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Combined replacement effects of human modified β -hexosaminidase B and GM2 activator protein on GM2 gangliosidosis fibroblasts



Keisuke Kitakaze^a, Chikako Tasaki^a, Youichi Tajima^b, Takatsugu Hirokawa^c, Daisuke Tsuji^a, Hitoshi Sakuraba^d, Kohji Itoh^{a,*}

^a Department of Medicinal Biotechnology, Institute of Biomedical Sciences, Graduate School of Pharmaceutical Science, Tokushima University, 1-78-1, Tokushima 770-8505, Japan

^b Molecular Medical Research Project, Department of Genome Medicine, Tokyo Metropolitan Institute of Medical Science, 2-1-6 Kamikitazawa, Setagaya-ku, Tokyo 156-8506, Japan

^c Molecular Profiling Research Center for Drug Discovery (molprof), National Institute of Advanced Industrial Science and Technology (AIST), Tokyo 135-0064, Japan

^d Department of Clinical Genetics, Meiji Pharmaceutical University, Tokyo 204-8588, Japan

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ABSTRACT

GM2 gangliosidosis is an autosomal recessive lysosomal storage disease (LSD) caused by mutations in the *HEXA*, *HEXB* and *GM2A* genes, which encode the human lysosomal β -hexosaminidase (Hex) α - and β -subunits, and GM2 activator protein (GM2A), respectively. These diseases are associated with excessive accumulation of GM2 ganglioside (GM2) in the brains of patients with neurological symptoms. Here we established a CHO cell line overexpressing human GM2A, and purified GM2A from the conditioned medium, which was taken up by fibroblasts derived from a patient with GM2A deficiency, and had the therapeutic effects of reducing the GM2 accumulated in fibroblasts when added to the culture medium. We also demonstrated for the first time that recombinant GM2A could enhance the replacement effect of human modified HexB (modB) with GM2-degrading activity, which is composed of homodimeric altered β -subunits containing a partial amino acid sequence of the α -subunit, including the GSEP loop necessary for binding to GM2A, on reduction of the GM2 accumulated in fibroblasts derived from a patient with Tay-Sachs disease, a HexA ($\alpha\beta$ heterodimer) deficiency, caused by *HEXA* mutations. We predicted the same manner of binding of GM2A to the GSEP loop located in the modified HexB β -subunit to that in the native HexA α -subunit on the basis of the x-ray crystal structures. These findings suggest the effectiveness of combinational replacement therapy involving the human modified HexB and GM2A for GM2 gangliosidosis.

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1. Introduction

GM2 gangliosidosis, including Tay-Sachs disease (TSD, variant B), Sandhoff disease (SD, variant O), and GM2 ganglioside (GM2) activator protein (GM2A) deficiency (variant AB), are neurodegenerative lysosomal storage diseases (LSDs) associated with excessive accumulation of GM2 [1]. GM2A is known as a co-factor of β -hexosaminidase A (HexA, $\alpha\beta$ heterodimer) involved in the

degradation of GM2 [2–4]. Recently, intravenous enzyme replacement therapy involving recombinant human lysosomal enzymes produced by CHO and human HT1080 cell lines was clinically applied for several LSDs [5,6], so GM2A-based protein replacement therapy should be applicable to patients with variant AB. Previously, we developed a modified human HexB (modB) [7] composed of homodimeric altered β -subunits containing amino acid substitutions, the DL (β 452–453) to NR (α 423–424) sequences, which are necessary for anionic substrate recognition by the native α -subunit, and the RQNK (β 312–315) to GSEP (α 280–283) loop sequence, which is required for interaction with GM2A. We also revealed that the modB has GM2-degrading activity in fibroblasts derived from TSD and SD patients. Here, we first demonstrated that co-administration of recombinant GM2A could significantly enhance the reduction of intracellular GM2 accumulated in TSD fibroblasts mediated by modB in the culture system.

Abbreviations: CI-M6PR, cation-independent M6P receptor; CM, conditioned medium; ERT, enzyme replacement therapy; GM2, GM2 ganglioside; GM2A, GM2 activator protein; Hex, β -hexosaminidase; LAMP-1, lysosomal associated membrane protein 1; LSD, lysosomal storage disease; M6P, mannose-6-phosphate; modB, modified HexB; SD, Sandhoff disease; TSD, Tay-Sachs disease

* Corresponding author.

E-mail address: kitoh@tokushima-u.ac.jp (K. Itoh).

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2. Materials and methods

2.1. Antibodies

Anti-GM2 mouse monoclonal antibodies (GMB28: IgM) were established previously [8].

2.2. Cells

The CHO-K1 cell line was provided by the RIKEN BioResource Center (Ibaraki, Japan). The use of cultured skin fibroblasts from patients with TSD (F218) [7] and variant AB (F582) [9], and normal ones (F258) were approved by the ethics committee of our institution. The fibroblasts were cultured in nutrient mixture Ham's F-10 (Sigma-Aldrich, St. Louis, MO, USA) medium containing 10% (v/v) fetal bovine serum (FBS; Thermo Fisher Scientific, Waltham, MA, USA), 100 µg/mL streptomycin (Sigma-Aldrich), and 70 µg/mL penicillin G (Sigma-Aldrich) at 37 °C in a humidified incubator continuously flushed with a mixture of 5% CO₂–95% air.

2.3. Establishment of CHO cell lines expressing GM2A

A DNA fragment encoding GM2A with a histidine-tag (10xHis) and the signal sequence of human lysosomal α-galactosidase A (GLA) was ligated into the pCXN₂-Hygro and pCXN₂-Neo vectors [10]. Then each vector was used to transform MAX Efficiency DH5α Competent Cells (Life Technologies, Carlsbad, CA, USA). Plasmid DNA-Lipofectamine 2000 (Life technologies) complexes were transfected into CHO cells according to the manufacturer's instructions. Drug-resistant cell lines were established by double selection with hygromycin (Wako, Osaka, Japan) and G418 (Sigma-Aldrich). The CHO cell line stably expressing GM2A was cultured in EX-CELL ACF CHO medium (Sigma-Aldrich). The conditioned medium (CM) derived from each cell line was collected.

Immunoblotting for the expressed GM2A was performed with anti-hGM2A polyclonal antibodies (HPA008063, Sigma-Aldrich). Briefly, aliquots of GM2A fractions were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) on 12.5% (w/v) acrylamide gels. The proteins were transferred to Immobilon[®]-P PVDF membranes (Merck, Darmstadt, HE, Germany). After blocking with 50% (v/v) Blocking One (Nacalai Tesque, Kyoto, Japan) in TBS [25 mM Tris (Sigma-Aldrich), 137 mM NaCl, 2.7 mM KCl, pH 7.4] at room temperature (rt) for 1 h, each membrane was treated with anti-hGM2A antibodies diluted with Blocking One/TBS (1:1,000 dilution) overnight at 4 °C. After washing with TBS containing 0.1% (v/v) Tween 20 (Sigma-Aldrich), the membrane was treated with horseradish peroxidase (HRP)-linked anti-rabbit IgG antibodies (#7074, Cell Signaling Technology, Danvers, MA, USA, 1:1000 dilution) at rt for 1 h. After washing, detection of antibody binding was carried out with ECL (PerkinElmer, Waltham, MA, USA) according to the manufacturer's instructions. The protein levels were determined by the DC[™] Protein Assay or Bio-Rad Protein Assay (Bio-Rad, Hercules, CA, USA) with BSA (Sigma-Aldrich) as a standard.

For assaying of the presence of N-glycans attached to recombinant GM2A, CM containing GM2A was treated overnight with or without PNGase F (New England Biolabs, Hitchin, Herts, UK) according to the manufacturer's protocol, and then immunoblotting with anti-hGM2A antibodies was performed.

2.4. Purification of recombinant GM2A

For Ni-column chromatography, 5N NaOH (Sigma-Aldrich) was added to the CM to adjust the pH to 8.0–8.3, and then the CM was filtered with a Minisart[®] Syringe Filter 0.45 µm (Sartorius, Göttingen, NI, Germany). Then samples were applied to Ni Sepharose

6 Fast Flow (GE Healthcare, Little Chalfont, BKM, UK) equilibrated with 25 mM sodium phosphate buffer (NaPB), 0.3 M NaCl (pH 8.0). After washing with 25 mM NaPB, 0.3 M NaCl (pH 7.0 and 6.0), the bound proteins were eluted with 50 mM sodium acetate buffer, 0.3 M NaCl (pH 4.0). Each fraction was subjected to SDS-PAGE on a 12.5% (w/v) acrylamide gel and silver staining with a Dodeca Silver Stain Kit (Bio-Rad). The molecular weight of GM2A was calculated based on those of the APRO markers (APRO Science, Tokushima, Japan).

2.5. Preparation of modified HexB

CHO cell line stably expressing modified HexB [7] was cultured in EX-CELL ACF CHO medium and the CM was collected. Then samples were applied to a TOYOPEARL AF-Blue HC-650 column (Tosoh, Tokyo, Japan), and the bound proteins were eluted with 0.6 M NaCl (Nacalai tesque)/0.1 M Tris-acetate buffer (pH 7.5). The eluate was applied to Phos-tag agarose (Wako) and the bound proteins were eluted with 0.2 M NaPB (pH 6.0). The eluate was applied to a HiTrap SP HP column (GE Healthcare). The eluted fractions containing β-Hex activity were collected.

2.6. Replacement assay for GM2 gangliosidosis fibroblasts

Each type of fibroblast was seeded onto a collagen type I-coated 35 mm dish (AGC Techno Glass, Shizuoka, Japan). GM2A (5 µM) was added, followed by incubation for 4 d. The cells were dissociated and sonicated with phosphate-buffered saline [PBS; 8.1 mM Na₂HPO₄ (Sigma-Aldrich), 1.5 mM KH₂PO₄ (Wako), 137 mM NaCl, 2.7 mM KCl, pH 7.4] containing 1% (v/v) Nonidet P-40 and protease inhibitors [1 µM pepstatin A (Peptide Institute, Osaka, Japan), 20 µM leupeptin (Peptide Institute), 2 mM EDTA (Sigma-Aldrich) and 200 µM PMSF (Wako)]. Then each sample (9 µg protein/lane) was subjected to SDS-PAGE, and immunoblotting with anti-hGM2A (1:1000 dilution) and anti-β-actin antibodies (A5316, Sigma-Aldrich, 1:2000 dilution). The Image J software program (Ver.1.46) [11] was used to quantify the signal intensity.

For immunostaining, variant AB fibroblasts (F582) were seeded onto 8-well Lab-Tek chamber slides (Thermo Fisher Scientific) coated with 0.05% (w/v) atelocollagen (Koken, Tokyo, Japan). Then, GM2A (5 µM) was added, followed by incubation for 7 d. The fibroblasts were fixed with 4% (w/v) paraformaldehyde (PFA, Wako). After blocking with 5% (v/v) goat serum (Cedarlane Labs, Burlington, Ontario, Canada), 1% (w/v) BSA/PBS at rt for 2 h, the intracellular GM2 and LAMP-1 were detected with anti-GM2 (1:20 dilution) and anti-LAMP-1 antibodies (ab24170, Abcam, Cambridge, MA, USA, 1:200 dilution) overnight at 4 °C. After washing, FITC-mouse IgG+M (ab47830, Abcam) and Cy3-rabbit IgG (111-165-006, Jackson ImmunoResearch, West Grove, PA, USA) were treated at rt for 1 h. Nuclei were stained with Hoechst33258 (Sigma-Aldrich). Specimens were viewed under the LSM700 (Carl Zeiss, Oberkochen, BW, Germany).

For GM2-ELISA [12], TSD fibroblasts were seeded onto a collagen type I-coated 96-well plate (AGC Techno Glass, 5 × 10³ cells/well), followed by incubation for 3 d. Then GM2A (4 µg) was added to the culture medium, followed by incubation for 2 d. After a medium change, modified HexB [7] (4-methylumbelliferyl-6-sulfo-N-acetyl-β-D-glucosaminide-degrading activity, 3 µmol h⁻¹) was added, followed by incubation for 2 d. The cells were fixed with 4% (w/v) PFA/PBS overnight at 4 °C. After washing with PBS, the cells were treated with 0.6% (v/v) H₂O₂ (Sigma-Aldrich)/PBS for 30 min, and 5% (v/v) goat serum/1% (w/v) BSA/PBS at 37 °C for 2 h. Then the cells were immunostained with anti-GM2 antibodies (1:100 dilution) at rt for 1 h. After washing, the cells were treated with biotin-conjugated anti-mouse IgG, IgM antibodies (31,807, Thermo Fisher Scientific, 1:1000 dilution) at rt for 1 h. After washing, the cells were treated with HRP-conjugated

streptavidin (P0397, Dako, Glostrup, Hovedstaden, Denmark, 1:2000 dilution) at rt for 1 h. After washing, a peroxidase assay kit for ELISA (Sumitomo Bakelite, Tokyo, Japan) was used with a microtiter plate reader at 450 nm.

2.7. Computation modeling of modified HexB/GM2A complex

The proposed model of the modB/GM2A complex was constructed by means of the protein-protein docking and homology

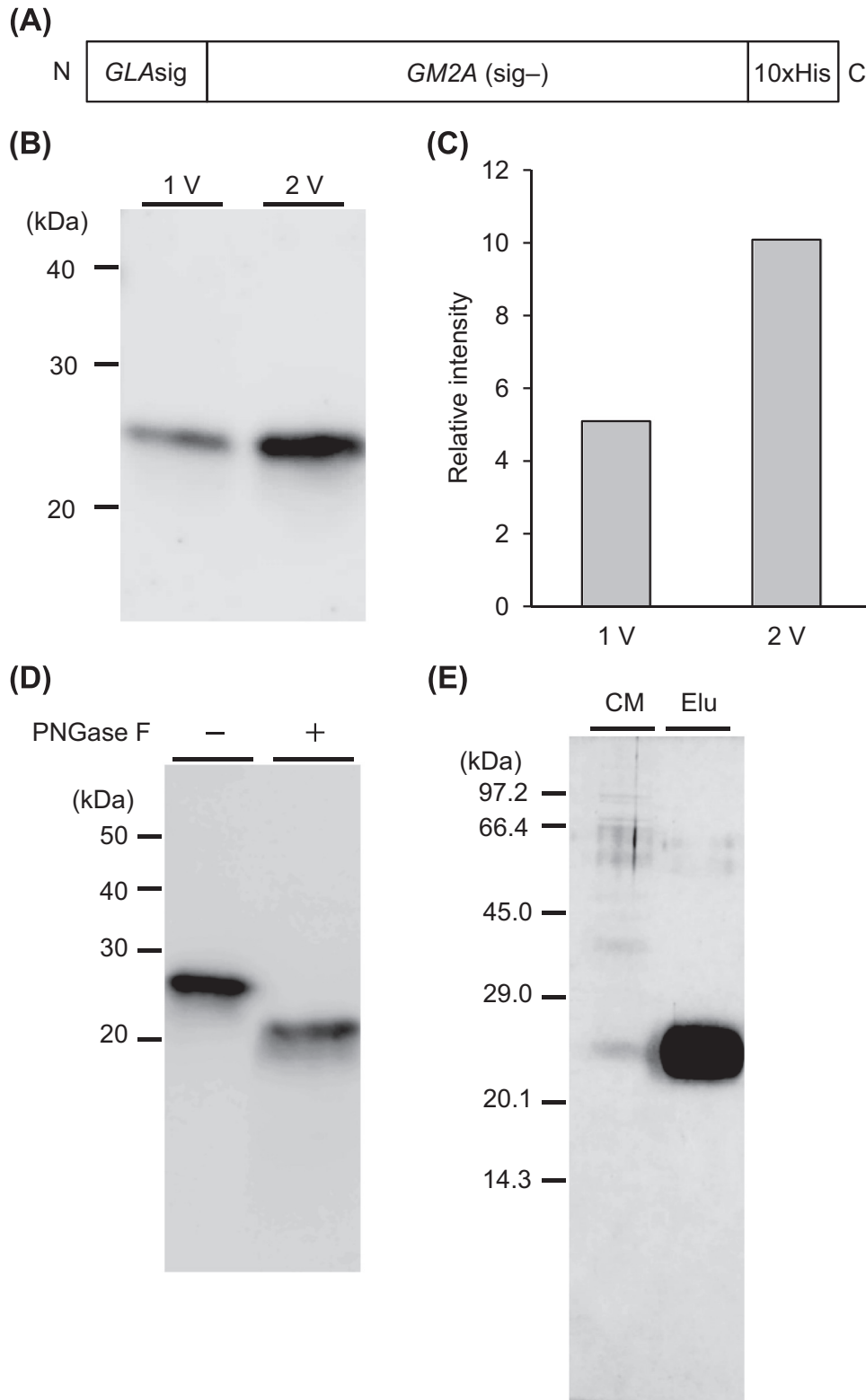


Fig. 1. Expression and purification of GM2A-His with CHO cell lines. (A) Schematic drawing of the GM2A construct. GLAsig: α -galactosidase A signal sequence, GM2A (sig-): GM2A without a signal sequence. (B) Immunoblotting of CM with anti-hGM2A antibodies. Each lane contained 20 μ L of CM. (C) GM2A-immunoreactivity indicated as relative signal intensity of GM2A. 1 V: one vector, 2 V: two vectors transfected. (D) Immunoblotting of N-glycosylated GM2A and the digested product with PNGase F. Each lane contained 50 μ L of CM. (E) Purification of GM2A by Ni-column chromatography. Each fraction was separated by SDS-PAGE, and then silver staining was performed. Each lane contained 5 μ g of protein. CM: conditioned medium. Elu: eluted fraction.

modeling. We first simulated protein-protein docking of the x-ray structure of GM2A (Protein Data Bank ID code: 1pub) with that of the HexA $\alpha\beta$ -heterodimer structure (PDB ID code: 2gjx, A &

B-chains). ZDOCK version 3.0.2 [13] was used to generate 2000 candidate conformations with the lowest docking energies. The best model was manually selected based on both the ZDOCK

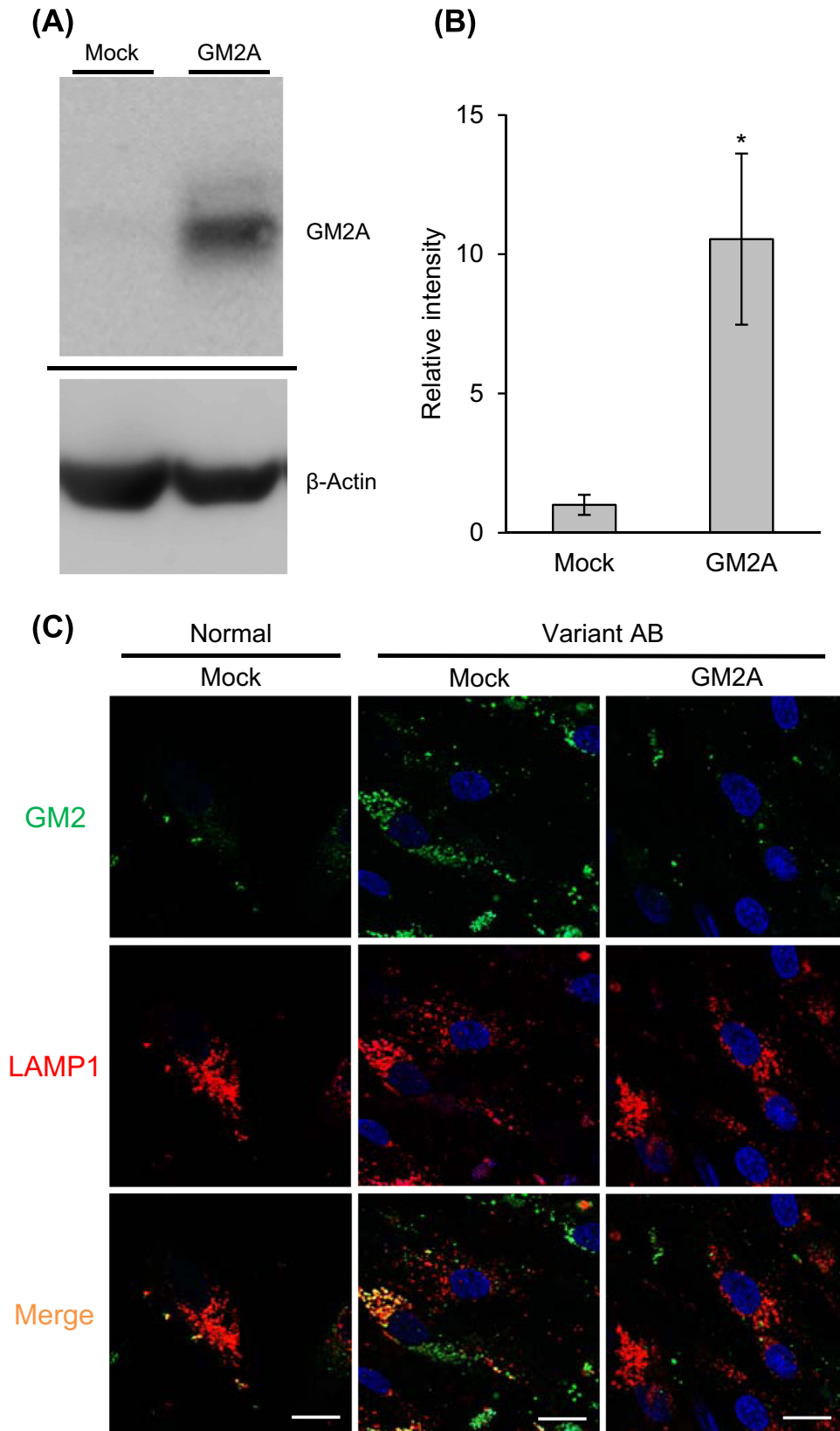


Fig. 2. GM2A replacement effect on variant AB fibroblasts. (A) Immunoblotting of cell extracts with anti-hGM2A antibodies after GM2A replacement. Each lane contained 9 μ g protein. (B) Relative signal intensities of GM2A. Error bars show means \pm SEM ($n=3$). Unpaired t -test, $*P < 0.05$. (C) Variant AB fibroblasts treated with GM2A were immunostained for GM2 (green) and LAMP-1 (red), and then examined by confocal laser scanning microscopy. Scale bars indicate 20 μ m.

score within the top 1%, and the molecular connectivity of GM2 between GM2A and the active site of the HexA α -subunit. The GM2 binding position was refined into the interface between GM2A and the HexA α -subunit with restricted docking to the reference position of the lipid molecule bound in GM2A using MOE (Chemical Computing Group, Montreal, Quebec, Canada). Finally, the HexA $\alpha\beta$ -heterodimer structure was replaced with modB including GM2A and GM2 molecules using the homolog modeling in MOE.

2.8. Statistics

Statistical analyses were performed using the SigmaPlot11 software program (Systat Software, San Jose, CA, USA), $P < 0.05$ being considered to be significant. For comparisons of three or more groups, we used one-way ANOVA with a Tukey *post-hoc* test. We used the 2-tailed unpaired *t*-test to compare two groups.

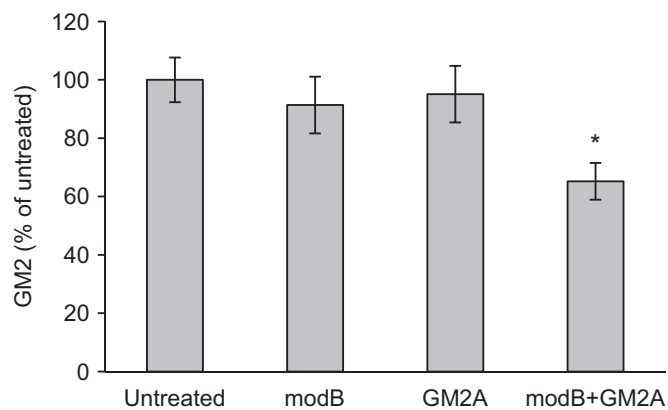


Fig. 3. Combined replacement effect of modified HexB and GM2A on TSD fibroblasts. Reduction of the GM2 accumulated in TSD fibroblasts was evaluated by GM2-ELISA after treatment with modB or GM2A. Error bars show means \pm SEM ($n=6-7$), ANOVA with a Tukey *post-hoc* test, * $P < 0.05$.

3. Results

3.1. Production of GM2A by CHO cell lines and its purification

We twice introduced the GM2A gene containing a 10xHis-tag and the GLA signal sequence by utilizing plasmid vectors containing different drug-resistance genes (Fig. 1(A)), and established CHO cell lines stably expressing GM2A by sequential selection with hygromycin and G418. Although the recombinant GM2A-His proteins were secreted from the CHO cells, marked increases in the GM2A level was observed on immunoblotting with anti-GM2A antibodies in serum-free CM derived from the CHO cell line repeatedly transfected with the GM2A expression vectors. The secreted GM2A-immunoreactivity in serum-free CM of the CHO cell line transfected with two different vectors (Fig. 1B, C, lane 2V) was about two times higher than that for the cell line transfected once (Fig. 1B, C, lane 1V). The expressed GM2A migrated to the 23 kDa position. After digestion with PNGase F, non-glycosylated GM2A gave a 20 kDa band, indicated the GM2A contains N-glycans (Fig. 1D). We purified the recombinant GM2A-His by Ni chromatography, and then subjected it to SDS-PAGE. We obtained 6.1 mg of purified GM2A-His per 1 L of CM (Fig. 1E).

3.2. GM2A replacement and GM2 reduction in patient fibroblasts

We evaluated the GM2A function in the culture system. Cultured fibroblasts derived from a variant AB patient were treated with recombinant GM2A, and then examined by immunoblotting with anti-GM2A antibodies and immunostaining with anti-GM2 antibodies. Significant restoration of the GM2A-immunoreactivity in a cell extract was observed after treatment with recombinant GM2A (Fig. 2A and B). We detected excessive accumulation of GM2 in lysosomes as punctate fluorescence, co-localized with LAMP-1, in untreated variant AB fibroblasts. After treatment with GM2A, the punctate fluorescence due to GM2 was markedly reduced (Fig. 2C).

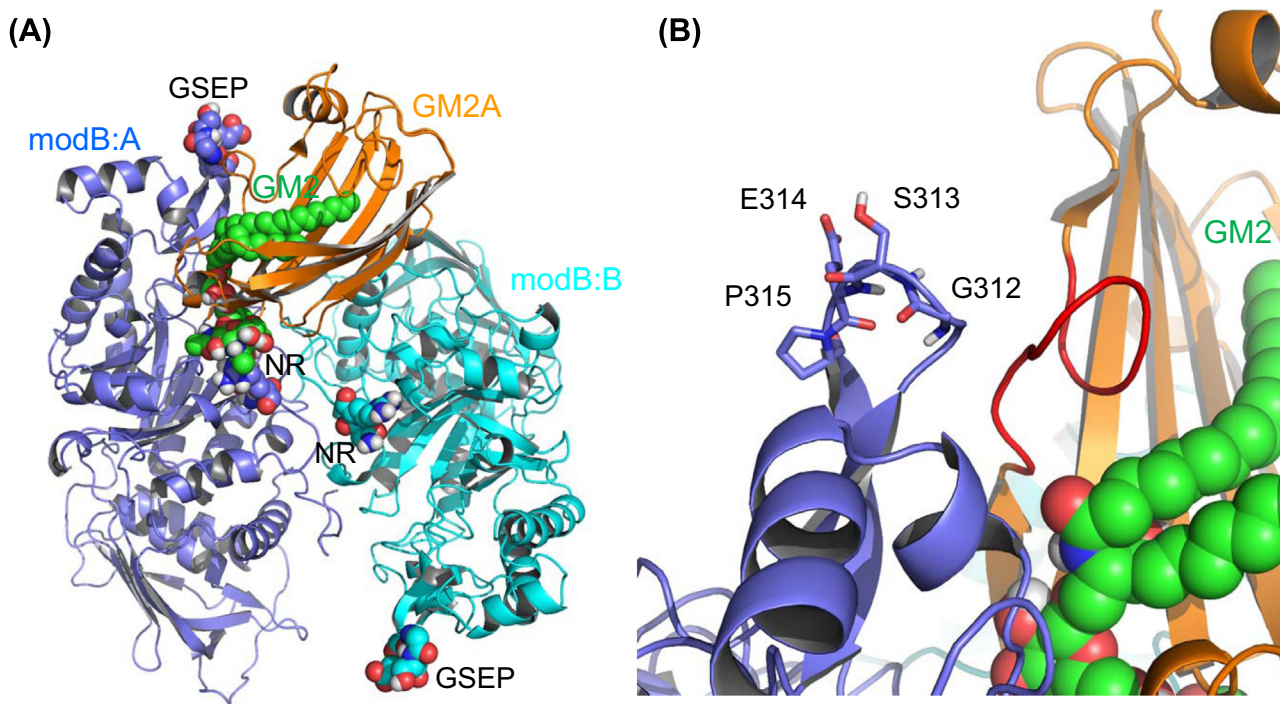


Fig. 4. Homology model of the modified HexB and GM2A complex. Proposed model for the modB/GM2A complex. (A) Overview of the model. Amino acid substitution regions (RQNK 312–315 GSEP and DL 452–453 NR) in modB are shown as sphere models with labels (GSEP and NR). (B) Close-up view of the substitution region of GSEP in the A chain of modB. The GSEP-interacting loop region (I66–C75) of GM2A is shown in red.

3.3. Combined replacement effects of modified HexB and GM2A on Tay-Sachs disease fibroblasts

Next, we evaluated the combinational replacement effect of modB and GM2A. TSD fibroblasts were treated with modB and GM2A, and then reduction of the GM2 accumulated in the cells after administration of the recombinant proteins was examined by GM2-ELISA. Accumulated GM2 was not significantly decreased by single replacement with modB or GM2A. In contrast, GM2 was significantly reduced by combinational replacement with modB and GM2A (Fig. 3).

3.4. In silico models of the modified HexB and GM2A complex

We constructed a complex model composed of modB and GM2A based on the crystal structures of human HexA (PDB ID: 2GJX) [14] and GM2A (PDB ID: 1PUB) [15] *in silico* (Fig. 4A and B), and predicted the quite similar manner of binding of the GSEP loop sequences located in the altered β -subunit of modB with the loop region (166–C75) of the mature GM2A compared to that in the Hex α -subunit [16].

4. Discussion

Recently, several therapeutic approaches have been developed for clinical application and trials for LSDs on the molecular bases of the enzymology and structural biology of lysosomal enzymes, including enzyme replacement therapy (ERT) [7,17], gene therapy [18–20], pharmacological chaperone therapy [21], and substrate reduction therapy [22,23]. However, these therapies are not clinically available for GM2 gangliosidoses (TSD, SD and variant AB) at present. In recent years, clinical trials of intrathecal and intracerebroventricular ERT as well as intracranial gene therapy for neurodegenerative LSDs, including mucopolysaccharidoses types I, II and IIIA, Batten disease and metachromatic leukodystrophy, have been performed in the U.S.A. and E.U. according to ClinicalTrials.gov (<https://clinicaltrials.gov/>, accessed March 3, 2016). These strategies are expected to be applicable to the GM2 gangliosidoses therapy.

GM2 is known to be physiologically degraded in lysosomes by HexA ($\alpha\beta$ heterodimer) in co-operation with GM2A [4]. Previously, we developed a genetically engineered human HexA carrying *N*-glycans with high contents of terminal mannose 6-phosphate (M6P) (*Om4HexA*) produced by an *MNN4*-introduced methylotrophic yeast *Ogataea minuta* strain [17]. We also designed a human modified HexB (modB), which contains substitutions of six amino acids in the α -subunit, based on homology modeling of human HexA ($\alpha\beta$ heterodimer, PDB ID: 2gix) and HexB ($\beta\beta$ homodimer, PDB ID: 1nou), and produced a recombinant modB with a CHO cell line, which can degrade GM2 through interaction with GM2A [7], for brain-directed ERT for TSD and SD. We also demonstrated that both *Om4HexA* and modB could reduce the GM2 accumulated in the brains of SD (*Hexb*^{-/-}) mice after intracerebroventricular administration.

In this study, we established a CHO cell line that stably expresses and secretes human GM2A (> 10 mg/L CM). The purified GM2A with terminal M6P residues was taken up mainly via CI-M6PR [24] by the fibroblasts derived from a variant AB patient (GM2A deficiency), and reduced the GM2 accumulated in lysosomes of the patient cells. However, in the presence of 5 mM M6P in the CM, not only was the intracellular GM2 reduction partly inhibited but also the GM2A protein level became lower (data not shown). Since there are two mechanisms involved in recapture of extracellular GM2A via CI-M6PR and sortilin receptor [25], the purified recombinant GM2A was considered to be incorporated by the variant AB fibroblasts via both receptors.

Although human GM2A is produced by *Escherichia coli* [26], methylotrophic yeast *Pichia pastoris* [27], insect cells [28], and the CHO cell line [9,25] and through chemical synthesis [29], as previously reported, the only form derived from the CHO cell line contains an *N*-glycan with M6P residues at the non-reducing termini [25]. So, we examined the combined replacement effect of recombinant modB and GM2A on TSD fibroblasts. It took about 7 days for the modB treatment to reduce the accumulated GM2 (data not shown). In contrast, combined replacement with modB and GM2A had a significant synergistic effect on the reduction of the GM2, it taking only 4 days.

We also predicted through *in silico* analysis the molecular interaction between the altered Hex β -subunit containing the substituted GSEP loop sequence and GM2A based on the structural information of the human HexA and GM2A complex model. Both of the GSEP loop structures in modB and HexA were suggested to be commonly recognized by the loop domain (Ile66–Cys75) of GM2A, and are responsible for the synergistic effect of co-administration of modB and GM2A on GM2 reduction in TSD fibroblasts. From these findings, we propose a novel combinational ERT involving co-administration of the modified HexB and GM2A for not only TSD and SD but also variant AB patients, although the latter GM2 gangliosidosis is known to be an exceptionally ultra-rare disease. Additional modifications of the Hex protein or GM2A to enhance the protein-protein interaction predicted on *in silico* analysis may be more effective for this combinational replacement therapy. Furthermore, the combination of GM2A protein replacement and modified *HEXB* single gene therapy may become an alternative therapy as a more effective therapy for GM2 gangliosidoses in the future.

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Appendix A. Transparency document

Transparency document associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.bbrep.2016.04.012>.

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