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神経移動障害を伴う筋疾患の病態解明と治療法実現に向けた技術集約的研究

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目 次

I. 総括研究報告

神経移動障害を伴う筋疾患の病態解明と
治療法実現に向けた技術集約的研究
萬谷 博

----- 1

II. 研究成果の刊行に関する一覧表

----- 4

III. 研究成果の刊行物・別刷

----- 5

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総括研究報告書

神経移動障害を伴う筋疾患の病態解明と治療法実現に向けた技術集約的研究

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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であることを示し、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。本研究では O-マンノース型糖鎖の機能を明らかにすることで、筋ジストロフィー症の病態解明から診断・治療法への応用を目指している。本年度は、他の O-マンノシル化蛋白質に関する知見を得る目的で、O-マンノース転移酵素の基質特異性から蛋白質の O-マンノシル化機構を検討し、特異的に O-マンノシル化されるアミノ酸配列があることを明らかにした。

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 POMGnT1 と POMT1 及び POMT2 が O-マンノース型糖鎖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。本研究事業では、平成 17 年度に POMT1 と POMT2 は小胞体で複合体を形成することにより O-マンノース転移酵素活性が発揮されることを明らかにした。さらに、平成 18 年度には、これまで全く機能が分からなかった FCMD の原因遺伝子産物 fukutin はゴルジにおいて POMGnT1 と結合し、POMGnT 活性に影響

していることを明らかにした。また、POMT と POMGnT1 の酵素活性測定法による MEB 及び WWS の簡易診断の可能性を示した。このように、O-マンノース型糖鎖の生合成過程に着目して α -ジストログリカノパチーの病態解明及び診断法への応用を検討してきた。

ところで、O-マンノース型糖鎖が修飾される蛋白質に関しては、これまでに α -ジストログリカンしか見つかっていないため、O-マンノース型糖鎖の機能については不明な点が多い。そこで本年度は、他の O-マンノシル化蛋白質に関する知見を得る目的で、O-マンノース転移酵素の基質特異性から蛋白質の O-マンノシル化機構を検討した。

B. 研究方法

α -ジストログリカンのムチン様領域およびムチンのタンデムリピート領域のアミノ酸配列に基づいて 20 残基程度の合成ペプチドを作製し、これらの合成ペプチドを基質とした時の O-マンノース転移活性を比較した。さらに、O-マンノシル化したペプチドを質量分析及びアミノ酸シーケンスにより分析し、O-マンノシル化のメカニズム

を詳細に検討した。

C. 研究結果

α -ジストログリカン由来の 2 つのペプチドが効率良く *O*-マンノシル化されることが分かった。興味深いことに、これら 2 つのペプチドのアミノ酸配列の相同性は高く、特にプロリンとスレオニンの配置がほぼ一致していた。さらに、これらのペプチドのアミノ酸置換体を作製し、酵素活性への影響を調べた結果、両ペプチドで保存されているアミノ酸配列が *O*-マンノシル化において重要であることが分かり、*O*-マンノシル化のコンセンサス配列であると考えられた。*O*-マンノシル化されたペプチドの質量分析とアミノ酸シーケンスの結果、複数のスレオニンが存在する場合、*O*-マンノシル化される順番には規則性が存在し、最初のマンノシル化が引き金となって、連鎖的にマンノシル化が起こるといった新たなメカニズムを明らかにした。

D. 考察

本研究により、*O*-マンノース転移酵素は特異的なアミノ酸配列を認識してマンノースを転移することが明らかとなった。 α -ジストログリカンは多くの *O*-マンノース型糖鎖により修飾を受けると予想されるが、その理由として、分子内に *O*-マンノシル化のコンセンサス配列が複数存在することと、そこを起点とした連鎖的 *O*-マンノシル化機構が存在することが考えられた。コンセンサス配列の発見は、 α -ジストログリカン以外の *O*-マンノシル化蛋白質を探索する上で重要であり、*O*-マンノース型糖鎖の機能解明及び糖鎖異常による先天性筋ジストロフィーの病態解明に繋がることが期待される。

E. 結論

α -ジストログリカンのアミノ酸配列から蛋白質 *O*-マンノシル化のコンセンサス配列を見出し、 α -ジストログリカンが特異的に *O*-マンノシル化されるメカニズムを明らかにした。

F. 研究発表

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研究成果の刊行に関する一覧表

雑誌

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研究成果の刊行物・別刷

Regulation of Mammalian Protein O-Mannosylation PREFERENTIAL AMINO ACID SEQUENCE FOR O-MANNOSE MODIFICATION^{*§}

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O-Mannosyl glycans are important in muscle and brain development. Protein O-mannosyltransferase (POMT) catalyzes the initial step of O-mannosyl glycan biosynthesis. To understand which serine (Ser) and threonine (Thr) residues POMT recognizes for mannosylation, we prepared a series of synthetic peptides based on a mucin-like domain in α -dystroglycan (α -DG), one of the best known O-mannosylated proteins in mammals. In α -DG, the mucin-like domain spans amino acid residues 316 to 489. Two similar peptide sequences, corresponding to residues 401–420 and 336–355, respectively, were strongly mannosylated by POMT, whereas other peptides from α -DG and peptides of various mucin tandem repeat regions were poorly mannosylated. Peptides 401–420 and 336–355 contained four and six Ser and Thr residues, respectively. Substitution of Ala residues for the Ser or Thr residues showed that Thr-414 of peptide 401–420 and Thr-351 of peptide 336–355 were prominently modified by O-mannosylation. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry and Edman degradation analysis of the mannosylated peptide 401–420 indicated that Thr-414 was the Thr residue that was most prominently modified by O-mannosylation and that O-mannosylation occurred sequentially rather than at random. Based on these results, we propose a preferred amino acid sequence for mammalian O-mannose modification.

O-Mannosyl glycans are important in muscle and brain development (1). We previously found that the glycans of

α -dystroglycan (α -DG)² predominantly include O-mannosyl glycan Sia α 2–3Gal β 1–4GlcNAc β 1–2Man (2). α -DG is a component of the dystrophin-glycoprotein complex that acts as a transmembrane linker between the extracellular matrix and intracellular cytoskeleton (3). Previously we reported that defects in O-mannosyl glycan cause a type of muscular dystrophy (4, 5). We have found that protein O-mannosyltransferase 1 (POMT1) and its homolog POMT2 are responsible for the catalysis of the first step in O-mannosyl glycan synthesis (6). Mutations in *POMT1* and *POMT2* genes are considered to be the cause of Walker-Warburg syndrome (WWS: OMIM 236670), an autosomal recessive developmental disorder associated with congenital muscular dystrophy, neuronal migration defects, and ocular abnormalities (7, 8). We have demonstrated that mutations in the *POMT1* gene abolish POMT activity (9, 10). Thus, O-mannosylation is indispensable for normal structure and function of α -DG in muscle and brain in human.

We recently demonstrated that formation of a POMT1-POMT2 complex was required for POMT activity (10). POMT1 and POMT2 are homologous to members of the family of protein O-mannosyltransferases (PMTs) in yeast. PMTs were shown to catalyze the transfer of a mannosyl residue from dolichyl phosphate mannose to Ser/Thr residues of certain proteins (11). Individual PMTs have different specificities for protein substrates (12, 13), suggesting the presence of some sequence for recognition by PMTs, but the sequence was not identified. On the other hand, in mammals, O-mannosylated proteins are rare and O-mannosylation may require a specific sequence because we detected POMT activity when a glutathione S-transferase-fused mucin-like domain of α -DG (amino acid residues 316–489) was used as an acceptor (6). Previous studies suggested that the mucin-like domain is highly glycosylated and that certain glycans of α -DG play an important role in its binding to ligand proteins such as laminin, neurexin, and agrin (2, 14–17). To address the biological function and regulation of O-mannosylation, it is important to determine whether

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§ The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Figs. S1–S5.

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² The abbreviations used are: α -DG, α -dystroglycan; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; PMT and POMT, protein O-mannosyltransferase; WWS, Walker-Warburg syndrome; Con A, concanavalin A; HPLC, high pressure liquid chromatography.

there is a preferential amino acid sequence. In this study we synthesized a series of peptides that fully covered the mucin-like domain of α -DG (Fig. 1). Then we examined whether these peptides worked as acceptors for protein *O*-mannosylation. Further, the enzymatic products were analyzed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) and Edman degradation to determine the number of mannose residues transferred and the sites to which they were transferred. Based on the results of these studies, we proposed a preferred sequence for mammalian *O*-mannosylation.

EXPERIMENTAL PROCEDURES

Chemicals—Synthetic peptides were purchased by made-to-order system on the web site of Sigma-Aldrich (www.genosys.jp, Tokyo, Japan), and the quality of the synthesis was ascertained by HPLC analysis and mass spectrometry. A series of peptides, whose average length is \sim 20 amino acids, fully covered the mucin-like domain of α -DG (Table 1). Triton X-100 was purchased from Nacalai Tesque (Kyoto, Japan). *n*-Octyl- β -D-thioglucoside was from Dojindo Laboratories (Kumamoto, Japan). Tritium-labeled or unlabeled mannosylphosphoryldolichol were purchased from American Radio-labeled Chemical, Inc. (St. Louis, MO).

Mannosyl-threonine was synthesized as follows. To a solution of phenyl 2,3,4,6-tetra-*O*-benzyl-1-thio- α -D-mannopyranoside (1.0 g, 1.6 mmol) and *N*-benzyloxycarbonyl-L-threonine benzyl ester (813 mg, 2.4 mmol) in toluene (30 ml) and CH_2Cl_2 (10 ml), molecular sieves 4A (2.0 g) was added, and the mixture was stirred for 1 h at room temperature and then cooled to 0 °C. The cooled mixture were added to *N*-iodosuccinimide (720 mg, 3.2 mmol) and trifluoromethanesulfonic acid (30 μ l, 0.32 mmol), and the mixture was further stirred for 2 h at 0 °C. The precipitates were filtered off through Celite and washed with CH_2Cl_2 . Combined filtrate and washings were washed with saturated Na_2CO_3 and saturated $\text{Na}_2\text{S}_2\text{O}_3$, dried, and concentrated. Column chromatography of the residue on silica gel (AcOEt:Hexane = 1:4) gave *O*-(2,3,4,6-tetra-*O*-benzyl- α -D-mannopyranosyl)-*N*-benzyloxycarbonyl-L-threonine benzyl ester (1.05 g, 77%). To a solution of *O*-(2,3,4,6-tetra-*O*-benzyl- α -D-mannopyranosyl)-*N*-benzyloxycarbonyl-L-threonine benzyl ester (500 mg, 0.58 mmol) in MeOH (50 ml) 10% Pd on activated carbon (500 mg) and acetic acid (50 μ l) were added. H_2 gas was bubbled into the mixture, which was stirred for 2 h at room temperature. The precipitates were filtered off and washed with water. The filtrate and washings were combined and concentrated to solid, which was chromatographed on Sephadex G-50 (eluent: water) to give *O*-(α -D-mannopyranosyl)-L-threonine (170 mg, quantitative). The structure was identified by $^1\text{H-NMR}$ and MALDI-TOF MS.

Mannosyl peptide 401–420(T414Man) (IRPTMTIPGYVEPT(Man)AVATPP) was synthesized essentially as described in our previous report (18). The structure was identified by $^1\text{H-NMR}$ and MALDI-TOF MS.

POMT Enzyme Source—The microsomal membrane fraction of human embryonic kidney 293T cells co-transfected by *POMT1* and *POMT2* was used for POMT enzyme source. A cDNA containing the most common splicing variant of human

Preferential Sequence for Protein *O*-Mannosylation

POMT1 (which lacks bases 700–765, corresponding to amino acids 234–255) and human *POMT2* cDNA were cloned into pcDNA 3.1 (Invitrogen) as described previously (6). Human embryonic kidney 293T cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin, and 50 $\mu\text{g}/\text{ml}$ streptomycin at 37 °C with 5% CO_2 . Expression plasmids were transfected into human embryonic kidney 293T cells using Lipofectamine plus reagent (Invitrogen) according to the manufacturer's instructions. The cells were incubated for 3 days at 37 °C to produce *POMT1* and *POMT2* proteins. The cells were homogenized in 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 250 mM sucrose, 1 mM dithiothreitol with a protease inhibitor mixture (3 $\mu\text{g}/\text{ml}$ pepstatin A, 1 $\mu\text{g}/\text{ml}$ leupeptin, 1 mM benzamide-HCl, 1 mM phenylmethylsulfonyl fluoride). After centrifugation at $900 \times g$ for 10 min, the supernatant was subjected to ultracentrifugation at $100,000 \times g$ for 1 h. The precipitate was used as the microsomal membrane fraction. Protein concentration was determined by BCA assay.

Assay for POMT Activity—POMT activity was based on the amount of [^3H]mannose transferred from mannosylphosphoryldolichol to synthetic peptides. The reaction mixture containing 20 mM Tris-HCl, pH 8.0, 100 nM mannosylphosphoryldolichol (125,000 dpm/pmol), 2 mM dithiothreitol, 10 mM EDTA, 0.5% *n*-octyl- β -D-thioglucoside, 0.25–4 mM synthetic peptide; 40 μg of microsomal membrane fraction in 20 μl of total volume was incubated for 60 min at 25 °C. After boiling for 3 min, the mixture was analyzed by reversed-phase HPLC with a Wakopak 5C18–200 column (4.6 \times 250 mm; Wako Pure Chemical Industries, Ltd., Osaka, Japan) or a Mightysil RP-18GP Aqua column (4.6 \times 250 mm; Kanto Chemical Co., Inc., Tokyo, Japan). 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 1–50% solvent B. The peptide separation was monitored continuously at 215 nm, and the radioactivity of each fraction was measured using a liquid scintillation counter.

Peptide Sequencing and Mass Spectrometry—A mannosylated peptide 401–420 was prepared using concanavalin A (Con A)-Sepharose 4B beads (GE Healthcare) as follows. The reaction mixture containing 20 mM Tris-HCl, pH 8.0, 300 μM unlabeled mannosylphosphoryldolichol, 2 mM dithiothreitol, 10 mM EDTA, 0.5% *n*-octyl- β -D-thioglucoside, 1 mM peptide 401–420; 120 μg of microsomal membrane fraction in 30 μl of total volume was incubated for 15–120 min at 25 °C. The reaction mixture was mixed with 400 μl of 20 mM Tris-HCl, pH 7.4, 0.5 M NaCl, 1 mM MnCl_2 , 1 mM CaCl_2 , and 1 mM MgCl_2 and centrifuged at $10,000 \times g$ for 10 min. The supernatant was loaded onto the Con A-Sepharose column (100 μl of bed volume) and washed with 2 ml of the same buffer. The bound fraction was obtained by eluting with 250 μl of 200 mM α -methylmannoside in 20 mM Tris-HCl, pH 7.4, 0.5 M NaCl, 1 mM MnCl_2 , 1 mM CaCl_2 , and 1 mM MgCl_2 and separated by reversed-phase HPLC as described above.

Con A-purified mannosyl peptide preparation was fractionated by reversed-phase HPLC on an Inertsil ODS-3 column

Preferential Sequence for Protein O-Mannosylation

A

1 MRMSVGLSLL LPLWGRFTLL LLSVVMQAQSH WPSESEAVR DWENQLEASM HSVLSLDLHEA
 61 VPTVVGIPDG TAVVGRSFRV TIPTDLIASS GDIKVSAAK KEALPSWLHW DSQSHTLEGL
 121 PLDTDKGVHY ISVSATRLGA NGSHIPQTSS VFSIEVYPED HSDLQSVRTA SPDPGEVSS
 181 ACAADEPVTV LTVILDADLT KMPKQRIDL LHRMRSFSEV ELHNMKLVVP VNNRLFDSA
 241 FMAGPGNPKK VVENGALLSW KLGCSLNQNS VPDHGVVEAP AREGAMSAQL GYPVVGWHIA
 301 NKKPPLPKRV RRQIHATPTP VTAIGPPTA IQEPPSRIVE TPTSPAIAPP TETMAPFVRD
 361 PVPGKPTVTI RTRGAIQTP TLGPIQPTRV SEAGTTVPGQ IRPTMTIPGY VEPTAVATPP
 421 TTTTKKPRVS TPKPATPSTD STTTTTRRPT KKPRTPRPVP RVTKVSIIR LETASPPTRI
 481 RTTTSVPRG GEPNQRPELK NHIDRVDAWV GTFYEVKIPS DTFYDHEDTT TDKLKLTLKL
 541 REQQLVGEKS WVQFNSNSQL MYGLPDSSHV GKHEYFMHAT DKGGLSAVDA FEIHVHRRPQ
 601 GDRAPARFKA KFGVDPALVL NDIHKKIALV KKLAFAGDR NCSTITLQNI TRG

B

name	peptide
(mucin-like domain of α -DG)	
316-337	ATPTFVTAIGPPTTAIQEPPSR
336-355	SRIVPTTSPAIAPPETMA
364-383	GKPTVTIRGAIQTPTLG
385-403	IQPTRVSEAGTTVPGQIRP
401-420	IRPTMTIPGYVEPTAVATPP
418-440	TPPTTTTKKPRVSTPKPATPSTD
435-454	ATPSTDSTTTTTRRPTKKPR
451-470	KKPRTPRPVRVTKVSIIR
469-488	ITRLETASPPTRIRTTTSGVP
(mucin tandem-repeat region)	
MUC1	AHGVTSAPDTRPPPGSTAPP
MUC2	PTTTPITTTTTVTPPTPTGTQT
MUC3	HSTPSFTSSITTTETTS
MUC4	ATPLPVTDTSASTGH
MUC7	TTAAPPTPSATTPAPPSSAPPE
MUC11	SGLSEESTTSHSSPGSTHTT
MUC20	SSESSASSDGPHPVITPSR

FIGURE 1. Sequences of acceptor peptides. A, amino acid sequence of human DG. Amino acids are indicated by the single-letter amino acid codes. The mucin-like domain (316–489) is indicated by boldface. The underlines indicate acceptor peptides. Dashed line indicates a signal sequence. B, summary of acceptor peptides from mucin-like domain of α -DG and mucin tandem repeat region.

(1 × 100 mm; GL Sciences Inc., Tokyo) with a model 1100 series liquid chromatography system (Agilent Technologies, Waldbronn, Germany). Mannosyl peptides were eluted at a flow rate of 20 μ l/min using a linear gradient of 0–50% solvent B, where solvents A and B were 0.085% (v/v) aqueous trifluoroacetic acid and 0.075% (v/v) trifluoroacetic acid in 80% (v/v) acetonitrile, respectively. Three peptides thus obtained were subjected to Edman degradation using a Procise HT protein sequencing system (Applied Biosystems, Foster City, CA). “Cart pulsed liquid” and “Flask normal” were used for default programs of reaction and conversion cycle, respectively. At proline residues (3rd, 8th and 13th), the “Cart PL Proline” program was used for reaction cycles. Except for 8-mm peptide supports (Beckman Coulter Inc., Fullerton, CA), all sequence programs and reagents were supplied by Applied Biosystems. To reduce chemicals and background peaks, the chromatogram of the previous cycle was subtracted from one of the current cycle with the 610A data analysis system (Applied Biosystems) using the “Subtraction Mode”. To

336–355 (Fig. 2). The POMT activity was 4.03 pmol/h/mg total protein against peptide 401–420 and 2.32 pmol/h/mg total protein against peptide 336–355. The POMT activities against the other peptides were less than peptide 401–420 and 336–355 (<0.63 pmol/h/mg total protein), suggesting the occurrence of α -DG peptide-specific O-mannosylation.

Mucin type O-linked glycosylation is initiated by the action of a family of UDP-GalNAc, polypeptides N-acetylgalactosaminyltransferase (pp-GalNAc-T), which catalyze the transfer of GalNAc from the nucleotide sugar UDP-GalNAc to the hydroxyl group of either Ser or Thr. Peptides derived from the tandem repeat region of mucins, which have a high density of Ser/Thr, were utilized efficiently by all pp-GalNAc-Ts (19). Therefore, we examined whether or not several peptides of mucin tandem repeat regions worked as an acceptor of O-mannosylation (Fig. 1B). Among these peptides, MUC2 and MUC3 showed slight POMT activities (0.7 and 0.49 pmol/h/mg total protein, respectively). These results indicated that mucin peptides worked as poor acceptors for

identify mannosyl-threonyl residue, 100 pmol of synthetic mannosyl-threonine was subject to protein sequencer. The peaks of 3-phenyl-2-thiohydantoin-mannosyl-threonine and related molecule were eluted on the first cycle of the sequencer. A MALDI-TOF mass spectrum was obtained on a Ultraflex mass spectrometer (Bruker Daltonics, Bremen, Germany) in a reflector mode using 2,5-dihydroxybenzoic acid as a matrix. For MS/MS spectra, parent ion was selected \pm 15 Da from the observed MH⁺ value using time gate and re-accelerated (LIFT mode).

RESULTS

Detection of POMT Activity against Various Peptides—We previously detected POMT activity when POMT1 and POMT2 were co-expressed in human embryonic kidney 293T cells using glutathione S-transferase- α -DG as an acceptor (6). In the present study, we examined whether or not synthetic peptides worked as an acceptor. We prepared nine ~20-amino acid peptides that covered the mucin-like domain of α -DG (Fig. 1). When we measured the POMT activity using these peptides, these peptides were mannosylated to different degrees. The peptide sequence 401–420 worked as a prominent acceptor, followed by the peptide sequence

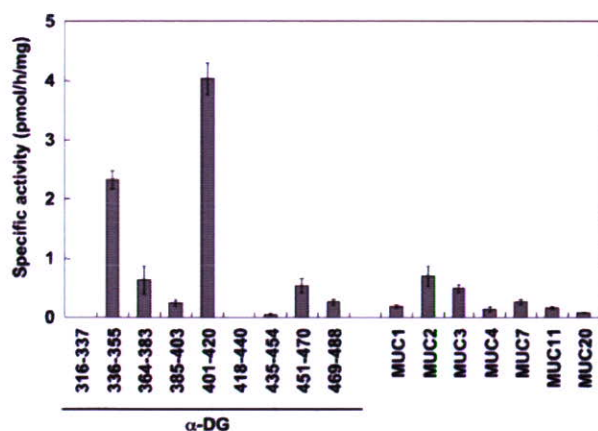


FIGURE 2. POMT activities against various peptides. POMT activity was measured in a 20- μ l reaction volume containing 20 mM Tris-HCl, pH 8.0, 100 nM mannosylphosphoryldolichol (125,000 dpm/pmol), 2 mM dithiothreitol, 10 mM EDTA, 0.5% *n*-octyl- β -D-thioglycoside, 1 mM synthetic peptide, and 40 μ g of microsomal membrane fraction. After incubation at 25 $^{\circ}$ C for 1 h, the peptide was separated by reversed-phase HPLC, and then the incorporation of [3 H]mannose was measured with a liquid scintillation counter. Average values \pm S.D. of three independent experiments are shown.

TABLE 1
Kinetics analysis of POMT activity against various peptides

The kinetic parameters were analyzed by means of Hanes-Woolf plotting (as shown in supplemental Fig. S1) based on the POMT reactions performed using various substrate concentrations, 0.25–4 mM of the acceptor peptide.

Peptide	K_m	V_{max}	K_m/V_{max}
	mM	pmol/h/mg	
336–355	0.63	3.98	0.16
364–383	9.70	3.98	2.44
385–403	2.96	0.70	4.23
401–420	0.73	7.11	0.10
451–470	2.98	1.82	1.64
MUC2	4.00	2.97	1.34
MUC3	2.11	1.39	1.52

O-mannosylation, suggesting that there are preferred protein sequences for O-mannosylation.

Kinetics Analysis of POMT Activity against Various Peptides—Among seven peptides (peptides 336–355, 364–383, 385–403, 401–420, 451–470, MUC2, and MUC3) examined, peptides 336–355 and 401–420 had the lowest K_m values (0.63 and 0.73 mM, respectively) (Table 1 and supplemental Fig. S1). The K_m/V_{max} values for peptides 336–355 and 401–420 were 0.16 and 0.10, respectively, and the K_m/V_{max} values for other peptides were 8 times higher than that of peptide 336–355. These results suggest that the affinities of POMT for peptides 336–355 and 401–420 are comparable and that peptides 336–355 and 401–420 are the most suitable acceptors for POMT reaction under these experimental conditions.

Substrate Specificities and Effect of Change of Amino Acids of Peptides—Peptide 336–355 and peptide 401–420 have six and four potential O-mannosylated Ser and Thr residues, respectively (Fig. 1B). To elucidate which Ser or Thr was preferentially modified by O-mannosylation, each Ser or Thr of the peptides was changed to Ala one by one and then the O-mannosylation efficiencies of the mutated peptides were examined. Six mutated peptides were prepared from peptide 336–355 and four peptides were prepared from peptide 401–420 (Fig. 3A). For peptide 336–355, changing Ser-336 or Ser-344 to Ala did not affect the acceptor efficiency of the peptides, whereas

Preferential Sequence for Protein O-Mannosylation

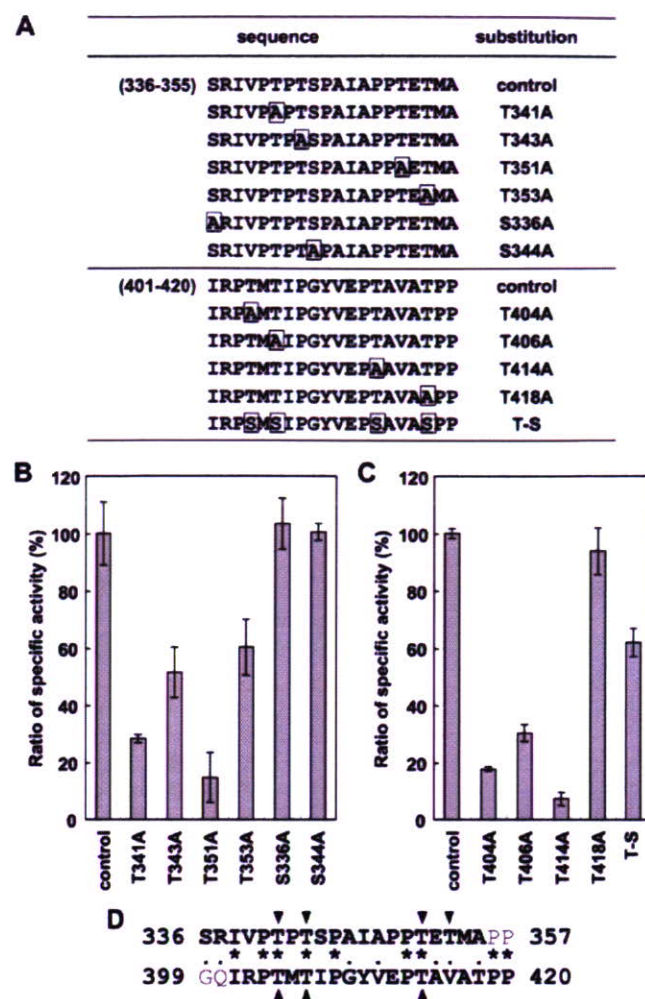


FIGURE 3. Effect of substitution of amino acids of peptides. A, summary of the peptides 336–355 and 401–420 in which boxed A and S residues indicate substitutions for Thr. B and C, relative activities of the mutated peptide 336–355 (B) and peptide 401–420 (C) were evaluated by the POMT reactions using 1 mM acceptor peptide. Average values \pm S.D. of three independent experiments are shown. D, alignment of peptides 336–355 and 401–420 was generated by the GENETYX-Mac program (GENETYX Corp., Tokyo, Japan) based on Lipman-Pearson's method. The asterisks indicate identical amino acids, and the dots indicate similar amino acids. The arrowheads indicate Thr residues whose substitution reduced the POMT activity. Residues shown in nonbold are residues flanking the peptides.

changing Thr-341 or Thr-351 to Ala greatly reduced the acceptor efficiency (by 71.7 and 85.1%, respectively) and changing Thr-343 or Thr-353 to Ala moderately reduced the acceptor efficiency (by 48.5 and 39.8%, respectively) (Fig. 3B). For peptide 401–420, changing Thr-418 to Ala slightly affected the acceptor efficiency, whereas changing Thr-404, Thr-406, or Thr-414 to Ala greatly reduced the acceptor efficiency (by 82.3, 69.7, and 92.7%, respectively) (Fig. 3C). The results indicate that Thr-351 of peptide 336–355 and Thr-414 of peptide 401–420 were prominently modified by O-mannosylation. Next, to elucidate the preference of O-mannosylation of Ser and Thr residues, four Thr residues of peptide 401–420 were changed to Ser (Fig. 3A, peptide T-S) and then the O-mannosylation efficiency of peptide T-S was examined. Changing Thr to Ser reduced the acceptor efficiency by 62.2% (Fig. 3C). This result indicates that

Preferential Sequence for Protein O-Mannosylation

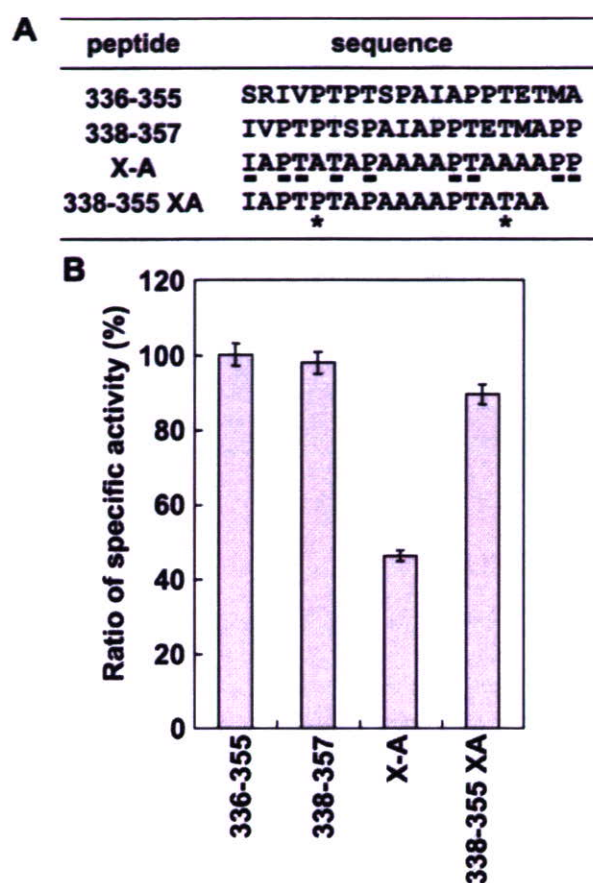


FIGURE 4. Sequence of preferential acceptor peptide for POMT. A, summary of the acceptor peptides. The *underlines* indicate identical amino acids between peptide 336–355 and peptide 401–420 as shown in Fig. 3D. The *asterisks* indicate the reverted Ala to Pro-342 and Thr-353. B, relative activities were evaluated by the POMT reactions using 1 mM acceptor peptide. Average values \pm S.D. of three independent experiments are shown.

the POMT O-mannosylates both Thr and Ser but prefers Thr to Ser.

Interestingly, peptides 336–355 and 401–420 have similar sequences. In an alignment analysis of these peptides and adjacent amino acid residues (Fig. 3D), the two sequences have nine identical amino acids (indicated by *asterisks*) and seven similar amino acids (indicated by *dots*). Taken together, these results indicate that the similar O-mannosylation efficiencies of the two peptides are due to the similarity of their sequences.

Next, we tried to identify a consensus sequence for O-mannosylation. First, to examine the necessity of C-terminal Pro-Pro and N-terminal Ser-Arg, we prepared peptide 338–357 (Fig. 4A). The POMT activity against peptide 338–357 was almost the same as the activity against peptide 336–355 (by 97.8%) (Fig. 4B). This result indicates that the C-terminal Pro-Pro and N-terminal Ser-Arg have a negligible effect on O-mannosylation efficiency. Then we examined the contribution of nonidentical amino acids between peptides 336–355 and 401–420 on O-mannosylation. To address this question, we prepared peptide X-A in which Ala was substituted for all nonidentical amino acids (Fig. 4A). The mutated peptide X-A reduced the acceptor efficiency by 46.2% (Fig. 4B), suggesting that nonidentical amino acids also are important for efficient O-manno-

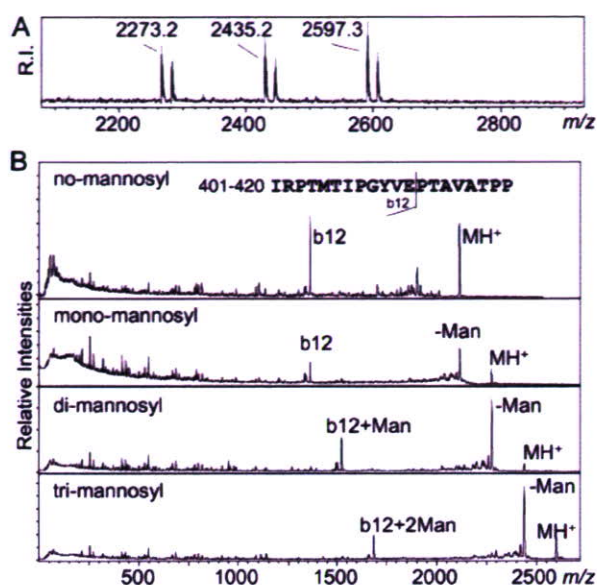


FIGURE 5. MALDI-TOF MS and MS/MS spectra of mannosyl peptides. A, MS spectrum of Con A-purified mannosyl peptides. Due to oxidation of the methionyl residue, +16-Da peaks were detected (m/z 2289.2, 2451.2, and 2613.3). B, MS/MS spectra of non-, mono-, di-, and tri-mannosylated peptides. The calculated average mass value of b12 fragment ion is 1359.6. Each observed mass = m/z (b12)1360.2 (*non-mannosyl* spectrum), 1361.1 (*mono-mannosyl* spectrum), (b12+Man)522.6 (*di-mannosyl* spectrum), (b12+2Man)1684.5 (*tri-mannosyl* spectrum).

sylation. Then we prepared the peptide 338–355 XA in which Ala-342 was reverted to Pro and Ala-353 was reverted to Thr (indicated by *asterisks*). The reverted peptide 338–355 XA restored the acceptor efficiency to 89.3% of peptide 336–355. Taken together, these results suggest that the identical amino acid sequence between peptide 401–420 and peptide 336–355 is necessary for efficient O-mannosylation and that (LXPT(P/X)TXPXXXXPTX(T/X)XX) is a consensus sequence for O-mannosylation.

MALDI-TOF MS Analysis and Edman Degradation Analysis—The enzymatic products of peptide 401–420 were analyzed by MALDI-TOF MS and Edman degradation to determine the number of attached mannose residues and their sites of attachment. The mannosylated peptides from the POMT reaction using peptide 401–420 as an acceptor were purified by Con A-Sepharose column followed by reversed-phase HPLC. The POMT reaction mixture showed several peaks around 22–23 min (supplemental Fig. S2A). A large peak at 23 min derived from the original peptide 401–420 was obtained from the unbound fraction of the Con A-Sepharose column (supplemental Fig. S2B, indicated by *closed triangle*). Three peaks around 22–23 min recovered from the bound fraction of the Con A-Sepharose column appear to be mannosyl peptides (supplemental Fig. S2C, indicated by *open triangle*).

The purified mannosylated peptides were subjected to MS analysis (Fig. 5). The monoisotopic mass value calculated from the peptide 401–420 is 2111.1 for $(M+H)^+$. On the other hand, the spectrum of the products showed three m/z values for $(M+H)^+$ of 2597.3, 2435.2, and 2273.2, suggesting that three, two, and one mannosyl residues have been transferred to the peptide, respectively (Fig. 5A). Then each peak was subjected to

Preferential Sequence for Protein O-Mannosylation

MS/MS analysis to determine the sites of attachment by mannosyl. MS/MS with selection of the ion at m/z 2597 resulted in detection of a demannosylated ion m/z 2438 (peptide 401–420 + 2Man) and b-series ion m/z 1685 (b12, peptide 401–412 + 2Man). MS/MS with selection of the ion at m/z 2435 resulted in detection of ions m/z 2274 (peptide 401–420 + Man) and m/z 1522 (b12, peptide 401–412 + Man). MS/MS with selection of the ion at m/z 2273 resulted in detection of ions m/z 2113 (peptide 401–420) and m/z 1358 (b12, peptide 401–412) (Fig. 5B). These results indicated that the POMT enzymatic products of peptide 401–420 have three, two, and one Man residues.

Analysis of the amino acid sequences of these mannosylated peptides to determine the mannosylated Thr residues (supplemental Fig. S3) showed that the mono-mannosylated peptide was mannosylated mainly at Thr-414 and slightly at Thr-404 and Thr-406, the di-mannosylated peptide was mannosylated at Thr-406 and Thr-414, and the tri-mannosylated peptide was mannosylated at Thr-404, Thr-406, and Thr-414. These results indicate that 1) Thr-414 was the residue that was most frequently modified by O-mannosylation, 2) Thr-404 and Thr-406 were sequentially mannosylated after Thr-414, and 3) Thr 418 was not mannosylated. To confirm the conclusions, we performed a time course study of O-mannosylation. As shown in supplemental Fig. S4, mono-mannosylated peptide appeared at the early stage of the reaction (~15 min), and then the amount of di- and tri-mannosylated peptides increased at later stages. Taken together, these results indicate that O-mannosylation did not occur at random but occurred sequentially.

Effect of Mannosylation of Thr-414 on Substrate Efficiency—To examine whether the presence of mannose on the peptide affects O-mannosylation efficiency, we synthesized a mono-mannosyl peptide (IRPTMTIPGYVEPT(Man)AVATPP, named as peptide 401–420(T414Man)) and compared kinetic parameters with peptide 401–420 (supplemental Fig. S5). The K_m value for peptide 401–420(T414Man) (0.01 mM) was remarkably lower than that of peptide 401–420 (0.73 mM), and the V_{max} value for peptide 401–420(T414Man) (9.63 pmol/h/mg) was higher than that of peptide 401–420 (7.11 pmol/h/mg). These results indicate that the mannosylation of Thr-414 in peptide 401–420 facilitates subsequent mannosylation.

DISCUSSION

In this study, we identified a preferred amino acid sequence for mammalian O-mannosylation using a series of synthetic peptides whose sequences were derived from the mucin-like domain of human α -DG. Our data show that the peptide sequences 336–355 and 401–420 from α -DG are suitable acceptors for O-mannosylation, whereas other sequences including various mucin tandem repeat regions were poor acceptors. Interestingly, peptides 336–355 and 401–420 had very similar amino acid sequences and had comparable K_m values. These findings suggest that these peptides have the same affinity for POMT and have what appears to be a consensus sequence (LXPT(P/X)TXPXXXXPTX(T/X)XX) for mammalian O-mannosylation. The positions of Pro and Thr residues in the two peptides are especially similar. A BLAST search for proteins with this sequence turned up only α -DG, suggesting that the primal O-mannosylated protein is α -DG.

We found that Thr residues modified by O-mannosylation in the peptides 336–355 and 401–420 were mannosylated sequentially rather than at random. The substitution analysis showed that Thr-341, Thr-343, Thr-351, and Thr-353 of peptide 336–355 and Thr-404, Thr-406, and Thr-414 of peptide 401–420 are mannosylated (Fig. 3). Interestingly, the effectiveness of substituted Thr depended on the position; the third Thr residues from the N terminus of these peptides (Thr-351 and Thr-414) were most effective, followed by the first Thr residues (Thr-341 and Thr-404) and then the second Thr residues (Thr-343 and Thr-406). These results indicate that three Thr residues in the consensus sequence are necessary for O-mannosylation. Because the third and first Thr residues in the peptides 336–355 and 401–420 are located next to a Pro residue, POMT may prefer a PT motif as an acceptor. The result that the acceptor efficiency of peptide 338–355 XA is higher than that of peptide X-A (Fig. 4) also suggests the presence of a PT motif is necessary for preferential O-mannosylation. However, the PT motif by itself does not seem to be enough for effective O-mannosylation. Although several of the peptides shown in Fig. 1B have a PT motif, some of them did not work as an acceptor at all and others worked poorly as acceptors (Figs. 1B and 2, and Table 1). These results suggest that the preferential common sequence for effective O-mannosylation is present in peptides 336–355 and 401–420. Indeed, the presence of a Thr residue that is preferentially mannosylated was confirmed by MALDI-TOF MS analysis and amino acid sequence analysis of mannosylated peptides. We obtained mono-, di-, and tri-mannosyl peptides from peptide 401–420. Analysis of these products showed that a mono-mannosylated peptide was mannosylated mainly at Thr-414 and a di-mannosylated peptide was mannosylated at Thr-406 and Thr-414, and Thr-404 is the last of the Thr residues to be O-mannosylated. These results indicate that O-mannosylation of the Thr residues is highly ordered. Furthermore, peptide 401–420(T414Man) was a more effective acceptor than peptide 401–420, indicating that the mannosylation of Thr-414 leads to effective subsequent O-mannosylation. Therefore, α -DG appears to be a prominent acceptor of O-mannosylation because it has both the PT motif and the preferential sequences.

O-GalNAc glycosylation is the most common protein modification and is initiated by the action of a family of pp-GalNAc-Ts. Rules are thought to exist that specify which Ser and Thr residues become decorated with O-GalNAc. However, despite intense investigation, no consensus sequence has emerged that is both necessary and sufficient for O-GalNAc glycosylation to occur. Many nuclear and cytosolic proteins are O-GlcNAc glycosylated at a variety of attachment sites, but a consensus sequence for O-GlcNAc transferase has not been reported (20, 21). On the other hand, O-Fuc glycosylation exists in direct O-linkage to Ser or Thr residues in two different types of Cys knot motifs, epidermal growth factor-like repeats and thrombospondin type 1 repeats. The enzyme responsible for adding O-Fuc to epidermal growth factor repeats was identified as protein O-fucosyltransferase 1 (POFUT1), and the enzyme for adding to thrombospondin type 1 repeats was identified as POFUT2 (22, 23). A consensus sequence for O-Fuc glycosylation in epidermal growth factor is proposed,

Preferential Sequence for Protein O-Mannosylation

CysX₄₋₅(Ser/Thr)Cys between the second and third Cys residues, and a consensus sequence for O-Fuc in thrombospondin type 1 repeats is TrpX₅CysX_{2/3}Ser/ThrCysX₂G between the first and second Cys residues, respectively (24–26). Both POFUT1 and POFUT2 require a specific sequence for O-Fuc glycosylation. Our results indicate that α -DG has preferred sequences for O-mannosylation. However, it may be that the efficiency of O-mannosylation depends not only on the sequence but also the secondary and tertiary structures.

Protein O-mannosylation is an essential post-translational modification (5). In yeast and fungi, protein O-mannosylation is indispensable for cell wall integrity and normal cellular morphogenesis (11). Protein O-mannosylation has also been suggested to be involved in the endoplasmic reticulum quality control system. In yeast, protein O-mannosylation is necessary for intracellular protein trafficking (11, 27). For example, it was found that nonnative proteins are O-mannosylated in the endoplasmic reticulum, which causes them to be excreted from the cell without aggregation and accumulation of aberrant proteins in the endoplasmic reticulum (28, 29). These results suggest that O-mannosyltransferases can recognize proteins that have undergone a conformational change. Thus, the conformation of a protein is another factor for O-mannosylation.

O-Mannosylation is an uncommon type of protein modification in mammals, but it is important in muscle and brain development (5). Although highly glycosylated α -DG was found to be selectively deficient in the skeletal muscle of WWS (7, 8), little is known about the molecular pathomechanism of WWS. Our results show that two peptides derived from the mucin domain of α -DG are highly O-mannosylated by POMT but that little or no O-mannosylation occurs in mucin tandem repeat peptides, suggesting that α -DG has multiple O-mannosylated glycans but other proteins have little, if any. The binding of ligands to glycans is known to depend on how the glycans are clustered. Binding of extracellular matrixes such as laminin, neurexin, and agrin may require a cluster of O-mannosyl glycan on α -DG. Therefore, a defect of O-mannosyl glycans in patients with WWS may severely affect α -DG functions but not other protein functions. Future studies may reveal how α -DG glycosylation contributes to muscular dystrophy and neuronal migration disorder and how normal glycosylation can restore functions of DG. Such studies may lead to the development of therapies for incomplete glycosylation-induced dystroglycanopathies.

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FIGURE 5. MALDI-TOF MS and MS/MS spectra of mannosyl peptides. (A), MS spectrum of Con A-purified mannosyl peptides. Due to oxidation of the methionyl residue, +16Da peaks were detected (m/z 2289.2, 2451.2 and 2613.3). (B), MS/MS spectra of non-, mono-, di- and tri-mannosylated peptides. The calculated average mass value of b12 fragment ion is 1359.6. Each observed mass = m/z [b12]1360.2 (non-mannosyl spectrum), 1361.1 (mono-mannosyl spectrum), m/z [b12+Man]1522.6 (di-mannosyl spectrum), m/z [b12+2Man]1684.5 (tri-mannosyl spectrum).

SUPPLEMENTAL DATA

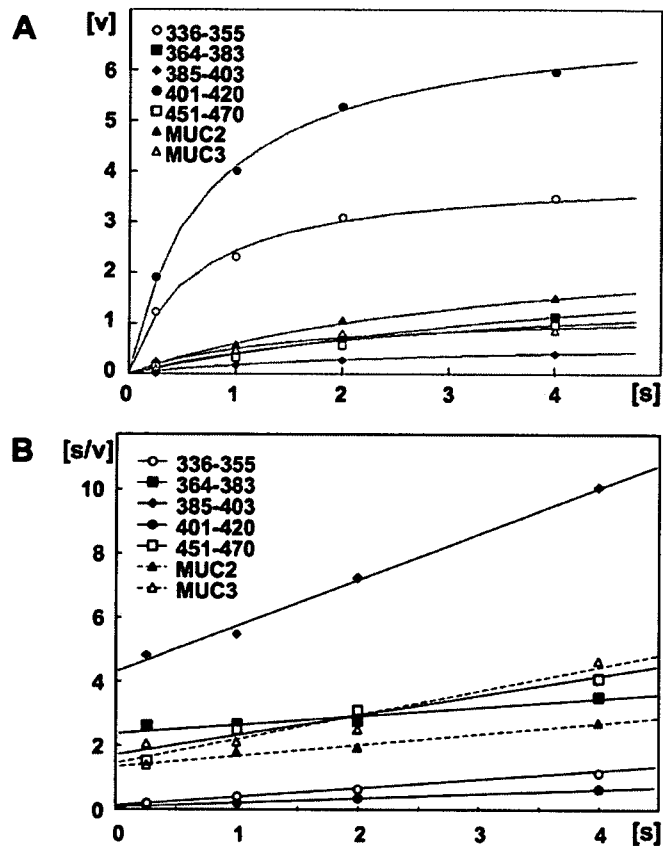
SUPPLEMENTARY FIGURE 1. Kinetics analysis of POMT activity against various peptides. Michaelis-Menten plots (A) and Hanes-Woolf plots (B) were based on the POMT reactions performed using various substrate concentrations: 0.25-4 mM of the acceptor peptide. s , concentration of acceptor peptide (mM); v , specific activity (pmol/h/mg total protein).

SUPPLEMENTARY FIGURE 2. Purification of mannosylated peptide 401-420 by sequential Con-A Sepharose column followed by reversed-phase HPLC. POMT reaction was performed in a 30 μ l reaction volume containing 20 mM Tris-HCl (pH 8.0), 300 μ M Dol-P-Man, 2 mM dithiothreitol, 10 mM EDTA, 0.5% *n*-octyl- β -D-thioglucoside, 1 mM peptide 401-420 and 120 μ g of microsomal membrane fraction at 25°C for 2h. The panels show reversed-phase HPLC (Wakopak 5C18-200 column) chromatograms of the reaction mixture (A), the fraction of the reaction mixture that did not bind to a Con A-Sepharose column (B) and the fraction of the reaction mixture that bound to the Con A-Sepharose column (C). The closed triangles indicate the original peptide 401-420 and the open triangles indicate the mannosylated products. Dashed line shows acetonitrile concentration.

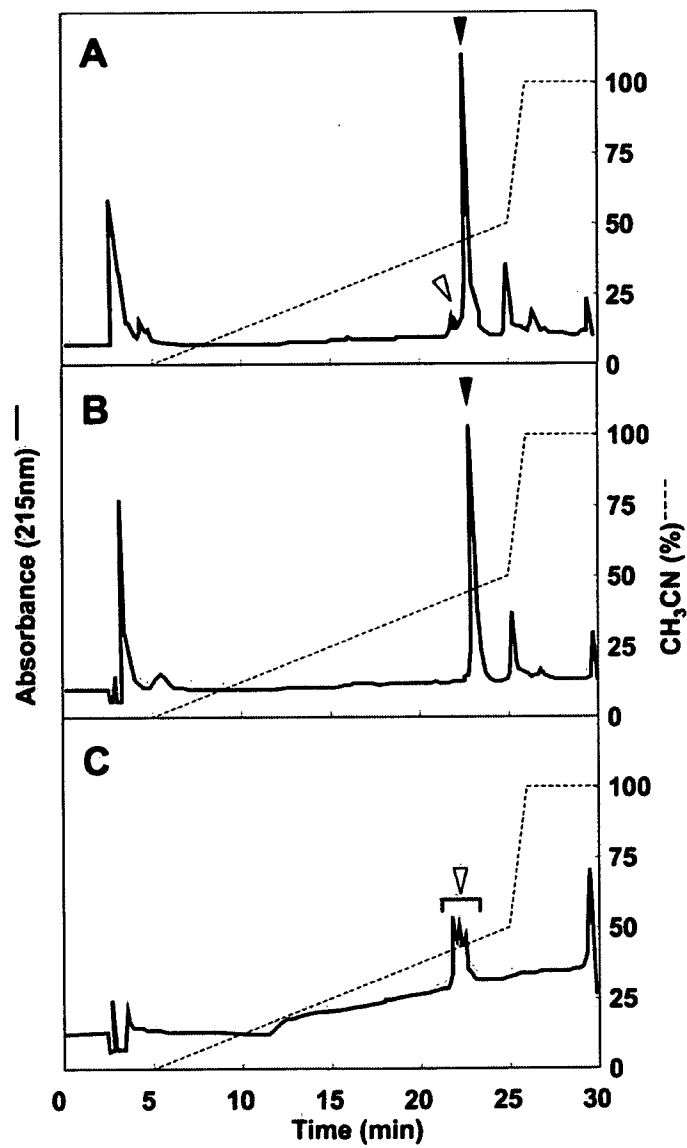
SUPPLEMENTARY FIGURE 3. Subtracted chromatograms of mannosyl peptide by Edman degradation. Mono-, di- and tri-mannosylated peptides were subjected to amino acid sequencing. The chromatograms of 4th, 6th, 14th or 18th cycles in Edman degradation were subtracted from those of the previous cycles. PTH-Thr (T) was eluted at 6 min, and mannosyl-Thr-related peaks (T-M) were eluted at 4.4 and 5.5 min. The retention times of these peaks were identical to those of peaks analyzed synthetic mannosyl threonine. The mono-mannosylated peptide was mannosylated mainly at Thr414, the di-mannosylated peptide was mannosylated at Thr406 and Thr414, and the tri-mannosylated peptide was mannosylated at Thr404, Thr406, and Thr 414.

SUPPLEMENTARY FIGURE 4. Sequential *O*-mannosylation of the peptide 401-420. POMT reaction was performed for 15-120 min under the condition as mentioned in supplemental Fig. 2. The panels show reversed-phase HPLC (Mightysil RP-18GP Aqua column) chromatograms of the fraction of the reaction mixture that bound to the Con A-Sepharose column. The arrowheads indicate the migration positions of (I) mono-, (II) di- and (III) tri-mannosylated peptides.

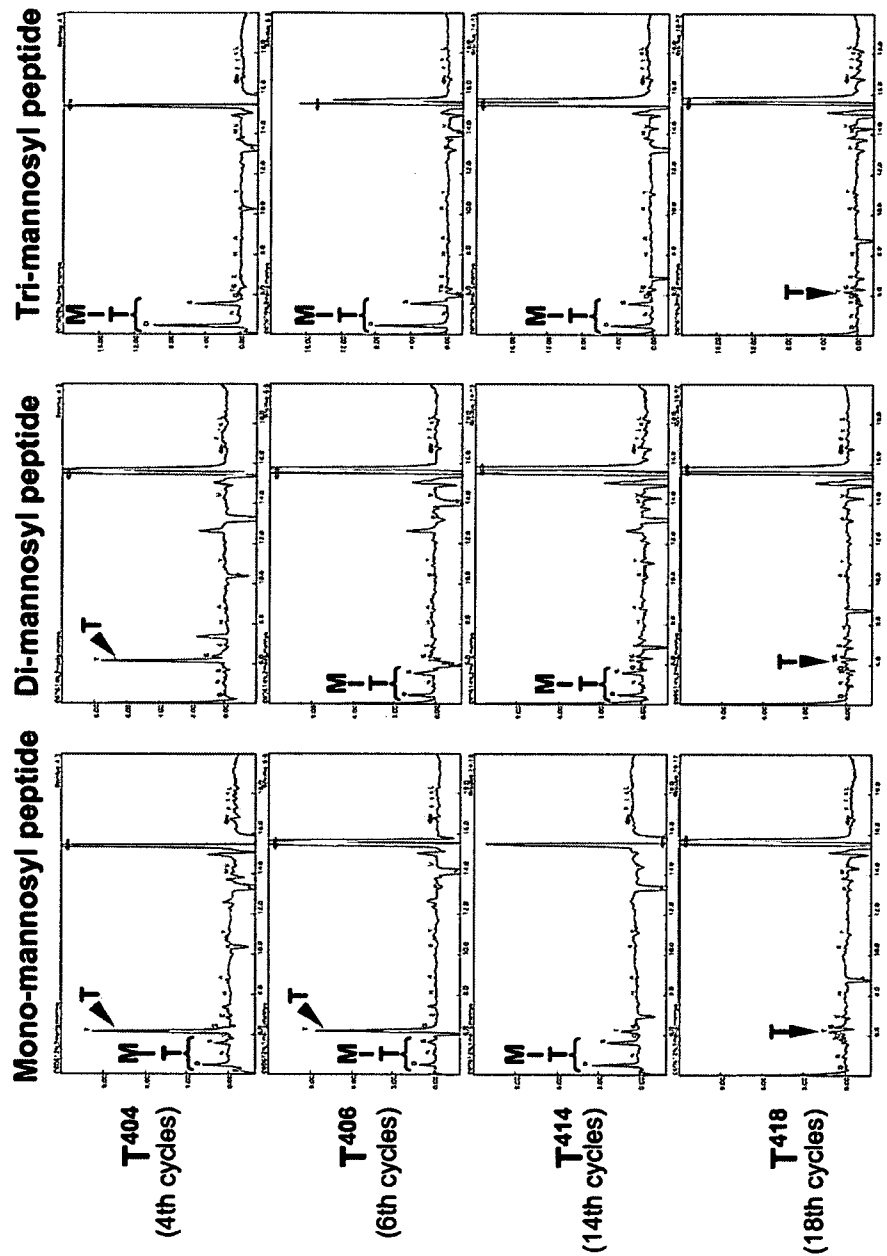
SUPPLEMENTARY FIGURE 5. Effect of Thr414-mannosylation on POMT activity. (A), Amino acid sequence of mono-mannosylpeptide 401-420(T414Man). Michaelis-Menten plots (B) and Hanes-Woolf plots (C) were based on the POMT reactions performed using various substrate concentrations: 0.025-1 mM of the acceptor peptide. s , concentration of acceptor peptide (mM); v , specific activity (pmol/h/mg total protein).



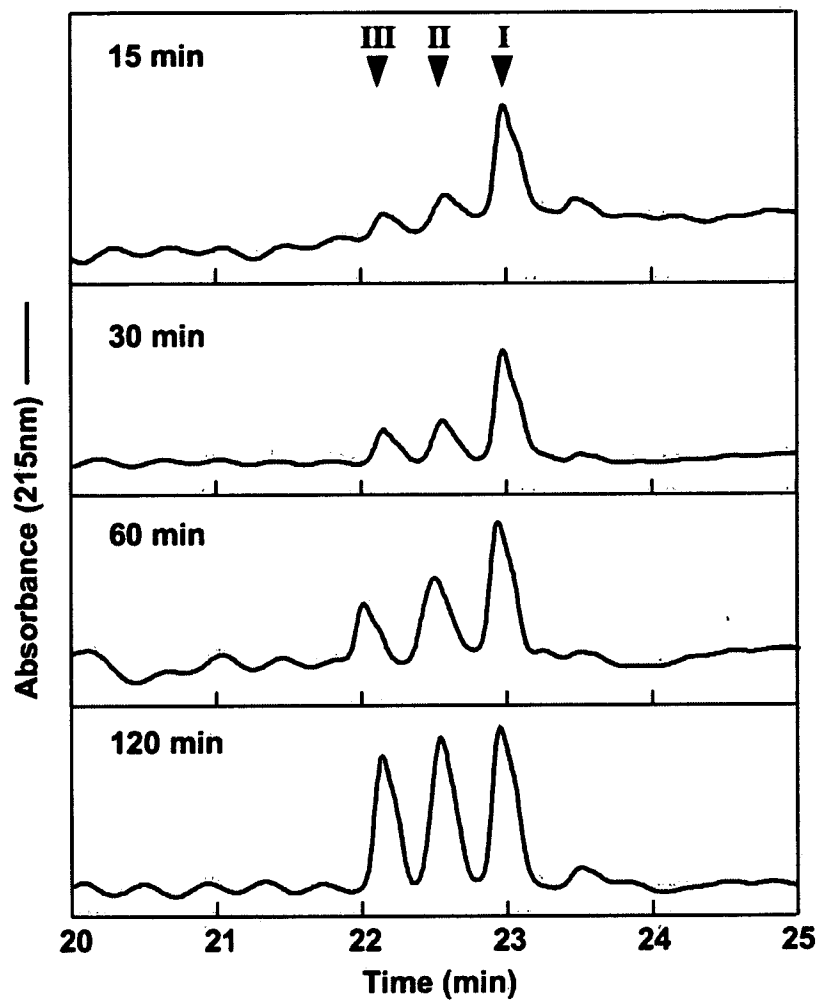
Supplementary Figure 1, Manya et al.



Supplementary Figure 2, Manya et al.

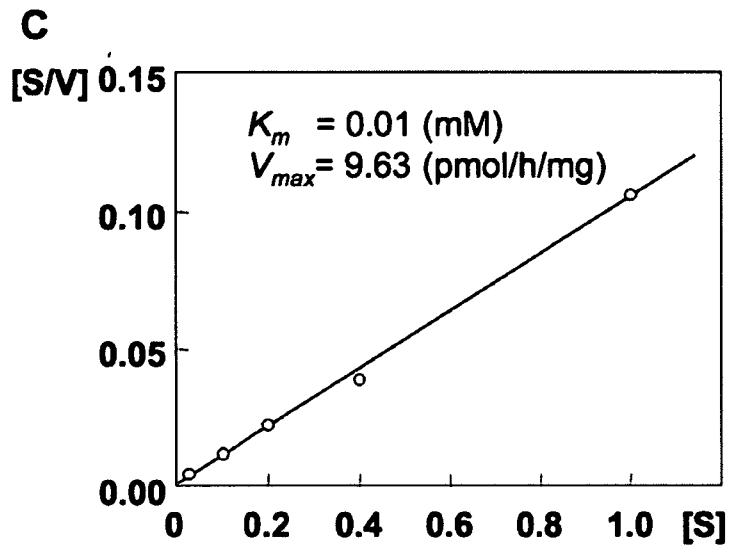
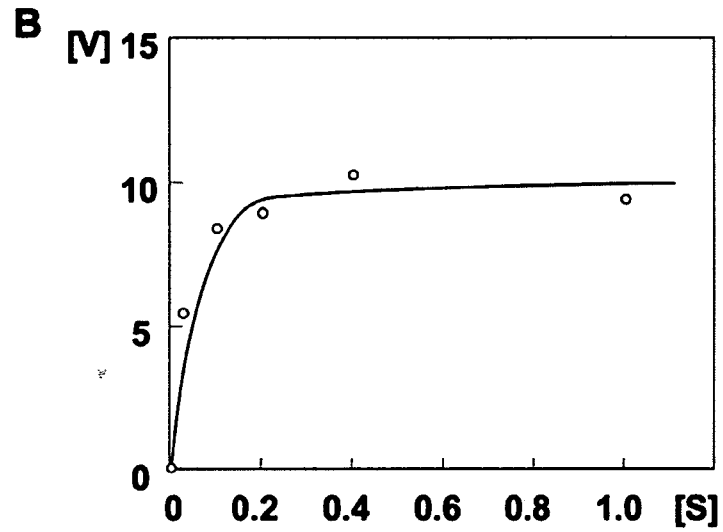


Supplementary Figure 3, Manyá et al.



Supplementary Figure 4, Manya et al.

A IRPTMTIPGYVEPT (Man) AVATPP



Supplementary Figure 5, Manya et al.