厚生労働科学研究費補助金

こころの健康科学研究事業

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

平成１８年度　総括研究報告書

主任研究者　　萬谷　博

平成１９（２００７）年　３

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主任研究者 萬谷　博、 ㈶東京都高齢者研究・福祉振興財団 東京都老人総合研究所、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。本研究では *O*-マンノース型糖鎖の機能を明らかすることで、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、WWS の原因遺伝子産物 POMT1 と POMT2 による *O*-マンノース転移酵素活性の発現機構ついて検討し、*O*-マンノース転移活性には POMT1-POMT2 複合体の形成が必要であることを明らかにした。また、福山型先天性筋ジストロフィーの原因遺伝子産物 fukutin の機能について検討し、fukutin は POMGnT1 に結合し、POMGnT1 活性に影響することを示した。さらに、酵素活性測定法による MEB と WWS の診断の可能性について検討し、先天性筋ジストロフィー患者から *POMGnT1*、*POMT1*、*POMT2* の新たな変異を発見した。

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。しかし、FCMD の原因遺伝子産物 fukutin の機能は未だ分かっていない。また、現在までに、α-ジストログリカノパチーに分類される疾患の原因遺伝子として、*fukutin, POMGnT1, POMT1, POMT2, FKRP, LARGE* の 6 種が報告されている。最近の研究から、これらの疾患の臨床症状の多様性と類似性が明らかになり、臨床所見による原因特定は困難であり、遺伝子診断の必要性が指摘されている。しかし、遺伝子診断は専門知識や技術が必要となり、さらに 6 種の候補遺伝子についてひとつずつ検索が必要となるので診断確定まで労力と時間を要する。

本年度は以下の３つの課題を中心に研究を行った。（1）POMT1 と POMT2 による *O*-マンノース転移酵素（POMT）活性の発現機構の解析：POMT1 と POMT2 は共発現することにより POMT 活性を示すことから、POMT1 と POMT2 が複合体を形成する可能性が示唆されていた。そこで、POMT 活性の発現における POMT1-POMT2 複合体形成の必要性を検討した。（2）fukutin の機能解析：未だ未解明である fukutin の機能について *O*-マンノース型糖鎖合成との関連性に注目し、fukutin が局在するゴルジ体に存在

する POMGnT1 との相互作用について検討した。

（3）診断法の開発：α-ジストログリカノパチーの診断の簡便迅速化を目指して、遺伝子診断に代わる MEB と WWS の診断法として、酵素活性測定法の可能性を検討した。

B. 研究方法

（1）POMT1 と POMT2 を HEK293T 細胞に発現させ、特異的抗体を用いた細胞染色により細胞内局在を調べた。強制発現細胞から膜画分を回収し、免疫沈降法により、POMT1-POMT2 複合体の検出を試みた。また、WWS 患者由来の変異を導入した変異 POMT1 を POMT2 と共発現させ複合体形成と酵素活性への影響を調べた。

（2）*fukutin* 遺伝子の強制発現細胞および遺伝子改変マウスを用いて、POMGnT1 との相互作用や変異による影響ついて検討した。

（3）α-ジストログリカノパチー患者由来リンパ球を EB ウイルスで不死化し、POMGnT1 活性と POMT 活性を測定した。健常者由来のリンパ球をコントロールとして、遺伝子診断により原因遺伝子が特定された患者由来のリンパ球、原因未特定の患者由来リンパ球について解析した。

C. 研究結果および考察

（1）POMT1 と POMT2 の細胞染色の結果、両分子の染色像は一致した。また、小胞体マーカーであるカルレティキュリンとも染色像が一致したことから、小胞体に局在することが明らかとなった。細胞より膜画分を調製し、POMT1 を免疫沈降した結果、免疫沈降画分から POMT2 の共沈が確認され、POMT1-POMT2 複合体の形成が明らかになった。さらに、免疫沈降物（POMT1-POMT2 複合体）から POMT 活性が検出されたことから、複合体の形成が POMT 活性の発現に必要であることが明らかとなった。POMT1 および POMT2 をそれぞれ単独発現させた細胞の混合膜画分を用いた免疫沈降では POMT1 と POMT2 の共沈は観察されなかった。この結果は、蛋白質合成の際に POMT1 と POMT2 が同時期に翻訳されることが複合体形成に必要であることを示唆している。また、今回調べた変異型 POMT1 は POMT2 と共発現しても酵素活性は検出されなかった。しかし、免疫沈降実験の結果、複合体を形成できることが分かった。これらの変異型 POMT1 では基質認識に異常が生じている可能性が考えられる

（2）fukutin と POMGnT1 を共発現させた細胞の細胞染色の結果、fukutin と POMGnT1 の染色像は一致し、ゴルジ体マーカーである GM130 と一致した。免疫沈降により、fukutin と POMGnT1 が共沈したことから、fukutin と POMGnT1 が結合することが明らかとなった。また、FCMD に見られる変異を導入した変異型 fukutin を POMGnT1 と共発現させた結果、変異型 fukutin と POMGnT1 の細胞内局在が小胞体に変化した。この結果から fukutin が POMGnT1 の細胞内局在を制御している可能性が考えられた。そこで、FCMD の変異を導入したヒト *fukutin* 遺伝子をノックインした遺伝子改変マウスを用いて POMGnT1 との関係を検討した。ノックインマウスでは α-ジストログリカンの分子量が減少しており、*O*-マンノース型糖鎖異常が確認された。また、野生型マウスに比較してノックインマウスでは POMGnT1 活性が減少していた。これらの結果から、fukutin が POMGnT1 活性の制御に関与することが示唆された。

（3）遺伝子診断で原因遺伝子の変異が特定され、MEB あるいは WWS であることが確定されている患者リンパ球では、POMGnT1 及び POMT 活性が顕著に低値であることが確認された。この結果は、*POMGnT1* および *POMT1*、*2* の変異が、実際の患者細胞においても糖転移活性を消失させていることを示している。さらに、遺伝子診断前の患者リンパ球から POMGnT1 及び POMT 活性が低い例がいくつか見つかった。これらの例について遺伝子診断を行った結果、*POMGnT1* と *POMT1*、*POMT2* 遺伝子の新たな変異が見つかった。今回の検討から、酵素活性測定法により MEB と WWS の診断が可能であることが示された。

D. 結論

（1）先天性筋ジストロフィー症 WWS の原因遺伝子産物 POMT1 と POMT2 は、POMT1-POMT2 複合体を形成することにより、*O*-マンノース転移酵素として機能することが明らかとなった。（2）FCMD の原因遺伝子産物 fukutin は MEB の原因遺伝子産物 POMGnT1 と結合し酵素活性に影響を及ぼすことを明らかにした。（3）MEB と WWS の酵素活性測定法による診断の可能性を示した。

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研究成果の刊行に関する一覧表

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Endo, T., Manya, H.	*O*-Mannosylation in Mammalian Cells.	Brockhausen, I.	Protocol for Methods in Molecular Biology 347	Humana Press Inc.	NJ, USA	2006	43-57,

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研究成果の刊行物・別刷

4

O-Mannosylation in Mammalian Cells

Tamao Endo and Hiroshi Manya

Summary

The *O*-mannosyl glycan is present in a limited number of glycoproteins of brain, nerve, and skeletal muscle. α-Dystroglycan is one of the *O*-mannosylated proteins and is a central component of the dystrophin–glycoprotein complex that has been shown to be related to the onset of muscular dystrophy. We have identified and characterized glycosyltransferases, protein *O*-mannose β1,2-*N*-acetylglucosaminyltransferase (POMGnT1) and protein *O*-mannosyltransferase 1 (POMT1), involved in the biosynthesis of *O*-mannosyl glycans. We subsequently found that loss of function of the *POMGnT1* gene is responsible for muscle–eye–brain disease (MEB). It has also been reported that the *POMT1* gene is responsible for Walker-Warburg syndrome (WWS). MEB and WWS are autosomal recessive disorders characterized by congenital muscular dystrophies with neuronal migration disorders. Therefore, the ability to assay enzyme activities of mammalian *O*-mannosylation would facilitate progress in the identification of other *O*-mannosylated proteins, the elucidation of their functional roles, and the understanding of muscular dystrophies. This protocol describes assay methods for the mammalian POMT and POMGnT.

Key Words: *O*-mannosylation; glycosyltransferase; POMGnT1; POMT1; POMT2; α-dystroglycan; Walker-Warburg syndrome; muscle-eye-brain disease; muscular dystrophy.

1. Introduction

O-mannosylation is a common type of glycosylation in fungi and yeast. These *O*-mannosyl glycans are neutral straight-chain glycans that are composed of one to seven mannose residues. Mammalian *O*-mannosylation is an unusual type of protein glycosylation (*see also* Chapter 5) and is present in a limited number of glycoproteins of brain, nerve, and skeletal muscle ***(1–6)***. We have previously found that the glycans of α-dystroglycan (α-DG) include *O*-mannosyl oligosaccharides, and that a sialyl *O*-mannosyl glycan, Siaα2-3Galβ1-4GlcNAcβ1-2Man, is very different from that of fungi and yeast ***(1)***. Our data also suggest

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that the sialyl *O*-mannosyl glycan is a laminin-binding ligand of α-DG ***(1)***. α-DG is a central component of the dystrophin–glycoprotein complex (DGC) isolated from skeletal muscle membrane and behaves as a connection between DGC and extracellular matrix molecules, such as laminin, agrin, and neurexin ***(7–9)***. DGC has a crucial role in linking the extracellular basal lamina to the cytoskeletal proteins for stabilization of sarcolemma.

Muscular dystrophies (MDs) are genetic diseases that cause progressive muscle weakness and wasting. Because the causative genes of several MDs have been identified from molecules associated with DGC, it is commonly believed that the dysfunction of DGC causes the development of MDs. Duchenne MD, as a famous case in point, results from mutations of the gene encoding dystrophin in DGC. Recently, scores of reports suggest that aberrant protein glycosylation of α-DG is the primary cause of some forms of congenital MD ***(8–10)***.

Muscle–eye–brain disease (MEB; MIM 253280) and Walker-Warburg syndrome (WWS; MIM 236670) are autosomal recessive disorders characterized by congenital MD, ocular abnormalities, and brain malformation (type II lissencephaly). We previously reported that MEB is caused by mutations in the gene encoding POMGnT1 uridine 5′-diphosphate (UDP)-*N*-acetylglucosamine: protein *O*-mannose β1,2-*N*-acetylglucosaminyltransferase ***(1)***. POMGnT1 is responsible for the formation of the GlcNAcβ1-2Man linkage of *O*-mannosyl glycan ***(11)***. We also demonstrated that protein *O*-mannosyltransferase 1 (POMT1) forms an enzyme complex with POMT2 and is responsible for the catalysis of the first step in *O*-mannosyl glycan synthesis ***(12)***. Mutations in the *POMT1* gene are considered to be the cause of WWS ***(13)***.

The GlcNAcβ1-2Man linkage of *O*-mannosyl glycan is identified only in mammals, and it was impossible to detect POMGnT1 activity by using acceptor substrates such as mannose, mannose–threonine, *p*-nitrophenyl-α-mannose, and mannose-2-aminobenzamide. Therefore, the synthesis of mannosylpeptide as acceptor substrate, derived from the α-DG sequence, enabled us to detect POMGnT1 ***(14)***.

POMT1 encodes a protein that is homologous to members of the family of protein *O*-mannosyltransferases (PMTs) in yeast. In yeast, PMTs catalyze the transfer of a mannosyl residue from dolichyl phosphate mannose (Dol-P-Man) to serine–threonine residues of certain proteins ***(15)***. However, using the same methods as those applied to yeast, POMT activity was not detected in mammalian tissues and cells. This difference between mammals and yeast may depend largely on the specificity of the acceptor peptide sequence and the effect of detergent. We established the method for POMT assay in mammals by using

recombinant α-DG expressed in *Escherichia coli* as acceptor substrate and *n*-octyl-β-D-thioglucoside (OTG) as detergent ***(12)***. This protocol describes assay methods for mammalian POMT and POMGnT.

2. Materials

2.1. Preparation of Enzyme Sources

1. *pcDNA3.1-POMGnT1*, *pcDNA3.1-POMT1*, and *pcDNA3.1-POMT2* expression plasmids: Human cDNAs encoding POMGnT1 ***(11)***, POMT1 ***(16)***, and POMT2 ***(17)*** are inserted into mammalian expression vectors, pcDNA3.1/Zeo, or pcDNA3.1/Hygro (Invitrogen Corp., Carlsbad, CA).
2. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), 100X penicillin–streptomycin–glutamine liquid (PC-SM-Gln, 10,000 U/mL of penicillin, 10,000 μg/mL of streptomycin, 29.2 mg/mL of glutamine), Lipofectamine transfection reagent and Plus reagent (Invitrogen).
3. Phosphate-buffered saline (PBS): prepare 10X stock with 1.37 *M* of NaCl, 27 m*M* of KCl, 80 m*M* of Na_2HPO_4, and 14.7 m*M* of KH_2PO_4 (adjust to pH 7.4 with HCl if necessary). Store at room temperature. Prepare working solution by dilution with water (1:9) and store at 4°C.
4. Silicone blade cell scraper (Sumilon, Sumotomo Bakelite Co., Tokyo, Japan).
5. Homogenization buffer: 10 m*M* of Tris-HCl (pH 7.4), 1 m*M* of ethylenediaminetetraacetic acid (EDTA), 250 m*M* of sucrose (SET buffer) with protease inhibitor cocktail (Complete, EDTA-free, Roche Diagnostics, Basel, Switzerland). SET buffer is stored at 4°C. Add protease inhibitor cocktail before use.

2.2. Preparation of Glutathione-S-Transferase-α-Dystroglycan

1. *pGEX-glutathione-S-transferase-α-dystroglycan (GST-α-DG):* Potential *O*-glycosylation sites of α-DG are predicted in the region corresponding to amino acids 313-483 ***(18)***. We amplified this region from mouse brain total ribonucleic acid (RNA) by reverse transcriptase polymerase chain reaction (RT-PCR) using the primer set 5′-GGGAATTCCACGCCACACCTACAC-3′ (sense) and 5′-GGGTC TAGAACTGGTGGTAGTACGGATTCG-3′ (antisense), and subcloned it into the pGEX-4T-3 vector to express the peptide as a GST-fusion protein (Amersham Biosciences Corp., Piscataway, NJ).
2. Luria-Bertani (LB) broth (Invitrogen) supplemented with 50 μg/mL of ampicillin.
3. LB agar plate (1.5% w/v agar) supplemented with 50 μg/mL of ampicillin.
4. Isopropyl-D-thiogalactopyranoside (IPTG; Invitrogen): prepare 1 *M* stock solution in water, sterilize by filtration, and store at –20°C.
5. Ampicillin sodium salt (Nacalai tesque, Kyoto, Japan): prepare 50 mg/mL stock solution in water, sterilize by filtration, and store at –20°C.
6. 1 mL of glutathione–sepharose column (GSTrap; Amersham).
7. Prepare 10 m*M* of reduced glutathione in PBS just before use.
8. 50 m*M* of $(NH_4)HCO_3$: prepare 1 *M* of stock solution (pH 7.0) in water and dilute 100 mL with 1900 mL of water for use.

2.3. POMT Assay

1. OTG and 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS; Dojindo Laboratories, Kumamoto, Japan): prepare 10% (w/v) stock solution in water and store at –20°C.
2. Triton X-100 (Nacalai tesque): prepare 20% (w/v) stock solution in water and store at room temperature.
3. POMT reaction buffer: 10 m*M* of Tris-HCl (pH 8.0), 2 m*M* of 2-mercaptoethanol (2-ME), 10 m*M* of EDTA, and 0.5% of OTG. Store at –20°C.
4. Mannosylphosphoryldolichol95: [Mannose-6-^{3}H] Dol-P-Man (1.48-2.22 TBq/mmol, American Radiolabeled Chemical, Inc., St. Louis, MO). 1.85 MBq of solution in chloroform and methanol is transferred into a screw-cap centrifugal tube and evaporated with a centrifugal evaporator (*see* **Note 1**). Add 1 mL of 20 m*M* Tris-HCl (pH 8.0) and 0.5% CHAPS, and dissolve by sonication with bath-type sonicator in ice-cold water (10 cycles of 15-s pulses with 30-s intervals). Measure radioactivity and then adjust to 40,000 cpm/μL with 20 m*M* of Tris-HCl (pH 8.0) and 0.5% CHAPS. Aliquot and store at –80°C.
5. PBS containing 1% Triton X-100 (1% Triton-PBS). Store at 4°C.
6. 0.5% Triton-*tris* buffer: 20 m*M* of Tris-HCl (pH 7.4) containing 0.5% Triton X-100. The buffer is stored at 4°C.
7. Glutathione–sepharose 4B (Amersham): Prepare a 25% slurry working suspension as follows. Suspension (1 mL, equivalent to 0.75-mL beads) is put in a centrifugal tube. 9 mL of water is added to the suspension and vortexed. After centrifugation at 1000*g* for 1 min the supernatant is removed by aspiration. The beads are rinsed with 10 mL of PBS and collected by centrifugation. 1% Triton-PBS (2.25 mL) is added and stored at 4°C.
8. Liquid scintillation cocktail: 0.4% (w/v) 2,5-Diphenyloxazole (Dojindo), 35% (w/v) polyethylene glycol *p*-isooctylphenyl ether (Nacalai tesque) in toluene.
9. Jack bean-α-mannosidase (Seikagaku Corp., Tokyo, Japan): 0.8 U of enzyme is dissolved in 50 μL of 0.1 *M* ammonium acetate buffer (pH 4.5). The enzyme solution is dried up with a centrifugal evaporator and stored at –20°C. The dried enzyme is dissolved with 50 μL of 1 m*M* $ZnCl_2$ before use.

2.4. POMGnT1 Assay

1. UDP-*N*-acetyl-D-glucosamine (UDP-GlcNAc; Sigma-Aldrich Corp., St. Louis, MO): Prepare a 1-m*M* stock solution in water and store at –20°C.
2. UDP-GlcNAc [glucosamine-6-^{3}H(N)] (UDP-[^{3}H]-GlcNAc, 0.74-1.66 TBq/mmol, PerkinElmer, Inc., Wellesley, MA). Store at –20°C.
3. Benzyl-α-D-mannopyranoside (Sigma-Aldrich): Prepare 100 m*M* of stock solution in 20% ethanol and store at –20°C.
4. Mannosylpeptide (Ac-Ala-Ala-Pro-Thr[Man]-Pro-Val-Ala-Ala-Pro-NH_2; *see* **Note 2**): prepare 2 m*M* of stock solution in water and store at –20°C.
5. POMGnT reaction buffer: 140 m*M* of methanesulfonic acid (MES; adjust pH to 7.0 with NaOH), 2% Triton X-100, 5 m*M* of adenosine 5′-monophosphate (AMP),

200 m*M* of GlcNAc, 10% glycerol, and 10 m*M* of $MnCl_2$. Store at –20°C without $MnCl_2$ ($MnCl_2$ is added just before use).
6. Reverse-phase column for high-performance liquid chromatography (HPLC): Wakopak 5C18-200 column (4.6 × 250 mm, Wako Pure Chemical Industries, Osaka, Japan).
7. 0.1% Trifluoroacetic acid (TFA) in water (Solvent A): Add 1 mL of TFA to 1000 mL of HPLC-grade water and degas with an aspirator before use.
8. 0.1% TFA in acetonitrile (Solvent B): Add 1 mL of TFA to 1000 mL of HPLC-grade acetonitrile and degas by sonication before use.
9. Liquid scintillation cocktail as described in **Subheading 2.3., item 8**.
10. Streptococcal β-*N*-acetylhexosaminidase (HEXaseI, Prozyme, San Leandro, CA): 50 mU of enzyme is dissolved with 50 µL of 0.3 *M* citrate phosphate buffer (pH 5.5) and stored at –20°C.
11. 0.05 *N* of NaOH, 1 *M* of $NaBH_4$, and 4 *N* of acetic acid solution in water.
12. AG-50W-X8 (H^+ form, Bio-Rad Laboratories, Hercules, CA).

2.5. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis and Western Blotting

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting are carried out in accordance with standard methods. Please refer to experimental guidebooks. Some points are described in the following items.

1. 4X Loading buffer (modified Laemmli *[19]* buffer): 250 m*M* of Tris-HCl (do not adjust pH), 8% (w/v) SDS, 40% (w/v) glycerol, 2.84 m*M* of 2-ME, and 0.005% (w/v) bromophenol blue. Store at –20°C.
2. Antibodies: Rabbit antisera specific to the human POMT1, POMT2, and POMGnT1 are produced by using synthetic peptides corresponding to residues 348–362 (YPMIYENGRGSSH) of POMT1, 390–403 (HNTNSDPLDPSFPV) of POMT2, and 649–660 (KEEGAPGAPEQT) of POMGnT1, respectively. Anti-rabbit IgG is conjugated with horseradish peroxidase (HRP; Amersham).
3. Coomassie Brilliant Blue R-250 (CBB): Prepare a 0.1% solution in methanol: acetic acid:water (40:10:50) and store at room temperature.
4. Enhanced chemiluminescent (ECL) reagent kit (Amersham).
5. Amplify fluorographic reagent (Amersham).
6. Hyperfilm ECL and Kodak BioMax MS X-ray film are purchased from Amersham.

3. Methods

The POMT activity is based on the amount of [^{3}H]-mannose transferred from Dol-P-Man to GST-α-DG *(12)*. The reaction product is purified with a glutathione–sepharose column, and the radioactivity of mannosyl GST-α-DG is measured by a liquid scintillation counter. The POMGnT1 activity is based on the amount of [^{3}H]GlcNAc transferred from UDP-GlcNAc to benzyl-α-mannose

(Benzyl-Man; *see* **ref. *20)*** or mannosylpeptide (Ac-Ala-Ala-Pro-Thr[Man]-Pro-Val-Ala-Ala-Pro-NH_2; *see* **ref. *14)***. The reaction product is purified with a reverse-phased HPLC and the radioactivity is measured. We also synthesized several mannosylpeptides derived from mucin box sequences of α-DG. These mannosylpeptides are not commercially available but it is possible to use Benzyl-Man, which is commercially available, as a substitute.

POMGnT1 and POMT activities are detected in various mammalian cells and mammalian tissues. This chapter describes the methods that use the microsomal membrane fraction of rat brain and human embryonic kidney 293T (HEK293T) cells as the enzyme source. To demonstrate that the gene products of *POMGnT1*, *POMT1*, and *POMT2* have enzymatic activity, the cells transfected with *POMGnT1* or *POMT1* and *POMT2* are used. Although whole cells instead of membrane fractions may be used as an enzyme source, we recommend using membrane fractions because mammalian tissues and cells have a low specific activity ***(12)***.

3.1. Preparation of Enzyme Sources

3.1.1. Cell Culture and Preparation of Cell Membrane Fraction

1. HEK293T cells are maintained in DMEM supplemented with 10% FBS, 2 m*M* of L-glutamine, and 100 U/mL of penicillin/50 μg/mL of streptomycin at 37°C with 5% CO_2.
2. The expression plasmids of human *pcDNA3.1–POMT1* and *pcDNA3.1–POMT2* are transfected into HEK293T cells using Lipofectamin PLUS reagent according to the manufacturer's instructions.
   a. The day before transfection, plate cells into a 100-mm culture dish with antibiotic-free 10% FBS-DMEM so that they are 60–70% confluent the day of transfection. Avoid antibiotics during transfection.
   b. Dilute 4 μg of DNA with 750 μL of serum-free DMEM, add the 20 μL of Plus reagent, and let stand at room temperature for 15 min (reagent A). In another tube, dilute 30 μL of Lipofectamin reagent with 750 μL of serum-free DMEM (reagent B).
   c. Mix reagent A with reagent B and let stand at room temperature for 15 min (reagent C).
   d. During **step c**, replace the medium on the cells with 5 mL of serum-free DMEM.
   e. Add reagent C to the cells from **step d** and incubate at 37°C with 5% CO_2 for 3 h.
   f. Add 5 mL of 20% FBS-DMEM to the cells from **step e** and culture for 2–3 d.
3. The culture supernatants are removed by aspiration and the cells are rinsed gently with cold PBS. Then 5 mL of cold PBS is added, and the cells are scraped into centrifugal tubes and washed with 10 mL of cold PBS. The cells are collected by centrifugation at 1000*g* for 10 min at 4°C (*see* **Note 3**).
4. The cell pellet is broken with a tip-type sonicator in 500 μL of homogenization buffer (*see* **Note 4**). After centrifugation at 900*g* for 10 min, the supernatant is

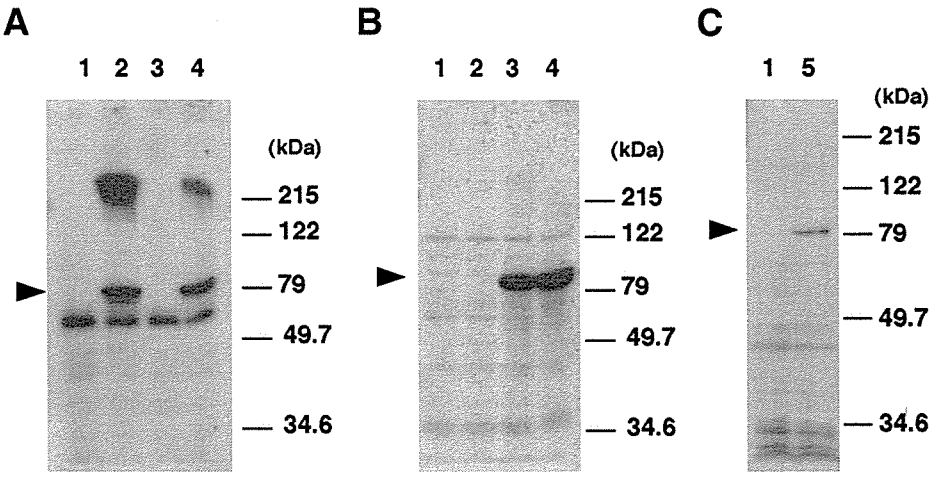

Fig. 1. Western blot analysis of (**A**) POMT1, (**B**) POMT2, and (**C**) POMGnT1 expressed in HEK293T cells. Lanes 1, cells transfected with vector alone; lanes 2, cells transfected with human *POMT1*; lanes 3, cells transfected with human *POMT2*; lanes 4, cells cotransfected with *POMT1* and *POMT2*; lane 5, cells transfected with human *POMGnT1*. The proteins (20 μg of membrane fraction) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% gel), and the separated proteins were transferred to a polyvinylidene difluorite membrane. The polyvinylidene difluorite membrane was stained with (**A**) anti-POMT1, (**B**) anti-POMT2, or (**C**) anti-POMGnT1 antibody. Arrowheads indicate the positions of the corresponding molecules. Molecular-weight standards are shown on the right. (Reprinted with permission from **ref. *12***. Copyright 2004 by National Academy of Sciences.)

dispensed in halves and subjected to ultracentrifugation at 100,000*g* for 1 h. The precipitates thus obtained are used as microsomal membrane fraction (*see* **Note 5**).

5. Half of the precipitates obtained in **step 4** are used to determine protein concentration and are subjected to Western blotting, and the remainder are used to assess the enzymatic activity.
6. Western blot is performed for detection of products (*see* **Fig. 1**). The microsomal fraction (20 μg) is separated by SDS-PAGE (10% gel) and proteins are transferred to a polyvinylidene difluorite membrane. The membrane, after blocking in PBS containing 5% skim milk and 0.5% Tween-20, is incubated with each antibody and then the membrane is treated with anti-rabbit IgG conjugated with HRP. Proteins bound to an antibody are visualized with ECL.

3.1.2. Preparation of Brain Membrane Fraction

1. The brain is harvested from a newborn rat (F344/N, Nihon SLC, Shizuoka, Japan) and rinsed with cold PBS. For every gram of brain, 9 mL of homogenization

buffer is immediately added and homogenized on ice using a potter's homogenizer at 800 rpm (8 strokes).
2. Nuclei, cellular debris, and connective tissues are removed by centrifugation at 900*g* for 10 min. For preparation of microsomal membranes, the postnuclear supernatant is subjected to ultracentrifugation at 100,000*g* for 1 h. The pellet fraction is aliquoted and stored at –80°C until used.

3.2. Preparation of GST-α-DG

1. BL21(DE3) *E. coli* cells are transformed with *pGEX-GST-α-DG*. Cultures are prepared by growing a single colony overnight in LB broth at 37°C. The overnight culture is then used to inoculate a fresh 50-mL culture, which is grown at 37°C to *A*620 = 0.5. At this point, 1 m*M* of IPTG is added to the culture in order to induce GST-α-DG expression. The induced cells are grown in parallel for an additional 4 h at 37°C, and harvested by centrifugation at 6000*g* for 15 min at 4°C.
2. The cell pellet is suspended in 10 mL of PBS (pH 7.4) and broken with a tip-type sonicator (*see* **Note 6**). The cell supernatant is recovered by ultracentrifugation at 100,000*g* for 1 h.
3. Recombinant GST-α-DG proteins are purified from the supernatant with a GSTrap column in a fast protein liquid chromatography system (Amersham) in the following manner: Pre-equilibrate the GSTrap column with 10 mL of PBS. Load the supernatant onto the column and wash with PBS at a flow rate of 0.2 mL/min. The absorbed recombinant GST-α-DG proteins are eluted with 10 mL of 10 m*M* reduced glutathione in PBS at a flow rate of 1 mL/min.
4. The purified GST-α-DG is dialyzed with 50 m*M* of $(NH_4)HCO_3$, pH 7.0.
5. Protein concentration is determined by bicinchonic acid (BCA) assay (Pierce, Rockford, IL), and the purity of GST-α-DG is checked by SDS-PAGE visualized with CBB (*see* **Subheading 2.5, item 3** and **Fig. 2A**).
6. The GST-α-DG aliquots are dispensed by 10 μg in microcentrifugal tubes, dried up with a centrifugal evaporator, and kept at –80°C.

3.3. POMT Assay

1. The POMT reaction buffer is added to the microsomal membrane fraction at a protein concentration of 4 mg/mL. The fraction is suspended by moderate pipetting and solubilized for 30 min on ice with occasional mild stirring.
2. 20 μL of the solubilized fraction and 2 μL of Dol-P-Man solution (from **Subheading 2.3., item 4**) are added to the dried GST-α-DG (**Subheading 3.2., step 6**), vortexed, and spun down gently. Immediately incubate the reaction mixture at 25°C for 1 h. The reaction is stopped by adding 200 μL of 1% Triton-PBS (*see* **Note 7**).
3. The reaction mixture is centrifuged at 10,000*g* for 10 min. The supernatant is transferred into a screw-cap tube with a packing seal (*see* **Note 8**). Mix 400 μL of 1% Triton-PBS and 40 μL of 25% slurry glutathione–sepharose beads with the supernatant and rotate with a rotary mixer at 4°C for 1 h.
4. After centrifugation at 1000*g* for 1 min, the supernatant is removed by aspiration and the beads are washed three times with 0.5% Triton-tris buffer. 2% SDS is

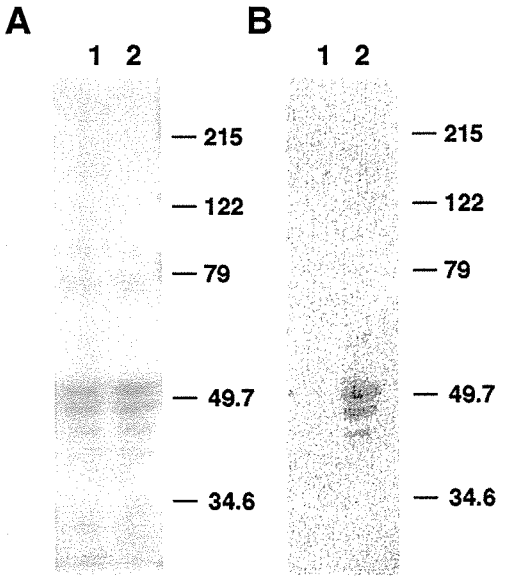

Fig. 2. Incorporation of [^{3}H]-mannose into glutathione-*S*-transferase-α-dystroglycan (GST-α-DG). Dol-P-[^{3}H]Man and GST-α-DG are incubated with HEK293T cell membrane fraction in POMT reaction buffer. After incubation, the products are recovered by the glutathione–sepharose 4B beads and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% gel). GST-α-DG was detected as triplet bands at around 50 kDa by CBB staining (**A**). Because all bands were stained with the anti-GST antibody (data not shown) the largest molecular-weight band was thought to be the full-length GST-α-DG, and the smaller bands were probably fragments of degraded GST-α-DG. The radioactivity of [^{3}H]-mannose was detected by autoradiography (**B**), and the radioactivity was incorporated into the GST-α-DG in the presence of both the membrane fraction and an acceptor. Lanes 1, incubation with GST-α-DG but without membrane fraction; lanes 2, incubation with membrane fraction and GST-α-DG. Molecular-weight standards are shown on the right. (Reprinted with permission from **ref. *12***. Copyright 2004 by National Academy of Sciences.)

added to the beads and boiled at 100°C for 3 min. The suspension is cooled down to room temperature and mixed with liquid scintillation cocktail. The radioactivity adsorbed by the beads is measured using a liquid scintillation counter (*see* **Fig. 3A**).

5. The incorporation of radioactive mannose into GST-α-DG can be detected by SDS-PAGE and subsequent autoradiography as follows. Instead of 2% SDS in **step 4**, add 20 μL of 2X loading buffer to the beads followed by boiling at 100°C for 3 min. After centrifugation at 1000*g* for 1 min, the supernatant is subjected to SDS-PAGE. Gel is stained with CBB to visualize GST-α-DG, soaked in amplify

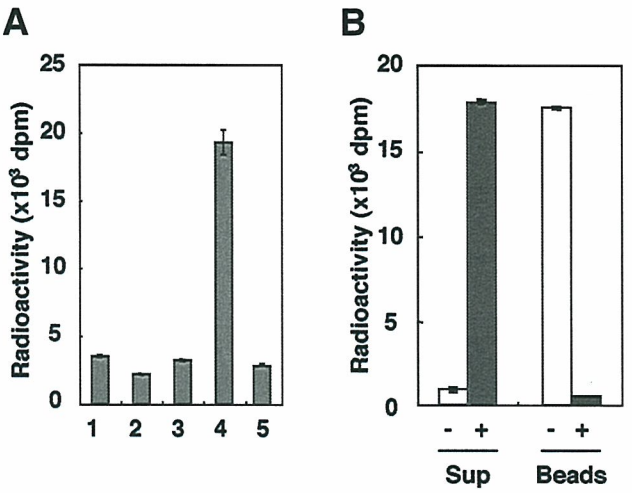

Fig. 3. (**A**) POMT activity of human POMT1 and POMT2 expressed in HEK293T cells. Bar 1, cells transfected with vector alone; bar 2, cells transfected with human *POMT1*; bar 3, cells transfected with human *POMT2*; bar 4, cells cotransfected with *POMT1* and *POMT2*; bar 5, a mixture of the membrane fractions from the *POMT1*-transfected cells and *POMT2*-transfected cells. (**B**) α-Mannosidase digestion of mannosyl-GST-α-DG. Glutathione–sepharose 4B beads bearing [^{3}H]-mannosyl-GST-α-DG were incubated with jack bean-α-mannosidase for 60 h. The radioactivities of the supernatant (Sup) and the beads (Beads) were measured by liquid scintillation counting. Closed bars, active α-mannosidase; open bars, inactive (heat-treated) α-mannosidase. The radioactivity released to the supernatant by active α-mannosidase is shown. (Reprinted with permission from **ref. *12***. Copyright 2004 by National Academy of Sciences.)

fluorographic reagent for 30 min to enhance detection efficiency of tritium, dried with a vacuum gel dryer, and exposed to X-ray film (*see* **Fig. 2B**).

6. The linkage of the mannosyl residue to peptide is determined as follows. Instead of 2% SDS in **step 4**, 50 µL of jack bean-α-mannosidase (0.8 U) is added to the beads and incubated at 37°C. Jack bean-α-mannosidase (0.8 U) is added fresh every 24 h and is incubated for up to 60 h. Inactivated jack bean-α-mannosidase, prepared by heating the enzyme for 5 min at 100°C, is used as a control. After incubation, the radioactivity of the supernatant and the beads is measured using a liquid scintillation counter (*see* **Fig. 3B**).

3.4. POMGnT1 Assay

1. 10 µL of 1 m*M* UDP-GlcNAc, 10 µL of UDP-[^{3}H]GlcNAc (100,000 dpm/nmol), and 10 µL of 2 m*M* mannosylpeptide (or 100 m*M* Benzyl-Man) are mixed in a microcentrifugal tube and dried with a centrifugal evaporator.

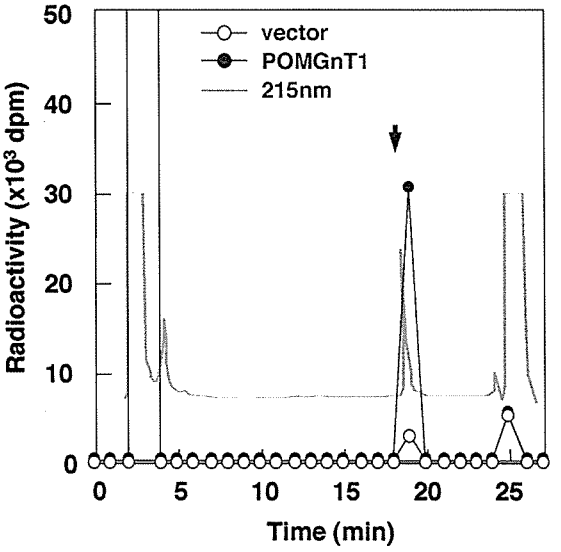

Fig. 4. POMGnT activity of human POMGnT1. UDP-[^{3}H]GlcNAc and mannosylpeptide were reacted with membrane fraction in POMGnT1 reaction buffer and then subjected to reversed-phase high-performance liquid chromatography. The mobile phase consists of (1) 100% A for 5 min, (2) a linear gradient to 75% A, 25% B for 20 min, (3) a linear gradient to 100% B for 1 min, and (4) 100% B for 5 min. The peptide separation was monitored at 214 nm and the radioactivity of each 1-mL fraction was measured by liquid scintillation counting. Arrow indicates the elution position of the mannosylpeptide. Vector (○), cells transfected with vector alone; POMGnT1 (●), cells transfected with human *POMGnT1*.

2. The POMGnT reaction buffer is added to the microsomal membrane fraction at a protein concentration of 2 mg/mL. The fraction is suspended with a bath-type sonicator on ice and solubilized by moderate pipetting until transparent. After centrifugation at 10,000*g* for 10 min, 20 μL of the supernatant is added to the dried substrate (prepared in **step 1**), vortexed gently, and incubated at 37°C for 2 h. The reaction is stopped by boiling at 100°C for 3 min. 180 μL of water is added to the reaction mixture and filtered with a centrifugal filter device.
3. The filtrate is analyzed by reversed-phase HPLC on the following condition: the gradient solvents are aqueous 0.1% TFA (solvent A) and acetonitrile containing 0.1% TFA (solvent B). The mobile phase consists of (1) 100% A for 5 min, (2) a linear gradient to 75% A, 25% B for 20 min, (3) a linear gradient to 100% B for 1 min, and (4) 100% B for 5 min. The peptide separation is monitored by measuring the absorbance at 214 nm, and the radioactivity of each 1-mL fraction is measured by liquid scintillation counting (*see* **Fig. 4**).
4. The reaction product is characterized by two different methods: (1) The product is dried up by an evaporator and then incubated with 50 mU of streptococcal