 4, recombinant human POMGnT1 purified by tandem HisTrap and Glutathione Sepharose columns.

(50,000 dpm/nmol), 0.25–8 mM substrate, 10 mM $MnCl_2$, 2% Triton X-100, 5 mM AMP, 200 mM GlcNAc, 10% glycerol and enzyme solution was incubated at 37 °C for 1 h. After boiling for 3 min, the mixture was analyzed by reversed phase HPLC with a Wakopak 5C18–200 column (4.6 × 250 mm). Solvent A was 0.1% trifluoroacetic acid in distilled water and solvent B was 0.1% trifluoroacetic acid in acetonitrile. The peptide was eluted at a flow rate of 1 ml/min using a linear gradient of 1–25% solvent B. The peptide separation was monitored continuously at 214 nm, and the radioactivity of each fraction was measured using a liquid scintillation counter. *Km* and *Vmax* values were calculated on the basis of Hanes–Woolf plot.

3. Results and discussion

To produce recombinant human POMGnT1 on a large scale, we expressed a soluble form of the enzyme using a baculovirus/insect cell expression system. The recombinant human POMGnT1 used in the study lacked the cytoplasmic tail and transmembrane domain and included an *Ig* kappa chain secretion signal and His-tag at N-terminus (Fig. 1A). In order to remove the His-tag after Ni^{2+}-affinity chromatography, a protease digestion sequence (PreScission protease cleavage site) was inserted behind the His-tag region. Recombinant soluble POMGnT1 was produced in insect *expres*SF+ cells (Fig. 1B, lane 1) and purified from the culture medium by Ni^{2+}-affinity chromatography (lane 2). After cleavage of the His-tag, the protein migrated as a 70 kDa protein (lane 3). The protein was then cleaned up with tandem HisTrap and Glutathione Sepharose columns (lane 4). Ni^{2+}-affinity chromatography was very effective, increasing the specific activity of POMGnT1 by about three orders of magnitude (Fig. 1C). The final activity was high (3.0 nmol/h/μg) despite the removal of amino acid residues 1–65 from human POMGnT1 (Fig. 1A). This was expected because the minimal catalytic domain of POMGnT1 is located between positions 299 and 651 [6].

To examine the effect of peptide length on substrate specificity, we synthesized a series of mannosyl peptides consisting of a single mannosylated threonine residue and three to 20 amino acids (Fig. 2). The tri-, tetra-, hexa-, hepta-, octa- and deca-peptides were derived from mannosyl nonapeptide. The latter was based on amino acid residues 316–324 of α-DG in which most of threonine residues were substituted with alanine. Acetyl and amino groups were added to the termini of some mannosyl peptides to prevent their degradation during incubation. Mannosyl icosapeptide is based on amino acid residues 401–420 of α-DG which was found to act as a good substrate for protein *O*-mannosyl transferase [7]. The alanine-substituted mannosyl nonapeptide was designed to examine the effect of amino acid sequence on the enzymatic activity. We also examined benzyl-α-D-mannose as a substrate [2].

POMGnT1 activities with the different substrates were determined in Hanes-Woolf plots (Fig. 3) to obtain *Km* and *Vmax* values. *Vmax* and the catalytic constant *Kcat* were found to increase and *Km* was found to decrease with increasing peptide lengths for lengths up to 8 amino acids, although the hexapeptide was an exception (Table 1). For peptides longer than 8 amino acids, however, the kinetics were similar to those of octapeptides. Adding an alanine residue to the C-terminus of the hexapeptide (to form heptapeptide-I) decreased *Km* and *Vmax*, while adding an alanine to the N-terminus of the hexapeptide (to form heptapeptide-II) decreased *Km* even more and changed *Vmax* only slightly. These results suggest that four amino acids at the N-terminus make a better acceptor than three amino acids at the N-terminus. The finding that *Km* of alanine-substituted mannosyl nonapeptide is almost twice that of the original mannosyl nonapeptide (Table 1) shows that the amino acid sequence is also important.

Benzyl-α-D-mannose has a smaller *Km* than mannosyl heptapeptides, although its *Vmax* and *Kcat* values were similar to those of mannosyl tripeptide (Table 1). Benzyl-α-D-mannose's small *Km* may be due to its smaller molecular size, which could make it more difficult to release the product, benzyl-α-D-mannose-GlcNAc, from the catalytic pocket of the enzyme. POMGnT1 transferred GlcNAc to benzyl-α-D-mannose [2] but, as we found previously, not to either mannose or *p*-nitrophenyl-α-mannose [1], which suggests that the hydrophobicity of the benzyl group helps it to be recognized by the catalytic pocket of the enzyme.

X-ray crystal structure studies of POMGnT1 are needed to better understand the catalytic domain of the enzyme. Such studies have been used to determine the structures of many glycosyltransferases including GnT-I [8]. To crystallize the enzyme, a large amount of pure recombinant protein is needed. The expression system

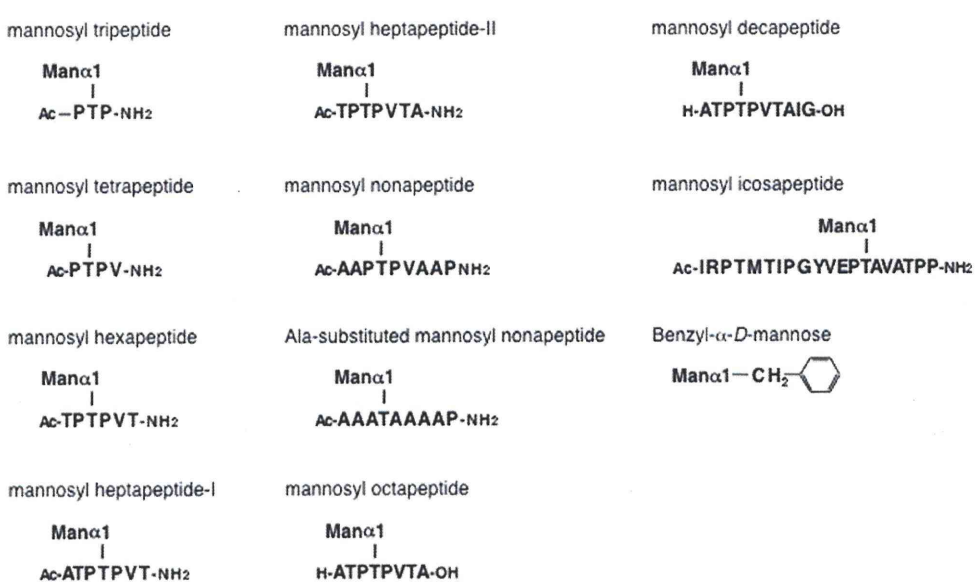

Fig. 2. POMGnT1 substrates used in this study. Ac and NH_2 indicate acetyl and amino groups of the N- and C-termini, respectively. H and OH indicate that the peptides are not blocked.

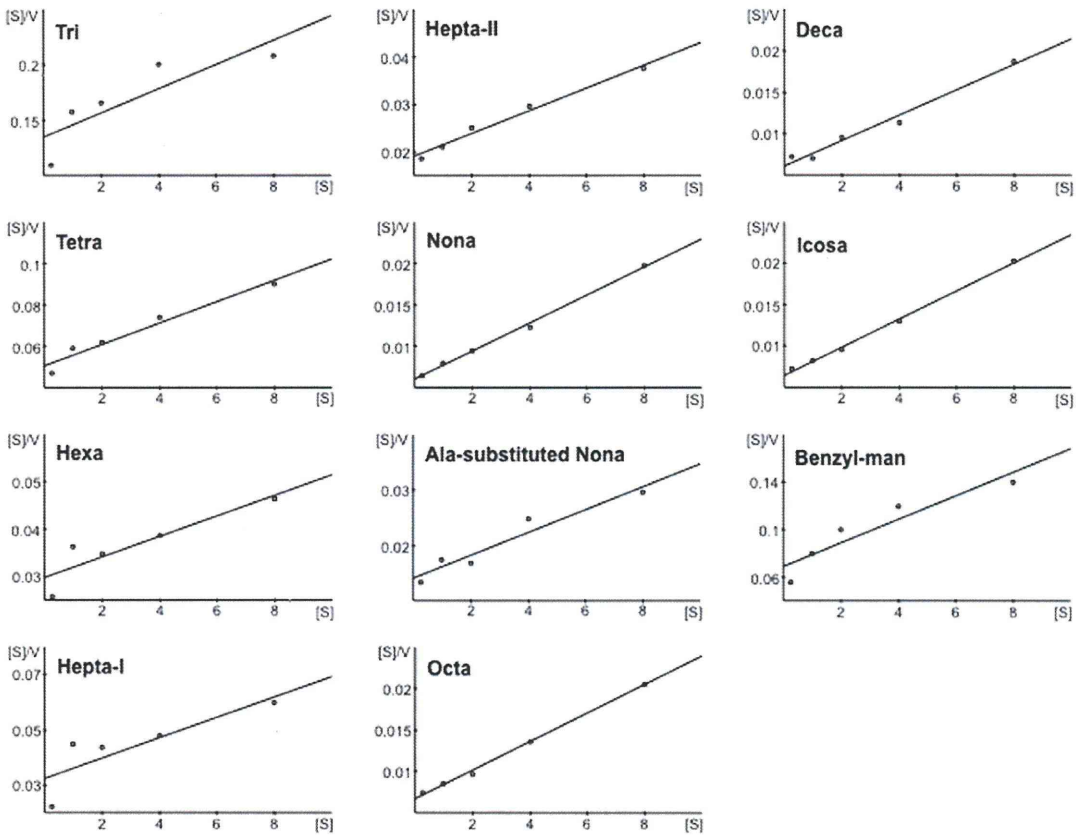

Fig. 3. Kinetic analysis of the purified recombinant POMGnT1 by Hanes-Woolf plot. The enzyme assay for POMGnT1 reaction is described in the Materials and methods. The kinetic parameters of POMGnT1 are summarized in Table 1. S, concentration of acceptor peptide (mM); V, specific activity (pmol/h/mg total protein).

Table 1
Kinetic parameters of POMGnT1 for various substrates.

Substrate	Sequence	MW	*Km* (mM)	*Vmax* (nmol/min/mg)	*Kcat* (1/s)
Tri	PT(Man)P	516	12.4	92	0.12
Tetra	PT(Man)PV	616	9.8	194	0.24
Hexa	TPT(Man)PVT	777	13.7	463	0.58
Hepta-I	TPT(Man)PVTA	848	8.8	271	0.34
Hepta-II	ATPT(Man)PVT	848	7.9	417	0.52
Ala-substituted Nona	AAAT(Man)AAAAP	917	6.8	483	0.60
Octa	ATPT(Man)PVTA	919	3.9	582	0.73
Nona	AAPT(Man)PVAAP	997	3.6	594	0.74
Deca	ATPT(Man)PVTAIG	1089	4.0	657	0.82
Icosa	IRPTMTIPGYVEPT(Man)AVATPP	2273	3.8	590	0.74
Benzyl-man		270	6.9	101	0.13

reported here, which can produce approximately 20 mg protein from 10 l of culture medium, is well suited for this task.

The regulatory mechanism of glycan processing is complicated and poorly understood. Some glycosyltransferases are known to modify specific regions of proteins. For example, two distinct *O*-fucosylation pathways exist [3,4]. EGF repeats in proteins are *O*-fucosylated by protein *O*-fucosyltransferase1 (Pofut1), and elongated by Fringe, a β1,3-*N*-acetylglucosaminyltransferase, while TSR repeats are *O*-fucosylated by a different *O*-fucosyltransferase, Pofut2, and then elongated by β1,3-glucosyltransferase [4,9]. These results indicate that these elongation enzymes recognize particular amino acid sequences or conformations. Our results (Table 1) also indicate that elongation of *O*-mannosyl glycans depends on the amino acid sequence. Recent studies have determined some of the *O*-mannosyl glycan structures and *O*-mannosylation sites on α-DG [10,11] but do not exclude the possibility that some *O*-mannose residues are not elongated. Further studies on all of the *O*-mannosyl glycan structures and *O*-mannosylation sites on α-DG will help to elucidate POMGnT1 substrate specificity.

Acknowledgments

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Different roles of the two components of human protein *O*-mannosyltransferase, POMT1 and POMT2

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ABSTRACT

Protein *O*-mannosyltransferase 1 (POMT1) and its homolog, POMT2, are responsible for the catalysis of the first step in *O*-mannosyl glycan synthesis. Mutations in their genes are associated with a type of congenital muscular dystrophy called Walker-Warburg syndrome. Arg^{64}, Glu^{78} and Arg^{138} in the N-terminus region of ScPmt1p, a POMT homolog in *Saccharomyces cerevisiae*, are important for transferase activity. Arg^{138} is also essential for complex formation with ScPmt2p. Here we examined the effects of replacing the corresponding residues in human POMT1 and POMT2 with Ala on complex formation and enzymatic activity. The human POMT1 mutants lost almost all transferase activity while the POMT2 mutants retained enzymatic activity. Neither mutant lost its ability to form complexes with the native counter component. These results indicate that ScPmtps and human POMTs have different mechanisms of complex formation. They also suggest that human POMT1 and POMT2 have discrete functions since the effect of amino acid substitutions on enzymatic activity are different.

1. Introduction

Protein *O*-mannosyltransferase 1 (POMT1) and POMT2 (POMT, EC 2.4.1.109) catalyze the initial step of the biosynthesis of *O*-mannosyl glycans, in which a mannosyl residue is transferred from dolichyl phosphate mannose (Dol-P-Man) to Ser/Thr residues of certain proteins [1]. Complex formation of POMT1 and POMT2 is indispensable for POMT activity [1,2]. α-Dystroglycan (α-DG) is predominantly glycosylated by *O*-mannosyl glycan Siaα2-3Galβ1-4GlcNAcβ1-2Man [3]. α-DG consists of a dystrophin–glycoprotein complex that acts as a transmembrane linker between the extracellular matrix and intracellular cytoskeleton [4]. Defects in *O*-mannosyl glycans on α-DG are reported to cause several α-dystroglycanopathies, which are a group of congenital muscular dystrophies that include Walker-Warburg syndrome (WWS: OMIM 236670) [5,6]. Mutations in the *POMT1* and *POMT2* genes were identified in patients with WWS [7–10], an autosomal recessive developmental disorder associated with congenital muscular dystrophy, neuronal migration defects and ocular abnormalities. We previously demonstrated that these mutations almost abolished POMT activity and α-DG glycosylation [2,9]. However, how mutations cause a loss of POMT activity remains unclear.

O-Mannosylation is an essential protein modification that is evolutionarily conserved from eukaryotes to mycobacteria [11,12]. *O*-Mannosylation is essential for maintaining cell shape and cell integrity [13] and contributes to quality control of proteins in the ER in yeast [14,15]. Reduction of protein *O*-mannosyltransferases in *Drosophila melanogaster* resulted in defects in embryonic muscle development [16–19] and *POMT1* deletion in mouse resulted in embryonic death [20]. Protein *O*-mannosyltransferases in *Saccharomyces cerevisiae* (ScPmtps) are composed of at least six members (Scpmtp1–6) that form hetero- and homo-complexes in various combinations [21,22]. ScPmtp1 has been proposed to consist of seven transmembrane helices [23]. The ScPmtp1 N-terminus and loops 2, 4 and 6 are located in the cytoplasm, and the C-terminus and loops 1, 3 and 5 are located in the ER lumen. Two hydrophilic regions (loops 5 and 1) are important for enzymatic activity [21,23,24]. We recently constructed models in which human POMT1 and POMT2 have seven- and nine-transmembrane helices respectively and in which the C-termini and loops 1, 3 and 5 are located in the ER lumen [25]. This is similar to the topology of ScPmtp1. In ScPmtp1, Arg^{64}, Glu^{78} and Arg^{138} have been shown to be essential for enzymatic activity [24].

Here, we investigated whether amino acids of human POMT1 and POMT2 corresponding to Arg^{64}, Glu^{78} and Arg^{138} in ScPmtp1 are required for transferase activity and complex formation.

2. Materials and methods

2.1. Vector construction of POMT1 and POMT2 mutants

Human POMT1 cDNA was used for site-directed mutagenesis and was cloned into pcDNA 3.1/Zeocin (−) (Invitrogen Corp.,

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