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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

平成１７－１９年度　総合研究報告書

主任研究者　萬谷　博

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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

主任研究者 萬谷　博、 (財)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　　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。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酵素であることを明らかにしている。そこで本研究では、α-ジストログリカノパチーの病態解明および診断・治療法への応用を目的として、原因遺伝子産物および *O*-マンノース型糖鎖の機能解明を目指した。平成 17 年度には *O*-マンノース転移酵素の活性発現機構の解析から、活性発現には小胞体で POMT1 と POMT2 の複合体形成が必要であることを明らかにした。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。平成 19 年度には、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を解析し、*O*-マンノシル化のコンセンサス配列を明らかにした。本研究により *O*-マンノース型糖鎖生合成における厳密な制御システムの存在が示された。本研究成果は今後のα-ジストログリカノパチー研究の基盤となりその進展に大きく寄与することが期待される。

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する。MEBとWWSの原因遺伝子産物POMGnT1とPOMT1およびPOMT2は*O*-マンノース型糖鎖合成酵素であり、FCMDの原因遺伝子産物fukutinの機能はまだ分かっていない。本研究では、これらの疾患の原因遺伝子産物および*O*-マンノース型糖鎖の機能を明らかにすることで、病態の解明から診断・治療法への応用を目的とした。

B. 研究方法

❶POMGnTおよびPOMT活性の臓器分布の解析：診断に適した組織・細胞を調べるため、ラット臓器およびヒト由来培養細胞株のPOMGnTおよびPOMT活性を測定した。❷*O*-マンノース型糖鎖のガラクトース転移に関わる酵素の検討：ヒトのガラクトース転移酵素β1,4GalTIーVIIのFLAG-tag融合蛋白質を作製し、*O*-マンノース型糖鎖の合成能を比較した。また、ヒトの各組織における各β1,4GalT mRNAの発現量を定量し比較した。❸fukutinの機能解析：fukutinの強制発現細胞およびFCMD変異型fukutinのノックインマウスについて、*O*-マンノース型糖鎖関連の酵素活性を調べた。❹POMTの機能解析：POMT1、POMT2の強制発現細胞を用いて、複合体形成と酵素活性の関係を調べた。❺MEBとWWSの診

断法の開発：EBウイルスで不死化した患者由来リンパ球を用いて酵素活性測定による診断の可能性を検討した。❻蛋白質*O*-マンノシル化機構の解析：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に基づいた合成ペプチド用いてPOMTに対する基質特異性を調べた。

（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から承認を受けた。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実験安全委員会から承認を受けた。診断法開発に用いた患者由来リンパ球細胞は、協力研究者のP.Guicheney博士（Inserm U582, Institut de Myologie, Paris）らにより細胞株化され、個人情報等もすべて管理されているため、当研究機関では倫理的問題は生じない

C.研究結果

❶我々の開発した高感度活性測定法により、生体組織における内在性POMGnT1とPOMT活性測定が可能になった。この方法により検体採取が簡便なリンパ球細胞で酵素活性を測定できることが明らかとなり、診断法開発の可能性を示した。❷*O*-マンノース型糖鎖合成に対するガラクトース転移活性はβ1,4GalT-IIが最も高かった。また、mRNAの発現量についてはβ1,4GalT-IIが脳で最も高いことが分かった。このことから、β1,4GalT-IIが脳における*O*-マンノース型糖鎖合成に関与することが示された。❸免疫沈降実験からfukutinとPOMGnT1は結合してゴルジ体に局在することが示された。また、FCMD変異型fukutinのノックインマウスではPOMGnT活性が減少しており、fukutinがPOMGnT活性に関与することが示唆された。❹共発現および免疫沈降実験からPOMT1とPOMT2の活性発現にはERにおけるPOMT1-POMT2複合体の形成が必要であることが明らかとなった。またWWS変異型POMT1は複合体形成には影響せず酵素活性のみを消失させることが分かった。❺遺伝子診断が確定したMEBとWWS患者由来細胞では、POMGnT1およびPOMT活性がそれぞれ消失していた。また、遺伝子診断前の患者由来細胞からもPOMGnT1およびPOMT活性の低下を示す例がみつかった。遺伝子解析の結果は活性測定の結果に一致したことから、酵素活性測定法がMEBとWWSの診断に有効であることを示している。❻α-ジストログリカン由来の2つのペプチドが効率良く*O*-マンノシル化された。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。アミノ酸置換の影響の解析から、両ペプチドで保存されているアミノ酸配列の重要性が示され、*O*-マンノシル化のコンセンサス配列である可能性が示された。

D.考察

❶高感度酵素活性測定法により内在性POMGnT1とPOMT活性測定が可能になった。両酵素活性の組織分布を示した世界最初の例である。また、本測定法による診断法の確立により、遺伝子診断の必要が減少し、診断時間の短縮、コスト削減が期待される。少量の血球系細胞を検体とすることにより患者さんの負担が軽減できる。原因遺伝子産物や関連酵素の機能を解明していくことで新たな診断・治療法開発への応用が期待できる。❷これまでに、ガラクトース転移酵素の異常による筋ジストロフィーは報告されていないが、*O*-マンノース型糖鎖合成にβ1,4GalT-IIの関与が示されたことは、今後、筋ジストロフィー症との関連を調べる必要性を示している。❸❹これまで全く分かっていなかったfukutinの機能について、POMGnT1の酵素活性や局在の制御に関与することが示され、また、WWSの原因遺伝子産物POMT1とPOMT2の酵素活性にはPOMT1-POMT2複合体の形成が必要で

あることが明らかとなった。これらの知見はO-マンノース型糖鎖の生合成が複数の分子複合体により厳密に制御されていることを示唆している。❺WWSおよびMEB患者由来細胞でPOMTおよびPOMGnT活性がほぼ完全に消失していたことから、ヒトのO-マンノース型糖鎖合成おいてはPOMT1-POMT2およびPOMGnT1が唯一の責任酵素であることが明らかとなった。❻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に繋がることが期待される。

E.結論

❶血球系のリンパ球から高感度に酵素活性を検出することを可能にした。検体採取時の患者の負担軽減を考慮する上で重要である。❷O-マンノース型糖鎖合成に関与するガラクトース転移酵素はβ1,4GalT-IIであることが示された。❸FCMD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することを明らかにした。❹WWSの原因遺伝子産物POMT1とPOMT2の酵素活性にはPOMT1-POMT2複合体の形成が必要であることを明らかにした。❺MEBとWWSの酵素活性測定法による診断の可能性を示した。❻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

I.論文発表

1. Fumiaki Saito, Martina Blank, Jörn Schröder, Hiroshi Manya, Teruo Shimizu, Kevin P. Campbell, Tamao Endo, Makoto Mizutani, Stephan Kröger, Kiichiro Matsumura: Aberrant glycosylation of α-dystroglycan causes defective binding of laminin in the muscle of chicken muscular dystrophy. *FEBS Lett.*, 579(11), 2359-2363, 2005

2. Norihiko Sasaki, Hiroshi Manya, Reiko Okubo, Kazuhiro Kobayashi, Hideki Ishida,Tatsushi Toda, Tamao Endo, Shoko Nishihara: β4GalT-II is a key regulator of glycosylation of the proteins involved in neuronal development. *Biochem. Biophys. Res. Commun.*, 333(1), 131-137, 2005

3. Tatsushi Toda, Tomohiro Chiyonobu, Hui Xiong, Masaji Tachikawa, Kazuhiro Kobayashi, Hiroshi Manya, Satoshi Takeda, M Taniguchi, H Kurahashi, Tamao Endo: Fukutin and α-dystroglycanopahties. *Acta Myol.*, 16(2), 60-63, 2005

4. 遠藤玉夫、萬谷博: 筋ジストロフィー.「糖鎖科学の新展開」NTS inc., 159-165, 2005

5. 萬谷博、遠藤玉夫: O-結合マンノースβ1,2-Nアセチルグルコサミン転移酵素（POMGnT1）. 生体の科学, 56(5), 380-381, 2005

6. Hiroshi Manya, Atsuro Chiba, Richard U. Margolis, Tamao Endo: Molecular cloning and characterization of rat Pomt1 and Pomt2. *Glycobiology*, 16(9), 863-873, 2006

7. Keiko Akasaka-Manya, Hiroshi Manya, Ai Nakajima, Masao Kawakita, Tamao Endo: Physical and functional association of human protein O-mannosyltransferases 1 and 2. *J. Biol. Chem.*, 281(28), 19339-19345, 2006

8. Hui Xiong, Kazuhiro Kobayashi, Masaji Tachikawa, Hiroshi Manya, Satoshi Takeda, Tomohiro Chiyonobu, Nobuhiro Fujikake, Fan Wang, Akemi Nishimoto, Glenn E. Morris, Yoshitaka Nagai, Motoi Kanagawa, Tamao Endo, Tatsushi Toda: Molecular interaction between fukutin and POMGnT1 in the glycosylation pathway of α-dystroglycan, *Biochem. Biophys. Res. Commun.*, 350(4), 935–941, 2006

9. Tamao Endo, Hiroshi Manya: *O*-Mannosylation in Mammalian Cells. Protocol for Methods in Molecular Biology (Ed. Brockhausen, I), *Humana Press*, New Jersey, 347, 43-57, 2006

10. Tamao Endo, Hiroshi Manya: Defect in glycosylation that causes muscular dystrophy. (Ed. Fukuda, M.), *Methods Enzymol*. Elsevier, San Diego, 417, 137-152, 2006

11. Hiroshi Manya, Takehiro Suzuki, Keiko Akasaka-Manya, Hide-Ki Ishida, Mamoru Mizuno, Yasushi Suzuki, Toshiyuki Inazu, Naoshi Dohmae, Tamao Endo: Regulation of mammalian protein *O*-mannosylation: Preferential amino acid sequence for *O*-mannose modification. *J. Biol. Chem.*, 282(28), 20200-20206, 2007

12. Hiroshi Manya, Céline Bouchet, Akiko Yanagisawa, Sandrine Vuillaumier-Barrot, Susana Quijano-Roy, Yasushi Suzuki, Svetlana Maugenre, Pascale Richard, Toshiyuki Inazu, Luciano Merlini, Norma B. Romero, France Leturcq, Isabelle Bezier, Haluk Topaloglu, Brigitte Estournet, Nathalie Seta, Tamao Endo, Pascale Guicheney: Protein *O*-mannosyltransferase activities in lymphoblasts from patients with α-dystroglycanopathies. *Neuromuscul. Disord.*, 18(1), 45-51, 2008

II.学会発表

1. 萬谷博、赤阪啓子、遠藤玉夫: 哺乳類 *O*-マンノース転移酵素 POMT1 と POMT2 の複合体形成. 日本薬学会第 126 年会, 仙台, 2006.3.28-30

2.赤阪—萬谷啓子、萬谷博、遠藤玉夫: 哺乳類 *O*-マンノース転移酵素 POMT1 と POMT2 の複合体形成による触媒活性の発現. 第 28 回日本分子生物学会年会, 福岡, 2005.12.7-10

3. Masaji Tachikawa, Hui Xiong, Kazuhiro Kobayashi, Hiroshi Manya, Tomohiro Chiyonobu, Yoshitaka Nagai, Tamao Endo, Tatsushi Toda: Fukutin Interacts with and Modulates POMGnT1. 第 78 回日本生化学会大会, 神戸, 2005, 10, 19-22

4. Hiroshi Manya, Keiko Akasaka-M, Tamao Endo: Determination of consensus sites for protein *O*-mannosylation. 第 78 回日本生化学会大会, 神戸, 2005, 10, 19-22

5. Tamao Endo, Shoko Nishihara, Tatsushi Toda, Hiroshi Manya: Glycosyltransferases responsible for congenital muscular dystrophies. 第 78 回日本生化学会大会, 神戸, 2005, 10, 19-22

6.Tamao Endo, Keiko Akasaka, Shoko Nishihara, Tatsushi Toda, Hiroshi Manya: Congenital muscular dystrophies due to glycosylation defect of protein *O*-mannosylation. XVIII International Symposium on Glycoconjugates. Florence, Italy. September 4-9, 2005

7. 萬谷博、赤阪啓子、遠藤玉夫: 先天性筋ジストロフィー原因遺伝子 *POMT1* の変異による *O*-マンノース型糖鎖生合成への影響. 第 25 回日本糖質学会年会, 大津, 2005. 7. 20-22

8. 遠藤玉夫、戸田年総、萬谷博、鈴木明身、佐藤雄治: 新規老化マーカー糖タンパク質の探索. 日本ヒトプロテオーム機構 (JHUPO) 第 3 回大会, 横浜, 2005.8.1-2

9. Tamao Endo, Shoko Nishihara, Hiroshi Manya: Initiation of protein *O*-mannosylation and congenital muscular dystrophy. Sixth-French-Japanese Workshop on muscular Dystrophy "Further progress toward therapy for muscular dystrophies". Paris, France, July 1-2, 2005

10. Hiroshi Manya, Tamao Endo: Defect of protein *O*-mannosylation in Walker-Warburg Syndrome. Glycoproteomics-protein modifications for versatile functions, Dubrovnik, Croatia, June 28 - 30, 2005

11. Shoko Nishihara, Yoshiko Ohmae, Tomomi Ichimiya, Hiroshi Manya, Hideki Yoshida, Hidenao Toyoda, Tamao Endo, Ryu Ueda: Functional analysis of *Drosophila* glycosyltransferases using RNAi mutant flies. The 2nd Japan-The Netherlands Glycobiology Symposium, April 18-20, 2005, Utrecht, The Netherlands

12. 千葉厚郎、萬谷博、Richard U. Margolis、遠藤玉夫: Protein *O*-mannosyltransferase (POMT)遺伝子の脳組織内発現の検討. 第 47 回日本神経学会総会, 東京, 2006.5.11-13

13. Tamao Endo, Atsuro Chiba, Richard U. Margolis, Hiroshi Manya: Molecular cloning and characterization of rat Pomt1 and Pomt2. 20th IUBMB International Congress of Biochemistry and Molecular Biology, Tokyo, 2006.6.18-23

14. Hiroshi Manya, Keiko Akasaka-Manya, Tamao Endo: A requirement of direct binding of human POMT1 and POMT2 for protein *O*-mannosyltransferase activity. 20th IUBMB International Congress of Biochemistry and Molecular Biology, Tokyo, 2006.6.18-23

15. Tamao Endo, Keiko Akasaka-Manya, Hiroshi Manya: Initiation of protein *O*-mannosylation and Walker-Warburg syndrome. 5th International Symposium on Glycosyltransferases (GlycoT 2006), Tuskuba, 2006.6.25-28

16. Hiroshi Manya, Keiko Akasaka-Manya, Tamao Endo: A requirement of direct binding of human POMT1 and POMT2 for protein *O*-mannosyltransferase activity. XXIIIrd International Carbohydrate Symposium, Whistler, Canada, 2006.7.23-28

17. 萬谷博、千葉厚郎、Richard U. Margolis、遠藤玉夫: ラット *O*-マンノース転移酵素（POMT）の遺伝子クローニングと各組織における酵素活性の分布. 第 26 回日本糖質学会年会, 仙台, 2006.8.23-25

18. Hiroshi Manya, Keiko Akasaka-Manya, Tamao Endo: A requirement of direct binding of human POMT1 and POMT2 for protein *O*-mannosyltransferase activity. 11th International Congress of the World Muscle Society, Bruges, Belgium, 2006.10.4-7

19. Céline Bouchet, Hiroshi Manya, Akiko Yanagisawa, Svetlana Maugenre, Susana Quijano-Roy, Brigitte Estournet, Luciano Merlini, Haluk Topaloglu, Norma Romero, France Leturcq, Nathalie Seta, Tamao Endo, Pascale Guicheney: Combined approaches to diagnosis congenital muscular dystrophies with α-dystroglycan hypoglycosylation. 11th International Congress of the World Muscle Society, Bruges, Belgium, 2006.10.4-7

20. Yasushi Suzuki, Hiroshi Manya, Tamao Endo, Toshiyuki Inazu: Synthesis and Evaluation of Mannosyl Peptides as an Acceptor for *N*-Acetylglucosaminyltransferase (POMGnT-1). International Conference of 43rd Japanese Peptide Symposium and 4th Peptide Engineering Meeting (43JPS/PEM4), Yokohama, 2006. 11. 5-8

21. Hiroshi Manya, Céline Bouchet, Pascale Guicheney, Tamao Endo: Combined approaches to diagnosis congenital muscular dystrophies with a-dystroglycan hypoglycosylation. Glycobiology and Sphingobiology 2007 (GS2007), Tokushima, 2007. 2.

27-3. 1

22. 萬谷博、赤阪（萬谷）啓子、櫻井洋子、遠藤玉夫: 家族性アルツハイマー病にみられるアミロイド前駆体タンパク質の糖鎖変化. 第 30 回日本基礎老化学会, 札幌, 2007.6.20-22

23. Hiroshi Manya, Takehiro Suzuki, Keiko Akasaka-Manya, Naoshi Dohmae, Tamao Endo: Preferential amino acid sequence for protein *O*-mannosylation. XIX International Symposium on Glycoconjugates (GLYCO-19), Cairns, Australia, 2007.7.15-20

24. 萬谷博、堂前直、稲津敏行、遠藤玉夫: 哺乳類 *O*-マンノース転移酵素の基質特異性の解析. 第 27 回日本糖質学会年会, 福岡, 2007.8.1-3

25. 遠藤玉夫、赤阪（萬谷）啓子、櫻井洋子、萬谷博: 家族性アルツハイマー病におけるアミロイド前駆体タンパク質の *N* 型糖鎖の変化. 第 30 回日本神経科学大会, 横浜, 2007.9.10-12

26. Hiroshi Manya, Takehiro Suzuki, Keiko Akasaka-Manya, Naoshi Dohmae, Tamao Endo: Biochemical approach for α-dystroglycanopathies: Identification of consensus sequence for protein *O*-mannosylation. 12th International Congress of the World Muscle Society, taormina, Italy, 2007.10.17-20

27. Akiko Yanagisawa, Céline Bouchet, Hiroshi Manya, Susana Quijano-Roy, Peter Y. K. Van den Bergh, Louis Viollet, Jean-Marie Cuisset, France Leturcq, Norma B. Romero, Michel Fardeau, Nathalie Seta, Tamao Endo, Pascale Guicheney: Novel *POMT2* mutations associated with a marked reduction of POMT activity causing congenital muscular dystrophy with mental retardation and microcephaly. 12th International Congress of the World Muscle Society, Messina, Italy, 2007.10.17-20

28. Yuko Miyagoe-Suzuki, K Miyamoto, Fumiaki Saito, Kiichiro Matsumura, Hiroshi Manya, Tamao Endo, Shinichi Takeda: *POMGnT1-null* myoblasts poorly proliferate *in vitro*. 12th International Congress of the World Muscle Society, Messina, Italy, 2007.10.17-20

29. Hiroshi Manya, Céline Bouchet, Pascale Guicheney, Tamao Endo: Combined approaches to diagnosis congenital muscular dystrophies with α-dystroglycan hypoglycosylation. Biochemistry and Molecular biology 2007 (BMB2007), Yokohama, 2007.12.11-15

30. Hiroshi Manya, Naoshi Dohmae, Keiko Akasaka, Toshiyuki Inazu, Tamao Endo: Identification of preferential sequence for protein *O*-mannosylation. Biochemistry and Molecular biology 2007 (BMB2007), Yokohama, 2007.12.11-15

31. 坂恵利子、石川文啓、秋山真一、萬谷博、遠藤玉夫、田丸浩: ゼブラフィッシュ遺伝子発現系を用いたヒト POMGnT1 の解析. Biochemistry and Molecular biology 2007 (BMB2007), Yokohama, 2007.12.11-15

32. 遠藤玉夫、萬谷博、赤阪啓子: 先天性筋ジストロフィーと糖鎖異常. Biochemistry and Molecular biology 2007 (BMB2007), Yokohama, 2007.12.11-15

33. 萬谷博、Pascale Guicheney、遠藤玉夫: 糖転移酵素反応を利用した α-ジストログリカノパチーの診断の試み. 日本薬学会第 128 年会, 横浜, 2008.3.26-28

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書籍名	出版社名	出版地	出版年	ページ
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,

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Saito, F., Blank, M., Schröder, J., Manya, H., Shimizu, T., Campbell, K. P., Endo, T., Mizutani, M., Kröger, S., Matsumura, K.	Aberrant glycosylation of α-dystroglycan causes defective binding of laminin in the muscle of chicken muscular dystrophy.	FEBS Letter	579 巻 11 号	2359-2363	2005
Toda, T., Chiyonobu, T., Xiong, H., Tachikawa, M., Kobayashi, K., Manya, H., Takeda, S., Taniguchi, M., Kurahashi, H., Endo, T.	Fukutin and α-dystroglycanopahties.	Acta Myologica	16 巻 2 号	60-63	2005
Xiong, H., Kobayashi, K., Tachikawa, M., Manya, H., Takeda, S., Chiyonobu, T., Fujikake, N., Wang, F., Nishimoto, A., Morris, GE., Nagai, Y., Kanagawa, M., Endo, T., Toda, T.	Molecular interaction between fukutin and POMGnT1 in the glycosylation pathway of α-dystroglycan.	Biochemical and Biophysical Research Communications	350 巻 4 号	935–941	2006
Akasaka-Manya, K., Manya, H., Nakajima, A., Kawakita, M., Endo, T.	Physical and functional association of human protein *O*-mannosyltransferases 1 and 2.	Journal of Biological Chemistry	281 巻 28 号	19339-19345	2006
Manya, H., Chiba, A., Margolis, R.U., Endo, T.	Molecular cloning and characterization of rat Pomt1 and Pomt2.	Glycobiology	16 巻 9 号	863-873	2006
Manya H, Suzuki T, Akasaka-Manya K, Ishida H, Mizuno M, Suzuki Y, Inazu T, Dohmae N, Endo T	Regulation of mammalian protein *O*-mannosylation: Preferential amino acid sequence for *O*-mannose modification	Journal of Biological Chemistry	282 巻 28 号	20200-20206	2007
Manya H, Bouchet C, Yanagisawa A, Vuillau mier-Barrot S, Quijano-Roy S, Suzuki Y, Maugenre S, Richard P, Inazu T, Merlini L, Romero NB, Leturcq F, Bezier I, Topaloglu H, Estournet B, Seta N, Endo T, Guicheney P	Protein *O*-mannosyltransferase activities in lymphoblasts from patients with α-dystroglycanopathies.	Neuromuscular Disorders	18 巻 1号	45-51	2008

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

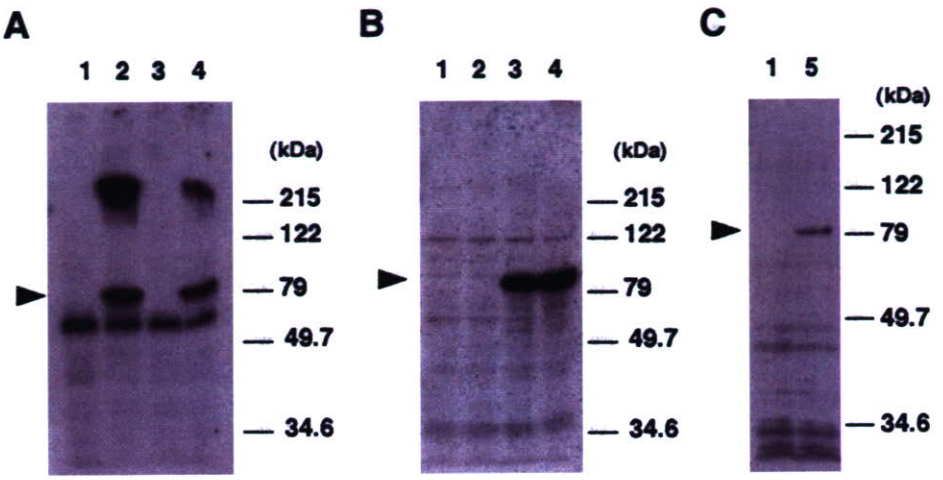

Fig. 1. Western blot analysis of (**A**) POMT1, (**B**) POMT2, and (**C**) POMGnT1 expressed in HEK293T cells. Lanes 1, cells transfected with vector alone; lanes 2, cells transfected with human *POMT1*; lanes 3, cells transfected with human *POMT2*; lanes 4, cells cotransfected with *POMT1* and *POMT2*; 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

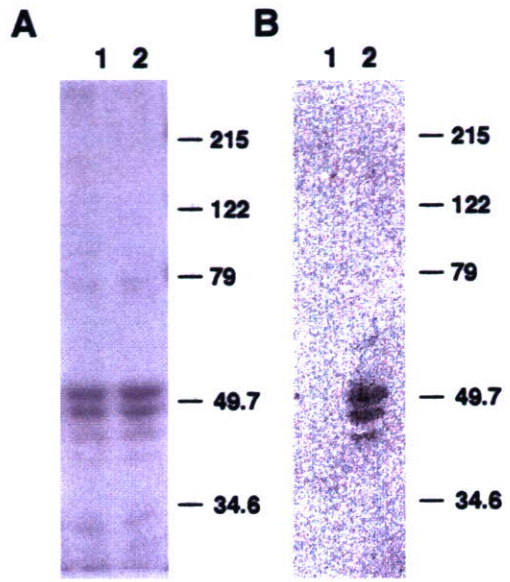

Fig. 2. Incorporation of [^{3}H]-mannose into glutathione-*S*-transferase-α-dystroglycan (GST-α-DG). Dol-P-[^{3}H]Man and GST-α-DG are incubated with HEK293T cell membrane fraction in POMT reaction buffer. After incubation, the products are recovered by the glutathione–sepharose 4B beads and subjected 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

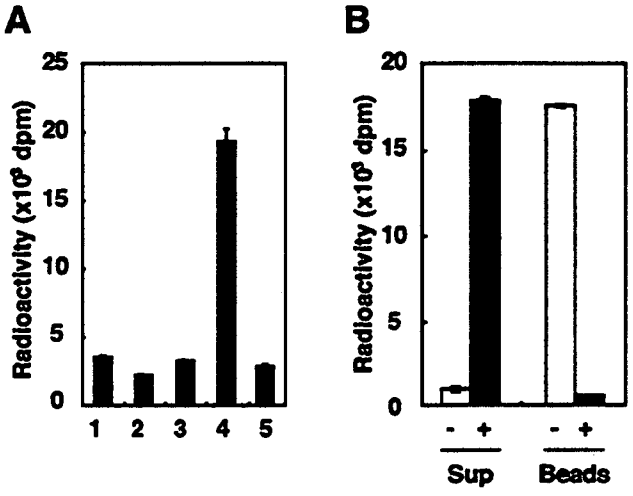

Fig. 3. (**A**) POMT activity of human POMT1 and POMT2 expressed in HEK293T cells. Bar 1, cells transfected with vector alone; bar 2, cells transfected with human *POMT1*; bar 3, cells transfected with human *POMT2*; bar 4, cells cotransfected with *POMT1* and *POMT2*; bar 5, a mixture of the membrane 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.