Published in final edited form as:
Biochem J. 2014 March 1; 458(2): 281–289. doi:10.1042/BJ20130845.

Insulin-like growth factor II peptide fusion enables uptake and lysosomal delivery of α-*N*-acetylglucosaminidase to mucopolysaccharidosis type IIIB fibroblasts

Shih-hsin Kan, **Larisa A. Troitskaya**, **Carolyn S. Sinow**, **Karyn Haitz**, **Amanda K. Todd**, **Ariana Di Stefano**, **Steven Q. Le**, **Patricia I. Dickson***, and **Brigette L. Tippin**
Department of Pediatrics, Los Angeles Biomedical Research Institute at Harbor-UCLA Medical Center, Torrance, CA, 90502 USA

Abstract

Enzyme replacement therapy for mucopolysaccharidosis type IIIB (MPS IIIB; Sanfilippo B syndrome) has been hindered by inadequate mannose 6-phosphorylation and cellular uptake of recombinantly produced human α-*N*-acetyl-glucosamindase (rhNAGLU). We expressed and characterized a modified, recombinant human NAGLU fused to the receptor binding motif of insulin-like growth factor-II (rhNAGLU-IGF-II) to enhance its ability to enter cells using the cation-independent mannose 6-phosphate receptor, which is also the receptor for IGF-II (at a different binding site). RhNAGLU-IGF-II was stably expressed in Chinese hamster ovary cells, secreted and purified to apparent homogeneity. The K_m and pH optimum of the fusion enzyme was similar to those reported for rhNAGLU. Both intracellular uptake and confocal microscopy suggested MPS IIIB fibroblasts readily take up the fusion enzyme via receptor-mediated endocytosis that was significantly inhibited ($p<0.001$) by monomeric IGF-II peptide. Glycosaminoglycan storage was reduced by 60% ($p<0.001$) to near background levels in MPS IIIB cells after treatment with rhNAGLU-IGF-II, with half-maximal correction at concentrations of 3–12 pM. Similar cellular uptake mechanism via the IGF-II receptor was also demonstrated in two different brain tumor-derived cell lines. Fusion of NAGLU to IGF-II enhanced its cellular uptake while maintaining enzymatic activity, supporting its potential as a therapeutic candidate for MPS IIIB.

To whom correspondence should be addressed: Patricia I. Dickson, Division of Medical Genetics, Department of Pediatrics, LA BioMed at Harbor-UCLA, 1124 W. Carson Street, Torrance, CA 90502, USA, Tel: 310-781-1399; Fax: 310-782-2999; pdickson@ucla.edu.

Treatment of mucopolysaccharidosis type IIIB is hindered by inadequate intracellular uptake of recombinantly-produced alpha-*N*-acetylglucosamindase (NAGLU). We generated an improved form of NAGLU by fusing it to insulin-like growth factor II, which is a natural ligand for the mannose 6-phosphate receptor.

Authors Contribution:
Brigette L. Tippin and Patricia I. Dickson developed the original concept and supervised the project. Shih-hsin Kan and Larisa A. Troitskaya conceived and designed the experiments. Shihhsin Kan, Carolyn S. Sinow, Karyn Haitz, Amanda K. Todd and Ariana Di Stefano made the construct and screened the expression clones. Shih-hsin Kan, Larisa A. Troitskaya, Carolyn S. Sinow and Steven Q. Le purified the protein and performed the biochemical assays. Shih-hsin Kan and Steven Q. Le performed and analyzed the in vitro studies. Shih-hsin Kan wrote the paper. Shih-hsin Kan, Brigette L. Tippin, Larisa A. Troitskaya, and Patricia I. Dickson proofread and edited the paper.

Keywords

Enzyme replacement therapy; lysosomal storage disease; Sanfilippo; mucopolysaccharidosis; insulin-like growth factor

Introduction

Sanfilippo syndrome type B (MIM 252920; mucopolysaccharidosis IIIB, MPS IIIB) is a rare autosomal recessive lysosomal storage disorder caused by the deficiency of α-N-acetylglucosaminidase (NAGLU; EC 3.2.1.50), an enzyme in the heparan sulfate degradation pathway. Cells lacking NAGLU accumulate heparan sulfate glycosaminoglycan (GAG) in their lysosomes, and the accumulation of undegraded GAG gives rise to adverse cellular and devastating clinical consequences. Individuals affected by this fatal disease exhibit severe central nervous system (CNS) degeneration with progressive cognitive impairment and aggressive behavioral problems, in addition to milder somatic symptoms [1]. There is currently no effective treatment for MPS IIIB.

Enzyme replacement therapy (ERT) is a therapeutic approach to lysosomal storage disorders in which the deficient enzyme is administered intravenously. ERT has been successfully applied to treat patients in the clinical setting for some storage disorders, including MPS I, II, and VI, Fabry, Pompe, and Gaucher diseases [2–7]. Enzyme can be administered into the cerebrospinal fluid to correct brain storage in animal models [8–13]. However, ERT for MPS IIIB was effective only in cells of the macrophage lineage in liver and spleen [14]. Uptake of exogenous ERT relies in most cases on binding of the mannose 6-phosphate residues on the enzyme to the 300 kDa, cationin-dependent mannose 6-phosphate receptor. Most cells, including neurons, depend on the M6P receptor system for uptake of exogenous lysosomal enzymes, whereas cells of the macrophage lineage use the mannose receptor. Previous attempts by several independent laboratories to generate recombinant NAGLU for ERT in multiple cell types were not successful, due in large part to inadequate mannose 6-phosphorylation of the enzyme and resultant poor intracellular uptake [15, 16]. Retroviral-mediated gene therapy and transduced hematopoietic stem cells have both shown improved pathology and lifespan in MPS IIIB mice [17–22]. However, cells transfected with vectors containing *NAGLU* cDNA are expected to secrete poorly phosphorylated enzyme, hampering their ability to cross-correct tissues away from the injection and implantation sites.

The M6P receptor also binds insulin-like growth factor-II and is henceforth referred to as the mannose 6-phosphate/insulin-like growth factor-II receptor (M6P/IGF-IIR) [23]. Here, we have employed a fusion protein strategy to enable recombinantly produced NAGLU to gain entry to cells through M6P/IGF-IIR which recognizes multiple ligands on its extracellular domain, including M6P-containing lysosomal enzymes, retinoic acid, and IGF-II via distinct binding sites on the receptor surface [24, 25]. Previous work by others showed that fusion proteins of lysosomal enzymes and IGF-II entered cells and lysosomes via the M6P/IGF-IIR on the cell membrane [26, 27]. We generated an expression construct of *NAGLU* and the receptor binding domain of *IGF-II* on the C-terminus to produce recombinant human

NAGLU-IGF-II (rhNAGLUIGF-II). In this study, rhNAGLU-IGF-II was expressed and purified from Chinese hamster ovary (CHO) cells for biochemical characterization and further tested for functional delivery to MPS IIIB cells and brain tumor-derived cell lines, and correction of GAG storage *in vitro*.

Experimental Procedures

Molecular cloning of *NAGLU-IGF-II*

An expression cassette containing the full-length cDNA coding for 743 amino acids of human *NAGLU* (NM_000263.3), a short unstructured linker and the c-myc epitope (EQKLISEED), followed by a portion of the *IGF-II* cDNA (NM_001007139.4) encoding amino acids 32–91 was synthesized using codon optimization for expression in CHO cells by Genscript USA Inc (Piscataway, NJ) and provided in the cloning vector pUC57. The DNA fragment was subcloned into pCI-neo (Promega Corporation, Madison, WI) at the *Xho*I and *Xba*I sites and transformed into XL1-Blue Supercompetent cells (Agilent Technologies, Santa Clara, CA) for amplification. The resulting expression vector was named pCI-NagGScIGF. Full length human *NAGLU* cDNA was provided in pCMV-NAGLU by Dr. E. F. Neufeld (University of California, Los Angeles, CA) and was subcloned into pCI-neo at the *Eco*RI and *Xba*I sites to form pCI-NAGLU without any other epitopes.

Cell culture and expression lines in Chinese Hamster Ovary cells

All chemical reagents were obtained from Sigma-Aldrich (St. Louis, MO), unless otherwise specified. CHO *dhfr*$^-$ cells were cultured in Ham's F12/DME (Irvine Scientific, Irvine, CA) supplemented with 10% fetal bovine serum (SAFC Biosciences, Lenexa, KS), 1 mM non-essential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, and antibiotics (100 units/ml penicillin G, 100 μg/ml streptomycin sulfate, Irvine Scientific, Irvine, CA) and 50 μg/ml gentamicin sulfate (EMD Chemicals Inc., Gibbstown, NJ) at 37°C in a 5% CO_2 air atmosphere. pCI-NagGScIGF (or pCINAGLU) was linearized with *Ahd* I (New England BioLabs, Ipswich, MA) and transfected into CHO *dhfr*$^-$ cells using PolyFect Transfection Reagent (Qiagen Inc., Valencia, CA). The stable lines were selected by their resistance to 700 μg/ml G-418 (EMD Chemicals Inc., Gibbstown, NJ), and colonies were formed after 7–14 days. Individual colonies were isolated and the highestyielding expressors of secreted rhNAGLU-IGF-II (5H10) or rhNAGLU (117-1.511) were identified by NAGLU activity assay. Stable CHO cell clones were maintained in Ham's F12/DME with supplements containing 250 μg/ml G-418.

For protein production, rhNAGLU-IGF-II clone 5H10 or rhNAGLU clone 117-1.511 were seeded into roller bottles and grown to confluence, at which time the medium was replaced with EX-CELL PF CHO serum-free medium supplemented with 4 mM L-glutamine, nucleosides (10 mg/l each of guanosine, adenosine, uridine, cytosine, hypoxanthine, and thymidine), 50 μg/ml gentamicin sulfate and 250 μg/ml G-418. Secreted NAGLU activity was monitored daily for 7–14 days until NAGLU expression reached a plateau, before the conditioned medium was harvested for enzyme purification.

Purification of modified recombinant NAGLU enzymes

rhNAGLU-IGF-II and rhNAGLU enzymes were purified from culture medium. Conditioned medium was filtered (0.2 μm), supplemented with methyl-α-D-glucopyranoside (10 mM) and stored at 4°C prior to purification (below).

Medium containing rhNAGLU-IGF-II was initially concentrated using an Amicon ultrafiltration concentrator with a YM30 membrane (EMD Millipore Corp., Billerica, MA) to its 20% volume, dialyzed against PBS, and loaded onto an 80 ml Concavalin A (Con A) Sepharose column (GE Healthcare Bio-Sciences Corp, Piscataway, NJ), pre-equilibrated with binding buffer (20 mM sodium phosphate pH 6.8; 300 mM NaCl; 10 mM methyl-α-D-glucopyranoside; 1 mM β- mercaptoethanol). The column was washed with one column volume of binding buffer followed by two column volumes of wash buffer (20 mM sodium phosphate pH 5.8; 10 mM methyl-α-D-glucopyranoside; 10 mM methyl α-D-mannopyranoside). RhNAGLU-IGF-II was eluted (20 mM sodium phosphate pH 5.8, 300 mM NaCl, 10 mM methyl-α-D-glucopyranoside, 500 mM methyl α-D-mannopyranoside) and collected in 15 ml fractions. NAGLU was identified in the fractions by enzymatic activity assay.

The fractions containing enzyme activity were pooled and concentrated to a final volume of 6 ml. The concentrate was mixed with 300 μl of c-myc affinity beads (50% slurry; Medical & Biological Laboratories, Nagoya, Japan) and incubated overnight at 4°C with gentle tumbling. The beads were washed with PBS (3 times, 6 ml each) and rhNAGLU-IGF-II was eluted by incubating the beads at 4°C with 1 ml of PBS containing 0.1 mM c-myc peptide in 3 rounds: 1 hour, 2 hours, and overnight. Purified rhNAGLU-IGF-II was sterile filtered (0.2 μm) and stored in aliquots at 4°C.

The purification of rhNAGLU was performed as described by Weber et al. [16] with some modifications. All column and reagents were from GE Healthcare Bio-Sciences Corp (Piscataway, NJ), unless specified otherwise. Production medium was concentrated, followed by buffer exchange (25 mM Tris-HCl, pH 8.4) using a PD10 column. The sample was then loaded on a 1 ml HiTrap Q Sepharose column and eluted with a NaCl step gradient (100-200-300-400-500 mM). Fractions with the highest NAGLU activity were pooled, buffer-exchanged (50 mM sodium acetate, pH 5.5; 50 mM NaCl), and loaded onto a 5 ml HiTrap Heparin HP column. A linear sodium chloride gradient (100–600 mM NaCl in 50 mM sodium acetate, pH 5.5) was applied for resin wash and protein elution. After concentrating, pooled fractions with the highest NAGLU activity were loaded onto a Sephacryl S-200 column (25×600 mm) and eluted with S buffer (25 mM sodium phosphate, pH 5.8; 200 mM NaCl). The purity of the enzyme was analyzed using SDS-PAGE stained with Coomassie Brilliant Blue R-250 (Bio-Rad Laboratories; Hercules, CA).

Activity assay and protein determination for rhNAGLU enzymes

Fluorometric measurements of NAGLU activity were performed essentially as described elsewhere with minor modifications [15, 28]. Briefly, medium or purified enzyme was incubated with 0.1 mM 4-methylumbelliferyl-2-acetamido-2-deoxy-α-D-glucopyranoside (4-MUNG; Toronto Research Chemicals Inc., North York, ON, Canada) in 50 μl of reaction

buffer (0.1 M sodium acetate, pH 4.3; 0.5 mg/ml BSA) at 37°C for 1 hour. Reactions were quenched by the addition of 1 ml of glycine carbonate buffer, pH 10.5. Fluorescence measurements were obtained using an RF-1501 spectrofluorophotometer (Shimadzu Scientific Instruments, Columbia, MD) at excitation and emission wavelengths of 360 nm and 450 nm, respectively. One activity unit equaled 1 nmol converted substrate per hour. Protein concentration was estimated using the Bradford method and bovine serum albumin was used as a standard (Bio-Rad Laboratories, Hercules, CA). Specific activity was defined as units of activity per mg of protein.

SDS-PAGE and Western blot

The purity of rhNAGLU-IGF-II was determined by 4–12 % Bis-Tris SDS-PAGE followed by staining with SYPRO Ruby (Life Technologies, Carlsbad, CA). The approximate molecular mass of the enzyme was estimated by comparison to Precision Plus Protein All Blue Standards (Bio-Rad Laboratories; Hercules, CA). Standard immunoblotting was performed with rabbit anti-NAGLU polycolonal antibody (1:2,500; a gift from Dr. E.F. Neufeld, UCLA), mouse anti-c-myc monoclonal antibody (1:2,500; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), or rabbit anti-human IGF-II antibody (1:1,250; Abcam, Boston, MA), diluted in 5% nonfat milk and incubated for 1–2 hours at room temperature. HRP conjugated secondary antibodies, goat anti-rabbit IgG or goat anti-mouse IgG (Southern Biotechnology, Birmingham, AL) was diluted at 1:5,000 in 5% nonfat milk, incubated for 1 hour, and detected with SuperSignal West Dura Substrate (Thermo Scientific, Waltham, MA).

Glycosylation analysis

Deglycosylation of the enzymes was performed by first denaturing in 0.5% SDS/40 mM dithiothreitol for 10 minutes at 100°C. Denatured rhNAGLU-IGF-II, 50 units, was incubated for 1 hour at 37°C with either 200 units of endoglycosidase H_f (Endo H_f; New England Biolabs; Ipswich, MA) in 50 mM sodium citrate (pH 5.5), or 2 units of glycopeptidase F (PNGase F; Sigma-Aldrich, St. Louis, MO) in 50 mM sodium phosphate (pH 7.5) plus 1% nonidet P-40. Recombinant α-L-iduronidase (Biomarin Pharmaceutical, Novato, CA) was used as an internal control for Endo H_f and PNGase F digestion (not shown). Digested products were resolved with SDS-PAGE as described above.

Substrate kinetics

K_m was determined at standard reaction conditions (50 μl reaction volume, pH 4.2, 1 hour incubation at 37°C) with 1.6 units (12.6 ng) of rhNAGLU-IGF-II. 4-MUNG substrate concentration ranging from 0.5 μM to 1 mM. Triplicate reactions were performed at each substrate concentration. Data were plotted as substrate concentration versus the average value for product formed and were best fit to a rectangular hyperbola using Sigma Plot 10.0 (Systat Software Inc, Chicago, IL) and analyzed by non-linear regression. Time course experiments were performed as described above except using a fixed 0.8 mM 4-MUNG substrate (8 times the K_m) concentration and varied incubations times (1 min to 6 hours). To assess the optimal pH range for the enzymatic activity of rhNAGLU-IGF-II, assays were

performed with 1.6 units of enzyme in 50 μl of 200 mM/100 mM sodium phosphate-citrate buffers over the pH range of 2.6–7.9 with 0.1 mM 4-MUNG at 37°C for 1 hour.

Uptake kinetics

Human MPS IIIB skin fibroblasts (GM1426; Coriell Institute Camden, NJ) were seeded in 6-well plates in 2 ml of Eagle's Minimum Essential Medium (EMEM) with supplements and antibiotics as described above at the concentration of 3.33×10^5 cells/well. After incubation for 24 hours or when confluence was reached, the medium was removed, and cells were incubated with 1 ml of EMEM without serum containing increasing amounts (10 to 320 units/ml) of purified rhNAGLU-IGF-II or rhNAGLU. Following incubation for 4 hours at 37°C and 5% CO_2, cells were harvested by trypsinization, and cell pellets were washed in PBS and resuspended in 60 μl PAD buffer (10 mM sodium phosphate, pH 5.8, 0.02 % sodium azide, 0.1 mM dithiothreitol, 0.1% Triton X-100). Cell lysates were then sonicated for 20 seconds and centrifuged at 13,000 rpm for 15 min. 25 μl of each supernatant was used to measure NAGLU activity as described above. Data were analyzed in Sigma Plot 12.0 using non-linear regression.

Uptake inhibition assay

Uptake inhibition assays were performed in MPS IIIB fibroblasts and two brain tumor-derived cell lines, Dao Y and Es (cerebellar medulloblastoma and glioblastoma, respectively, kindly provided by Dr. J Lasky, Los Angeles Biomedical Research Institute at Harbor-UCLA, Torrance, CA). Cells were seeded in 12-well plates and grown to confluence. Culture medium was replaced with 0.5 ml of EMEM without serum prior to the addition of uptake inhibitors. 5 μg/ml recombinant human IGF-II (R&D Systems; Minneapolis, MN) or 5 mM D-mannose 6-phosphate (Sigma-Aldrich; St. Louis, MO) was applied to the cells for 10 minutes prior to applying purified rhNAGLU-IGF-II at a final concentration of 160 units/ml. Following 4 hours incubation at 37°C and 5% CO_2, cells were harvested and intracellular NAGLU activity was measured as described above. The enzyme activity measured in MPS IIIB fibroblasts with rhNAGLU-IGF-II treatment alone was defined as 100% and the intracellular enzyme activity observed in the presence of inhibitor(s) was normalized to rhNAGLU-IGF-II activity. The endogenous intracellular enzyme activity measured in brain tumor-derived cell lines was defined as 100% and the enzyme uptake with or without inhibitor was normalized to this value.

GAG storage reduction

GAG in cultured fibroblasts were labeled with $H_2\,{}^{35}SO_4$ as described elsewhere [29, 30]. Briefly, MPS IIIB fibroblasts were grown in six-well plates until they reached confluence. The medium was removed and replaced with 1.5 ml serum-free EMEM supplemented with 1 mM pyruvate, 1 mM non-essential amino acids, and 25 μCi/ml $H_2\,{}^{35}SO_4$ (Perkin-Elmer, Waltham, MA). Purified rhNAGLU-IGF-II was applied to the cells at different concentrations (0, 0.05, 0.1, 0.5 and 1 units per ml), and cells were labeled for 72 hours at 37°C and 5% CO_2. The medium was removed and cells were rinsed with PBS twice before harvesting by trypsinization and centrifugation. GAG extraction was performed twice from the cell pellets by boiling briefly in 80% ethanol and centrifugation in a clinical centrifuge (15 min, at the highest speed). Pellets were resuspended in 10% sodium hydroxide and

neutralized with 2 M acetic acid, and radiolabeled GAG was measured via scintilliation counting (Tri-Carb 2800TR, Perkin-Elmer, Waltham, MA). Radioactive counts per minute were normalized to protein concentrations as determined using a Bio-Rad protein assay described above. Data were plotted as enzyme applied in the reaction versus the average ^{35}S cpm per mg protein detected and were best fit to an exponential decay using Sigma Plot. Half-maximal concentration for correction was calculated using the pharmacokinetics module (IC_{50}) in GraphPad Prism (GraphPad Software Inc., La Jolla, CA).

Confocal microscopy

For qualitative determination of enzyme uptake by confocal microscopy, MPS IIIB fibroblasts were grown on Millicell EZ slides (EMD Millipore Corp., Billerica, MA) in 200 μl EMEM. Cells were treated with 160 units/ml purified NAGLU-IGF-II with or without inhibitor (5 μg/ ml rhIGF-II peptide or 5 mM M6P) for 4 hours at 37°C and 5% CO_2. To determine whether purified enzyme could be delivered to lysosomes, 0.5 mg/mL dextran-Texas red (Life Technologies, Carlsbad, CA) was incubated with cells in the presence of 160 units/ml purified NAGLU-IGF-II for 4 h [31]. Cells were washed 3 times with PBS, fixed (4% paraformaldehyde in PBS), permeabilized (0.2% Triton X 100 in PBS), and blocked (1 hour, 10% goat serum in PBS). Rabbit polyclonal antibody against NAGLU (1:2,500) was applied and incubated overnight at 4°C. Cells were rinsed in PBS and incubated for 1 hour at room temperature with Alexa Fluor 488-conjugated goat anti-rabbit IgG at 1:2,500 (Life Technologies, Carlsbad, CA). After rinsing in PBS, cells were mounted onto glass microscope slides using Vectashield with DAPI (Vector Laboratories, Burlingame, CA). Uptake images of anti-NAGLU were captured using a Leica TCS SP2 or SP8 confocal microscope (Leica Microsystems, Bannockburn, IL).

Statistics

For each sample assayed biochemically, the mean of triplicate experiments was calculated along with standard deviations for each experimental point. Treatment groups were compared using ANOVA with post-hoc Tukey-Kramer test (SYSTAT13, Systat Corp., Chicago, IL).

Results

Characterization of NAGLU-IGF-II expressed in a CHO cell line

Purified rhNAGLU-IGF-II had a specific activity of 127,000 units/mg with 19.4% final recovery after the purification process (Table 1). The secreted full-length fusion protein was engineered to be 832 amino acids in length, with a predicted molecular weight of 91.6 kDa and theoretical pI of 5.96. However, the enzyme migrated at an apparent molecular weight of 105 kDa on SDS-PAGE (Fig 1A), likely due to N-glycosylation at one or more of the six potential N-glycosylation sites present on the NAGLU protein [32]. Western blotting with anti-NAGLU, anti-c-myc, and anti-IGF-II antibodies verified the presence of all recombinantly engineered peptide domains in the final, full-length purified product (Fig 1B). To verify the glycosylation pattern of rhNAGLU-IGF-II, we treated it with the enzymes PNGase F or Endo H_f to remove attached carbohydrate molecules (Fig 1C). PNGase F hydrolyzes almost all types of N-linked oligosaccharides, whereas Endo H_f cleaves high-

mannose N-linked oligosaccharides within the chitobiose core. The rhNAGLU-IGF-II was resistant to digestion with Endo H_f but sensitive to PNGase F, suggesting that the protein was N-glycosylated but lacked high-mannose residues.

We next verified that addition of the linker and IGF-II sequences did not interfere with normal enzymatic function of the NAGLU active site. We evaluated rhNAGLU-IGF-II for its ability to cleave the fluorogenic substrate analog 4-MUNG (Fig. 2A) as a function of increasing substrate concentration and fit the data to the hyperbolic Michaelis-Menten kinetic equation. The 4-MUNG substrate was utilized with a K_m value of 0.2 mM. The K_m value towards 4-MUNG substrate we obtained was comparable to previous reports for wild-type rhNAGLU produced in CHO cells that ranged from 0.22 – 5.34 mM [15, 16]. When rhNAGLU-IGF-II was provided an excess amount of 4-MUNG substrate (0.8 mM), the reaction followed first order kinetics, in which the cleavage product forms linearly with increased time (Fig. 2B).

The optimal pH for maximal lysosomal hydrolase activity is typically observed in the acidic range. We measured rhNAGLU-IGF-II activity towards 4-MUNG across a broad range of pH optimum (from pH 3.8 to 5.0) and found that it maintained a strong bias towards acidic environments (e.g. lysosomal pH). This is comparable to the pH optimum range reported previously for native endogenous human NAGLU (pH 4.1–4.5, [33–35]) as well as recombinantly produced wild-type NAGLU (pH 4.1–4.6, [15, 16]). However, at neutral pH (e.g. extracellular pH), very little enzymatic activity was detected.

rhNAGLU-IGF-II uptake into MPS IIIB fibroblasts

We cultured human MPS IIIB fibroblasts in medium containing various concentrations of purified rhNAGLU-IGF-II, to determine whether the IGF-II peptide improved NAGLU uptake into cells. The intracellular enzyme activity of rhNAGLU-IGF-II increased in a dose-dependent fashion with an apparent concentration for half-maximal uptake of 30 nM. For comparison, we also treated MPS IIIB cells with purified wild-type human rhNAGLU that had been overexpressed in CHO cells. Uptake of rhNAGLU lacking the IGF-II fusion was barely detectable, and the calculated concentration for half-maximal uptake of rhNAGLU was approximately 200 nM (Fig. 3).

rhNAGLU-IGF-II uptake via the M6P/IGF-II receptor

We performed an uptake inhibition assay to determine the extent to which rhNAGLU-IGF-II entered cells using the M6P/IGF-II receptor (Fig. 4A). After incubating cells with rhNAGLU-IGF-II for 4 hours, substantially greater intracellular NAGLU activity was detected compared to cells incubated with rhNAGLU or untreated. To elucidate the entry mechanism, we treated cells with enzyme in the presence of potential inhibitors to the distinct ligand binding domains of the M6P/IGF-IIR and measured intracellular rhNAGLU-IGF-II activity. The addition of IGF-II peptide (5 μg/ml) led to substantial (~80%; $p<0.001$) uptake inhibition of rhNAGLU-IGF-II. A partially inhibitory effect was also observed in the presence of M6P (5 mM). Both M6P and IGFII- containing peptides have been shown to bind simultaneously to the M6P/IGF-IIR, although binding of one ligand may effectively reduce affinity for the other via steric inhibition [25]. We also observed that the combined

presence of IGF-II peptide and M6P led to very little rhNAGLU-IGF-II uptake into MPS IIIB fibroblasts. We also performed double-labeling confocal microscopic imaging to investigate the delivery of rhNAGLU-IGF-II into lysosomal compartments with or without uptake inhibitor in cultured MPS IIIB fibroblasts. MPS IIIB fibroblasts treated with rhNAGLU-IGF-II exhibited punctuate fluorescent signals distributed in the cytosol. Intracellular NAGLU signal was significantly decreased at the presence of 5 μg/ml rhIGF-II peptide, but was not affected by the presence of 5 mM M6P (Fig. 4B).

Lysosomal localization and reduction of GAG

In order to identify whether rhNAGLU-IGF-II reaches lysosomes, we co-incubated the enzyme and dextran-Texas red (for lysosomal labeling) with MPS IIIB fibroblasts for 4 h [31]. We observed partial colocalization of rhNAGLU-IGF-II with dextran-Texas red (Fig. 5).

We next tested whether delivery of rhNAGLU-IGF-II to cells restored their impaired GAG degradation (Fig. 6). Untreated MPS IIIB fibroblasts accumulated consistently higher levels of ^{35}S-labeled GAGs than fibroblasts treated with rhNAGLU-IGF-II over a 72-hour period. The concentration required for half-maximal correction was 0.04–0.14 units/ml, or approximately 3–12 pM.

rhNAGLU-IGF-II uptake into brain tumor-derived cell lines

We further performed uptake and inhibition assays on two brain tumor-derived cell lines to determine whether rhNAGLU-IGF-II can also enter brain cells through the M6P/IGF-II receptor (Fig. 7). After incubating cells with rhNAGLU-IGF-II for 4 hours, substantially higher intracellular NAGLU activity was detected compared to their endogenous levels ($p<0.05$). In the presence of 5 μg/ml rhIGF-II peptide, intracellular NAGLU activity decreased to endogenous levels.

Discussion

The potential of recombinant NAGLU as ERT for MPS IIIB patients is limited by inadequate cellular delivery. The limitation is rooted in the inability to generate rhNAGLU that acquires M6P during post-translational processing in CHO cells or a human cell line [15, 16]. In this study, we improved the uptake of exogenously applied rhNAGLU into human cells by introducing an IGF-II binding site at the carboxyl-terminus of the recombinant enzyme. Purified rhNAGLU-IGF-II produced in CHO cells entered human cell lines via the IGF-II binding site on the M6P/IGF-IIR in a manner which is independent of the post-translational modification of the enzyme by mannose 6-phosphorylation. Purified rhNAGLU-IGF-II exhibited *in vitro* biochemical properties similar to that reported for rhNAGLU, which suggests that the additional C-terminal motif did not substantially change the inherent properties of the enzyme. In addition, the enzyme retained full activity when stored as harvested conditioned PF CHO medium (pH 7.2) in the presence of methyl-α-D-glucopyranoside, for at least 8 months. The concentration for halfmaximal uptake was approximately 30 nM, and the concentration for half-maximal correction was three orders of magnitude lower at 3–12 pM. A substantially lower enzyme requirement for correction of

lysosomal GAG accumulation than for uptake has also been observed with another α-L-hydrolase (iduronidase) that degrades GAG [30]. Intracellular GAG accumulation did not reach zero in the assay. One possible explanation for this phenomenon is that the in the presence of artificially high NAGLU concentrations within treated cells, other enzymes become rate-limiting for GAG degradation. This may also explain why the half-maximal concentration for correction is much lower than that for uptake. Experiments in two brain tumor-derived cell lines also showed that rhNAGLU-IGF-II used the IGF-II receptor for uptake. Uptake into brain tumor-derived cell lines is encouraging for future applications of rhNAGLU-IGF-II to treat CNS disease.

Within the CNS, the M6P/IGF-IIR is found in the brain and spinal cord, including the deep cortical layers, pyramidal and granule cell layers of the hippocampus, cerebellar Purkinje cells, and motor neurons of the brainstem and spinal cord [25]. Although ERT is typically administered intravenously, direct delivery of enzyme to the brain is possible with intrathecal or intraventricular injections [8–13, 36–41]. Fusion with the IGF-II domain may also improve the uptake of enzymes which are adequately mannose 6-phosphorylated when produced in recombinant form. An IGF-II directed targeting strategy was used previously with recombinant human β-glucuronidase (hGUS) and tested in a mouse model of MPS VII [27]. Intravenous hGUS-IGF-II targeted a broad range of tissues and improved reduction in GAG storage versus wild-type GUS. Currently, a Phase I clinical trial is underway to study recombinant acid α-glucosidase fused to the IGF-II domain for the treatment of Pompe disease (NCT01230801).

IGF-II is a mitogenic peptide that plays a vital role in development and growth [42]. It exerts a hormonal activation cascade through its interaction with the type I IGF receptor. IGF-II also binds with almost equal affinity to the M6P/IGF-IIR that transports it to the lysosome for degradation, thereby ending the growth potentiation signal [43, 44]. Our study did not address the potential interaction of NAGLU-IGF-II with the IGF-I receptor. However, the IGF-II peptide used in this study has an N-terminal deletion, which has been shown to abolish its relative affinity to human IGF-I receptor [26]. This design was adopted to possibly prevent unwanted adverse effects of stimulating the IGF-I receptor while specifically targeting the lysosomal pathway. Other safety concerns, such as immunogenicity, are also possible and need to be addressed with future *in vivo* studies.

Several groups have used adeno-associated viral (AAV) vectors to deliver *NAGLU* into the brain in preclinical studies [17, 45–47]. Treated mice and dogs show increased NAGLU activity and reduced GAG accumulation in the brain. As these studies employ the same cDNA that has been shown to produce recombinant NAGLU without mannose 6-phosphate, it is likely that the protein secreted by transduced cells also lacks mannose 6-phosphate and displays inefficient uptake. Gene therapy employing our *NAGLU-IGF-II* construct could be more effective with this improvement in cellular uptake. Future *in vivo* studies will be needed to determine whether recombinant enzyme and gene therapy with NAGLU-IGF-II will be a better alternative to unmodified NAGLU for the treatment of Sanfilippo B syndrome.

Acknowledgments

We thank Dr. E. F. Neufeld (University of California, Los Angeles, CA) for providing human NAGLU cDNA, rabbit polyclonal anti-hNAGLU antibody, and thoughtful reading of the manuscript, and Dr. Jon LeBowitz (BioMarin, Novato, CA) for helpful discussions regarding the characterization of rhNAGLU-IGF-II and Dr. J. Lasky (Los Angeles Biomedical Research Institute at Harbor-UCLA, Torrance, CA) for providing DaoY and Es cell lines. We also thank Megan Craig and Jon Scott for careful review of the manuscript.

Funding

Funding was provided by grants from the Lauren's Hope Foundation and the Canadian MPS Society (to P.I.D. and B.L.T.), and in part by National Institute of Health [grant number R21 NS078314-01A1 (to P.I.D.); T32 GM8243-27 (to S-h.K.)]. Confocal imaging was supported by the National Center for Advancing Translational Sciences through UCLA CTSI [grant number UL1TR000124 (to P.I.D)]. The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH.

Abbreviations

GAG	glycosaminoglycans
ERT	enzyme replacement therapy
MPS	mucopolysaccharidosis
M6P/IGF-II	mannose 6-phosphate/insulin-like growth factor-II
CHO	Chinese hamster ovary

References

1. Valstar MJ, Ruijter GJ, can Diggelen OP, Poorhuis BJ, Wilburg FA. Sanfilippo syndrome: a mini review. J Inherit. Metab. Dis. 2008; 31(2):240–252. [PubMed: 18392742]
2. Kakkis ED, Muenzer J, Tiller GE, Waber L, Belmont J, Passage M, Izykowski B, Phillips J, Doroshow R, Walot I, et al. Enzyme-replacement therapy in mucopolysaccharisosis I. N. Engl. J. Med. 2001; 344:182–188. [PubMed: 11172140]
3. Muenzer J, Wraith JE, Beck M, Giugliani R, Harmatz P, Eng CM, Vellodi A, Martin R, Ramaswami U, Gucsavas-Calikoglu M, et al. A phase II/III clinical study of enzyme replacement therapy with idursulfase in mucopolysaccharidosis II (Hunter syndrome). Genet. Med. 2006; 8:465–473. [PubMed: 16912578]
4. Harmatz P, Giugliani R, Schwartz I, Guffon N, Teles EL, Miranda MCS, Wraith JE, Beck M, Arash L, Scarpa M, et al. Enzyme replacement therapy for mucopolysaccharidosis VI: A phase 3, randomized, double-blind, placebo-controlled, multinational study of recombinant human N-acetylgalactosamine 4-sulfatase (recombinant human arylsulfatase B or rhASB) and follow-on, open-label extension study. J. Pediatr. 2006; 148:533–539. [PubMed: 16647419]
5. Eng CM, Guffon N, Wilcox WR, Germain DP, Lee P, Waldek S, Caplan L, Linthorst GE, Desnick RJ. the International Fabry Disease Study Group. Safety and efficacy of recombinant human α-galactosidase A replacement therapy in Fabry's disease. N. Engl. J. Med. 2001; 345:9–16. [PubMed: 11439963]
6. Kishnani PS, Corzo D, Nicolino M, Byrne B, Mandel H, Hwu WL, Leslie N, Levine J, Spencer C, McDonald M, et al. Recombinant human acid α-glucosidase: Major clinical benefits in infantile-onset Pompe disease. Neurology. 2007; 68:99–109. [PubMed: 17151339]
7. Barton NW, Brady RO, Dambrosia JM, Di Bisceglie AM, Doppelt SH, Hill SC, Mankin HJ, Murray GJ, Parker RI, Argoff CE, et al. Replacement therapy for inherited enzyme deficiency: macrophage-targeted glucocerebrosidase for Gaucher's disease. N. Engl. J. Med. 1991; 324:1464–1470. [PubMed: 2023606]

8. Dickson P, McEntee M, Vogler C, Le S, Levy B, Peinovich M, Hanson S, Passage M, Kakkis E. Intrathecal enzyme replacement therapy: successful treatment of brain disease via the cerebrospinal fluid. Mol. Genet. Metab. 2007; 91:61–68. [PubMed: 17321776]
9. Lee WC, Tsoi YK, Troendle FJ, DeLucia MW, Ahmed Z, Dicky CA, Dickson DW, Eckman CB. Single-dose intracerebroventricular administration of galactocerebrosidase improves survival in a mouse model of globoid cell leukodystrophy. FASEB J. 2007; 21:2520–2527. [PubMed: 17403939]
10. Hemsley KM, King B, Hopwood JJ. Injection of recombinant human sulfamidase into the CSF via the cerebellomedullary cistern in MPS IIIA mice. Mol. Genet. Metab. 2007; 90:313–328. [PubMed: 17166757]
11. Chang M, Cooper JD, Sleat DE, Cheng SH, Dodge JC, Passini MA, Lobel P, Davidson BL. Intraventricular enzyme replacement improves disease phenotypes in a mouse model of late infantile neuronal ceroid lipofuscinosis. Mol. Ther. 2008; 16:649–656. [PubMed: 18362923]
12. Dodge JC, Clarke J, Treleaven CM, Taksir TV, Griffiths DA, Yang W, Fidler JA, Passini MA, Karey KP, Schuchman EH, et al. Intracerebroventricular infusion of acid sphingomyelinase corrects CNS manifestations in a mouse model of Niemann-Pick A disease. Exp. Neurol. 2009; 215:349–357. [PubMed: 19059399]
13. Kondagari GS, King BM, Thomson PC, Williamson P, Clements PR, Fuller M, Hemsley KM, Hopwood JJ, Taylor RM. Treatment of canine fucosidosis by intracisternal enzyme infusion. Exp. Neurol. 2011; 230:218–226. [PubMed: 21575633]
14. Yu WH, Zhao KW, Ryazantsev S, Rozengurt N, Neufeld EF. Shortterm enzyme replacement in the murine model of Sanfilippo syndrome type B. Mol. Genet. Metab. 2000; 71:573–580. [PubMed: 11136549]
15. Weber B, Hopwood JJ, Yogalingam G. Expression and characterization of human recombinant and [alpha]-N-actylglucosaminidase. Prot. Expr. Purif. 2001; 21:251–259.
16. Zhao KW, Neufeld EF. Purification and characterization of recombinant human [alpha]-N-acetylglucosaminidase secreted by Chinese Hamster Ovary cells. Prot. Expr. Purif. 2000; 19:202–211.
17. Fu H, Samulski RJ, McCown TJ, Picornell YJ, Fletcher D, Muenzer J. Neurological correction of lysosomal storage in a mucopolysaccharidosis IIIB mouse model by adeno-associated virus-mediated gene delivery. Mol. Ther. 2002; 5:42–49. [PubMed: 11786044]
18. Cressant A, Desmaris N, Verot L, Brejot T, Froissart R, Vanier MT, Maire I, Heard JM. Improved behavior and neuropathology in the mouse model of Sanfilippo type IIIB disease after adeno-associated virus-mediated gene transfer in the striatum. J. Neurosci. 2004; 24:10229–10239. [PubMed: 15537895]
19. Zheng Y, Ryazantsev S, Ohmi K, Zhao HZ, Rozengurt N, Kohn DB, Neufeld EF. Retrovirally transduced bone marrow has a therapeutic effect on brain in the mouse model of mucopolysaccharidosis IIIB. Mol. Genet. Metab. 2004; 82:286–295. [PubMed: 15308126]
20. Fu H, Kang L, Jennings JS, Moy SS, Perez A, DiRosario J, McCarty DM, Muenzer J. Significantly increased lifespan and improved behavioral performances by rAAV gene delivery in adult mucopolysaccharidosis IIIB mice. Gene Ther. 2007; 14:1065–1077. [PubMed: 17460717]
21. McCarty DM, DiRosario J, Gulaid K, Muenzer J, Fu H. Mannitol-facilitated CNS entry of rAAV2 vector significantly delayed the neurological disease progression in MPS IIIB mice. Gene Ther. 2009; 16:1340–1352. [PubMed: 19587708]
22. Fu H, DiRosario J, Kang L, Muenzer J, McCarty DM. Restoration of central nervous system alpha-N-acetylglucosaminidase activity and therapeutic benefits in mucopolysaccharidosis IIIB mice by a single intracisternal recombinant adeno-associated viral type 2 vector delivery. J. Gene Med. 2010; 12:624–633. [PubMed: 20603889]
23. Braulke T, Bonifacino JS. Sorting of lysosomal proteins. Biochim. Biophys. Acta (BBA) - Mol. Cell Res. 2009; 1793:605–614.
24. Hille-Rehfeld A. Mannose 6-phosphate receptors in sorting and transport of lysosomal enzymes. Biochim. Biophys. Acta (BBA) - Rev. Biomembr. 1995; 1241:177–194.
25. Hawkes C, Kar S. The insulin-like growth factor-II/mannose-6-phosphate receptor: structure, distribution and function in the central nervous system. Brain Res. Rev. 2004; 44:117–140. [PubMed: 15003389]

26. LeBowitz JH, Grubb JH, Maga JA, Schmiel DH, Vogler C, Sly WS. Glycosylation-independent targeting enhances enzyme delivery to lysosomes and decreases storage in mucopolysaccharidosis type VII mice. Proc. Natl. Acad. Sci. USA. 2004; 101:3083–3088. [PubMed: 14976248]
27. Grubb JH, Vogler C, Sly WS. New strategies for enzyme replacement therapy for lysosomal storage diseases. Rejuv. Res. 2010; 13:229–236.
28. Chow P, Weissmann B. 4-Methylumbelliferyl 2-acetamido-2-deoxy-α-Dglucopyranoside, a fluorogenic substrate for N-acetyl-α-d-glucosaminidase. Carbohydr. Res. 1981; 96:87–93. [PubMed: 7296566]
29. Barton RW, Neufeld EF. The Hurler Corrective Factor. J. Biol. Chem. 1971; 246:7773–7779. [PubMed: 4257494]
30. Kakkis ED, Matynia A, Jonas AJ, Neufeld EF. Overexpression of the human lysosomal enzyme α-l-iduronidase in Chinese hamster ovary cells. Prot. Expr. Purif. 1994; 5:225–232.
31. Chen CB, Dellamaggiore KR, Ouellette CP, Sedano CD, Lizadjohry M, Chernis GA, Gonzales M, Baltasar FE, Fan AL, Myerowitz R, Neufeld EF. Aptamer-based endocytosis of a lysosomal enzyme. Proc. Natl. Acad. Sci. USA. 2008; 105:15908–15913. [PubMed: 18838694]
32. Zhao HG, Li HH, Bach G, Schmidtchen A, Neufeld EF. The molecular basis of Sanfilippo syndrome type B. Proc. Natl. Acad. Sci. 1996; 93:6101–6105. [PubMed: 8650226]
33. von Figura KF, Logering MF, Kresse H. Serum alpha-*N*-acetylglucosaminidase: determination, characterization, and corrective activity in Sanfilippo B fibroblasts. Z. Klin. Chem. Klin. Biochem. 1975; 13:285–289. [PubMed: 242129]
34. Rohrborn WF, von FK. Human placenta alpha-N-acetylglucosaminidase: purification, characterization and demonstration of multiple recognition forms. Hoppe Seylers Z. Physiol. Chem. 1978; 359:1353–1362. [PubMed: 102578]
35. Sasaki T, Sukegawa K, Masue M, Fukuda S, Tomatsu S, Orii T. Purification and partial characterization of α-N-acetylglucosaminidase from human liver. J. Biochem. 1991; 110:842–846. [PubMed: 1783617]
36. DeChiara TM, Efstratiadis A, Robertsen EJ. A growth-deficiency phenotype in heterozygous mice carrying an insulin-like growth factor II gene disrupted by targeting. Nature. 1990; 345:78–80. [PubMed: 2330056]
37. Hashimoto R, Fujiwara H, Higashihashi N, Enjoh-Kimura T, Terasawa H, Fujita-Yamaguchi Y, Inagaki F, Perdue JF, Sakano KI. N-terminal deletion mutants of insulin-like growth factor-II (IGF-II) show thr and leu important for binding to insulin and IGF-I receptors and leu critical for all IGF-II functions. J. Biol. Chem. 1995; 270:18013–18018. [PubMed: 7629109]
38. Nakae J, Kido Y, Accili D. Distinct and overlapping functions of insulin and IGF-I receptors. Endo. Rev. 2001; 22:818–835.
39. Sands MS, Vogler CA, Ohlemiller KK, Roberts MS, Grubb JH, Levy B, Sly WS. Biodistribution, kinetics, and efficacy of highly phosphorylated and nonphosphorylated β-glucuronidase in the murine model of mucopolysaccharidosis VII. J. Biol. Chem. 2001; 276:43160–43165. [PubMed: 11562370]
40. Dickson PI, Naylor D, Mlikotic A, Victoroff A, Chen A, Passage M, Le S. MPS I Intrathecal Research Collaborative. Intrathecal recombinant human alpha-Liduronidase alleviates spinal cord compression symptoms and is well-tolerated in attenuated MPS I patients. Abstract, Mol. Genet. Metab. 2007; 93:247.
41. Garcia, AR.; Pan, J.; Stronge, A.; Tonini, M.; Alessandrini, M.; Neal, C.; Lieb, J.; Lu, Y.; Wiles, M. Intrathecal delivery of iduronate 2-sulfatase to the CNS of cynomolgous monkeys. Abstract, American Society of Human Genetics 57th Annual Meeting; San Diego, California. 2007.
42. Muñoz-Rojas MV, Costa R, Canani SF, Jardim L, Vedolin L, Kakkis E, Dickson P, Vieira T, John AB, Raymundo M, Giugliani R. Intrathecal enzyme replacement therapy in a patient with mucopolysaccharidosis type I and symptomatic spinal cord compression. Am. J. Med. Genet. A. 2008; 146A:2538–2544. [PubMed: 18792977]
43. Hemsley KM, Luck AJ, Crawley AC, Hassiotis S, Beard H, King B, Rozek TRT, Fuller M, Hopwood JJ. Examination of intravenous and intra-CSF protein delivery for treatment of neurological disease. Eur. J. Neurosci. 2009; 29:1197–1214. [PubMed: 19302155]

44. Hemsley KM, Hopwood JJ. Delivery of recombinant proteins via the cerebrospinal fluid as a therapy option for neurodegenerative lysosomal storage diseases. Int. J. Clin. Pharmacol. Ther. 2009; 47:S118–S123. [PubMed: 20040322]

45. Heldermon CD, Ohlemiller KK, Herzog ED, Vogler C, Qin E, Wozniak DF, Tan Y, Orrock JL, Sands MS. Therapeutic efficacy of bone marrow transplant, intracranial AAV-mediated gene therapy, or both in the mouse model of MPS IIIB. Mol. Ther. 2010; 18:873–880. [PubMed: 20179679]

46. Ellinwood NM, Ausseil J, Desmaris N, Bigou S, Liu S, Jens JK, Snella EM, Mohammed EE, Thomson CB, Raoul S, et al. Safe, efficient, and reproducible gene therapy of the brain in the dog models of Sanfilippo and Hurler syndromes. Mol. Ther. 2011; 19:251–259. [PubMed: 21139569]

47. Fu H, DiRosario J, Killedar S, Zaraspe K, McCarty DM. Correction of neurological disease of mucopolysaccharidosis IIIB in adult mice by rAAV9 trans-bloodbrain barrier gene delivery. Mol. Ther. 2011; 19:1025–1033. [PubMed: 21386820]

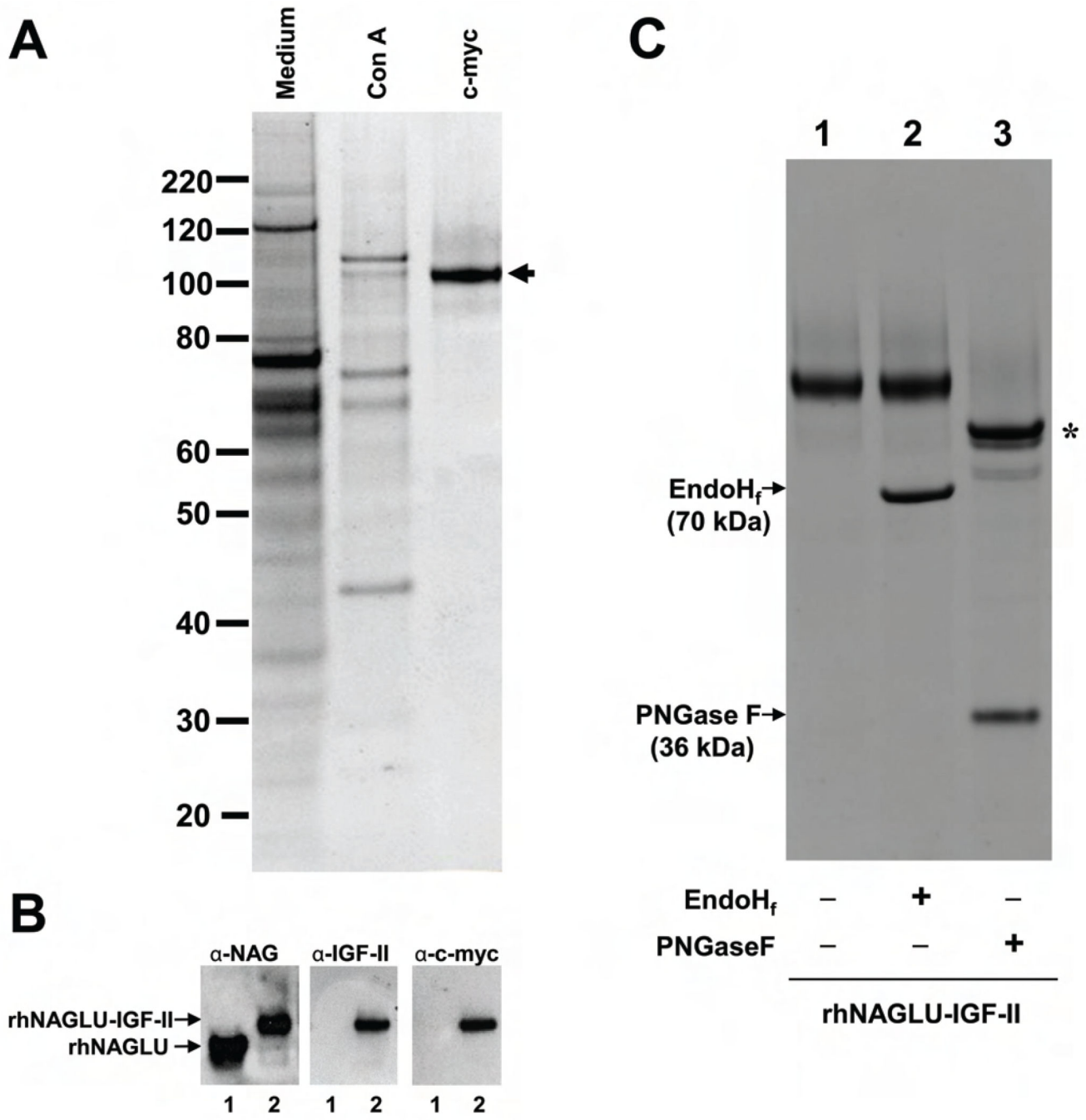

Fig. 1. Expression and glycosylation of rhNAGLU-IGF-II

A) The pCI-NagGScIGF construct generates a product of 832 amino acids with a predicted molecular weight of 91.6 kDa. A 4–12 % SDS-PAGE stained with SYPRO RUBY resolved protein products in different purification stages (culture medium (Medium), Concavalin A Sepharose column (Con A) and cmyc affinity column (c-myc)). After two-column purification, the enzyme migrated at an apparent molecular weight of 105 kDa (arrow). B) Western blots with anti-NAGLU (α-NAG), anti-IGF-II (α-IGF-II) and anti-c-myc (α-c-myc) antibodies. C) Purified rhNAGLU-IGF-II protein (lane 1) was denatured and treated with deglycosylation enzymes Endo H_f (lane 2) or PNGase F (lane 3). After PNGase F treatment, a lower molecular weight of

rhNAGLU-IGF-II of ~92 kDa is seen (asterisk). The enzymes Endo H_f and PNGase F appear in the treated lanes 2 and 3 as bands at 70 kDa and 36 kDa, respectively.

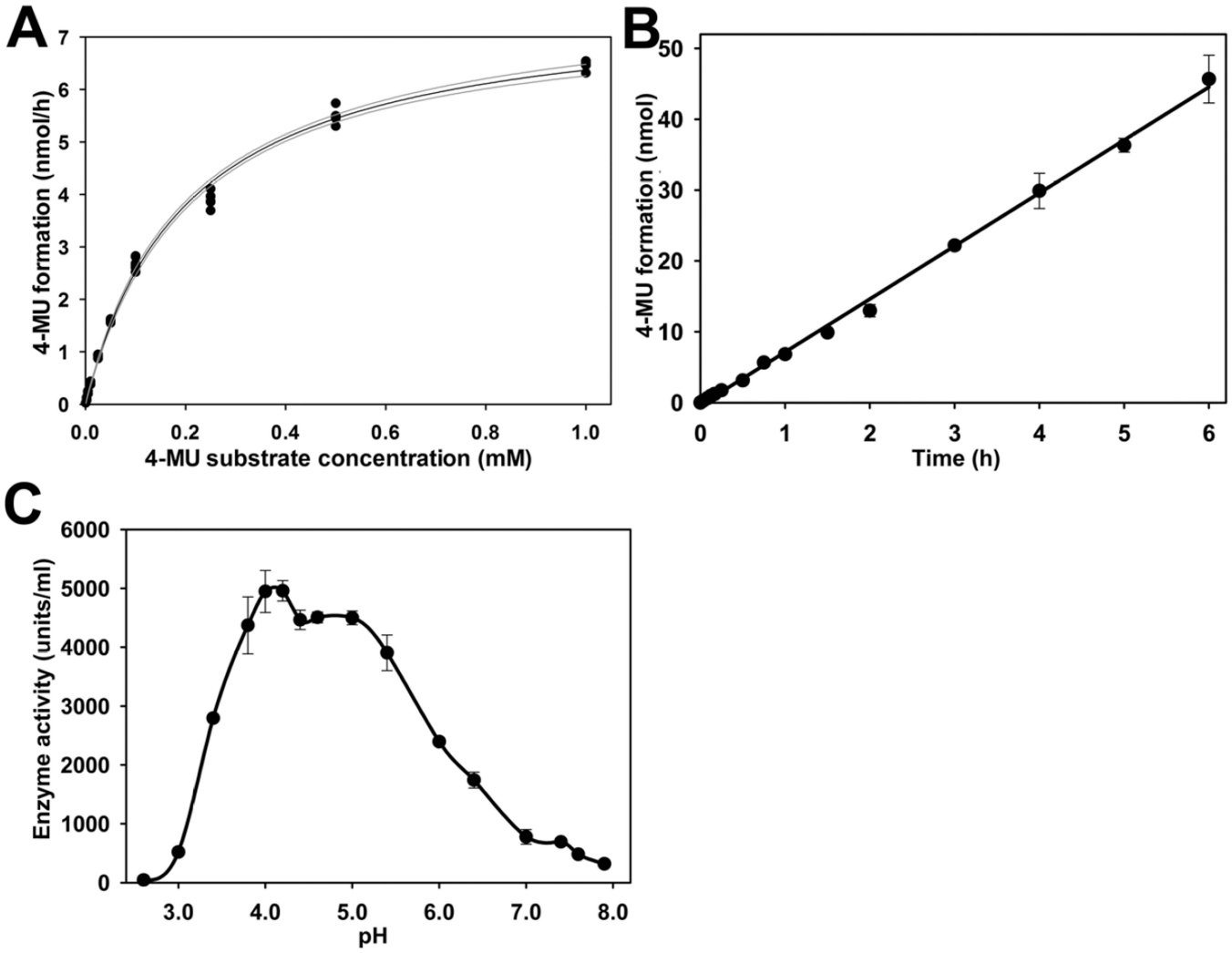

Fig. 2. Biochemical characterization of purified rhNAGLU-IGF-II

A) rhNAGLU-IGF-II activity towards the fluorogenic substrate 4-MUNG was measured as a function of increasing substrate concentration. The reaction followed Michaelis-Menten kinetics with a K_m value of 0.2 mM. The mean (black line) and 95% confidence intervals (gray lines) of four independent experiments are shown. B) In the presence of excess substrate (0.8 mM), rhNAGLU-IGF-II followed first order kinetics, where cleavage product concentration increased linearly with time at an apparent rate of 7.2 nmol of 4-MU product formed per hour. C) To assess the optimal pH range for the enzymatic activity of rhNAGLU-IGF-II, activity assays were performed in 200 mM/100 mM sodium phosphate-citrate buffer at a variety of pHs (2.6–7.9) at 37°C for 1 hour. Points and error bars represent the average and standard deviation respectively for triplicate experiments.

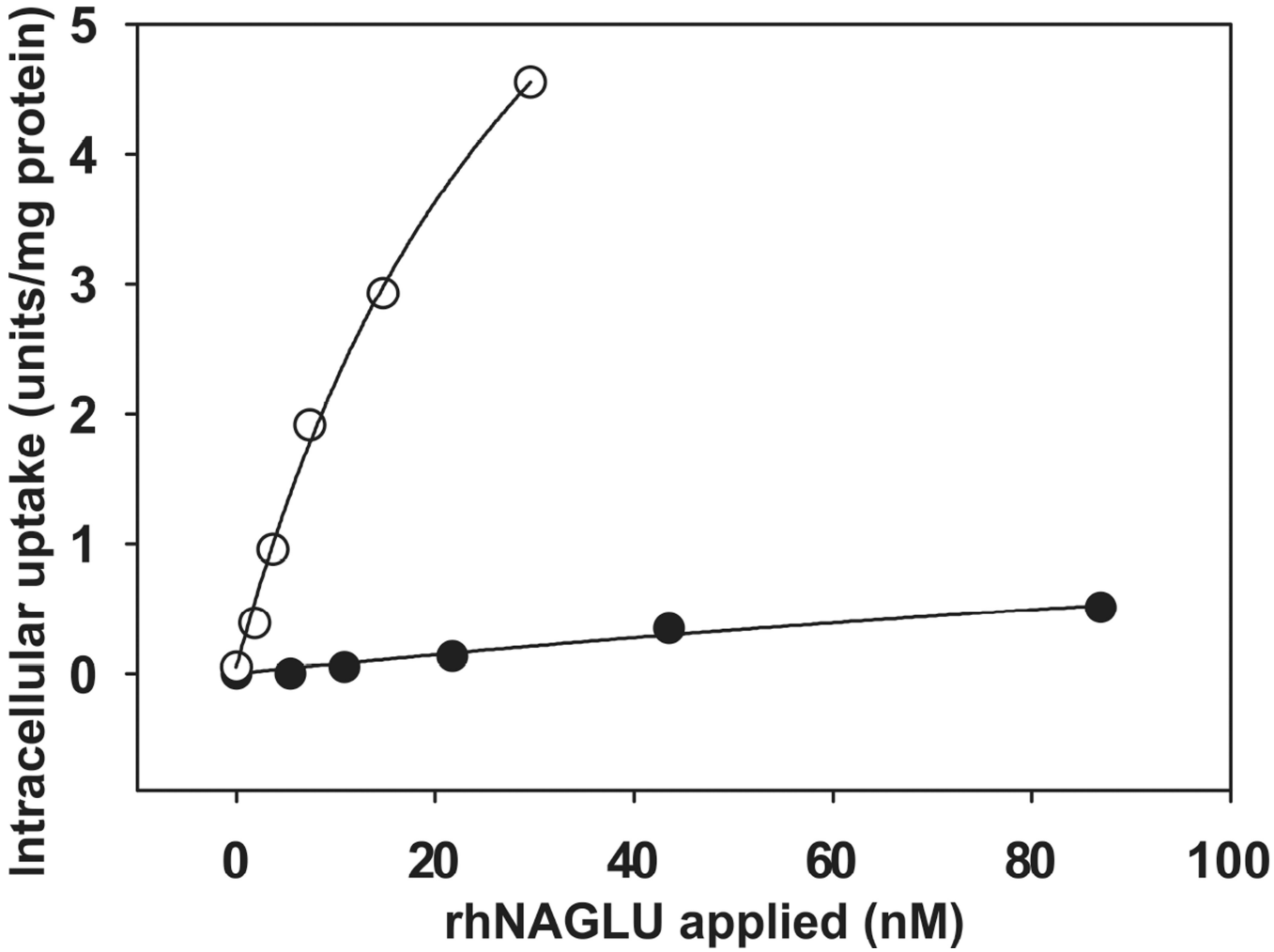

Fig. 3. Uptake kinetics of rhNAGLU-IGF-II

MPS IIIB fibroblasts were cultured for 4 hours with increasing units of purified rhNAGLU-IGF-II (●) or purified human rhNAGLU (○). Intracellular enzyme activity was determined using the substrate 4-MUNG at 0.1 mM at 37 °C for 1 hour as described in the methods. Molar concentrations were calculated using a molecular weight of 92 kDa for rhNAGLU-IGF2 and 82 kDa for rhNAGLU. A representative of at least three independent uptake experiments is shown for each enzyme.

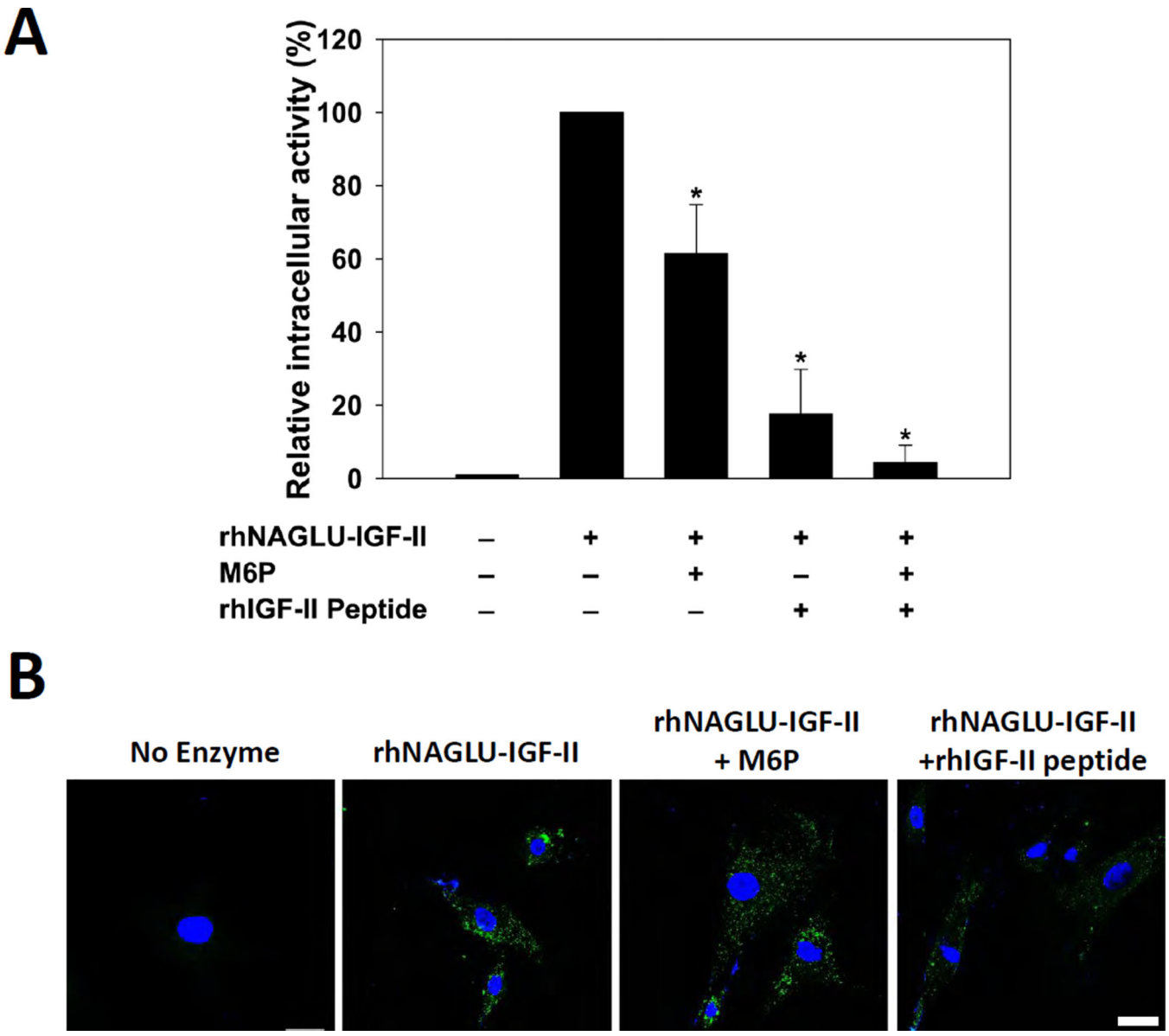

Fig. 4. Uptake inhibition assay in MPS IIIB fibroblasts

Inhibition of rhNAGLU-IGF-II uptake was performed in MPS IIIB fibroblasts. Recombinant human IGF-II (final concentration 5 μg/ml) and/or D-mannose 6-phosphate (M6P; 5 mM) were applied to cells for 10 min before purified rhNAGLU-IGF-II was added at the final concentration of 160 units/ml. A) After a 4- hour incubation, cells were harvested and NAGLU activity was estimated. The intracellular enzyme activity measured with rhNAGLU-IGF-II treatment alone was defined as 100%, and the intracellular enzyme activities observed with inhibitor(s) were normalized to this value. Asterisks (*) indicate a significant difference ($p<0.001$) compared to rhNAGLU-IGF-II treated group. The mean of triplicate experiments is shown. B) Confocal microscopy showing cellular enzyme uptake with or without inhibitor qualitatively. MPS IIIB fibroblasts were treated with 160 units/ml rhNAGLU-IGF-II and/or uptake competitors, 5 μg/ml rhIGF-II peptide or 5 mM M6P. rhNAGLU-IGF-II was detected with rabbit polyclonal antibody against human rhNAGLU and Alexa Fluor 488-labeled goat-anti-rabbit secondary antibody (green). Nuclei were stained with DAPI (blue). Scale bar is 25 μm.

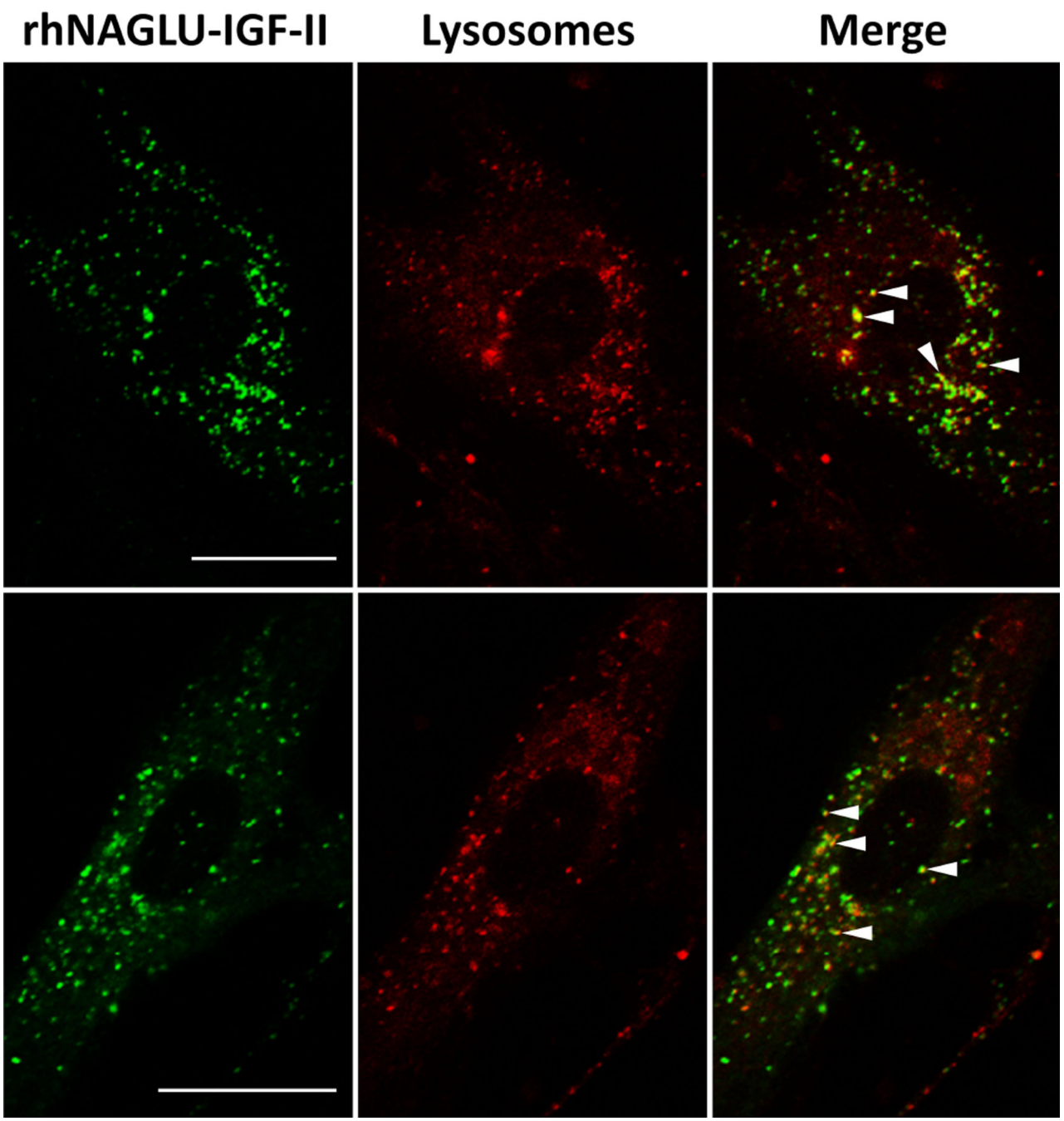

Fig. 5. rhNAGLU-IGF-II delivery to lysosomes in human MPS IIIB fibroblasts

Partial colocalization of internalized rhNAGLU-IGF-II with lysosomes after 4 h incubation. rhNAGLU-IGF-II was detected with rabbit polyclonal antibody against human rhNAGLU and Alexa Fluor 488-labeled goat-anti-rabbit secondary antibody (green, left) and lysosomes were labeled by dextran-Texas red (red, center). Arrowheads in the merged images (yellow, right) indicated overlap between green and red signals. Scale bar is 25 μm.

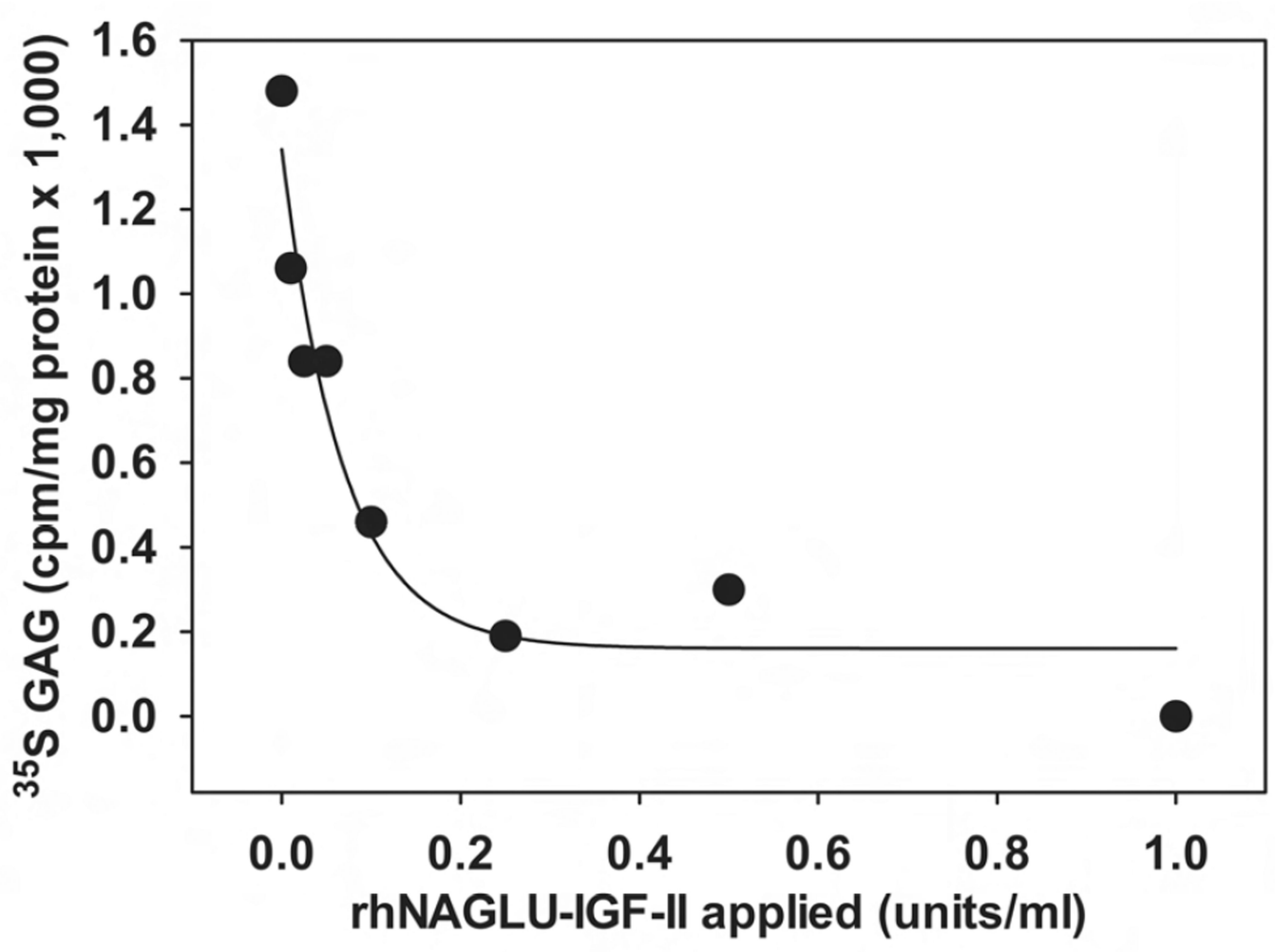

Fig. 6. rhNAGLU-IGF-II reduces GAG accumulation *in vitro*

MPS IIIB fibroblasts were labeled with 25 μCi/ml H_2 $^{35}SO_4$ in culture medium for 72 hours with different concentrations of purified rhNAGLU-IGF-II (0, 0.05, 0.1, 0.5 and 1 units per ml). The radio-labeled GAGs were extracted and measured via scintillation counting. Radioactive counts per minute were normalized to protein concentrations.

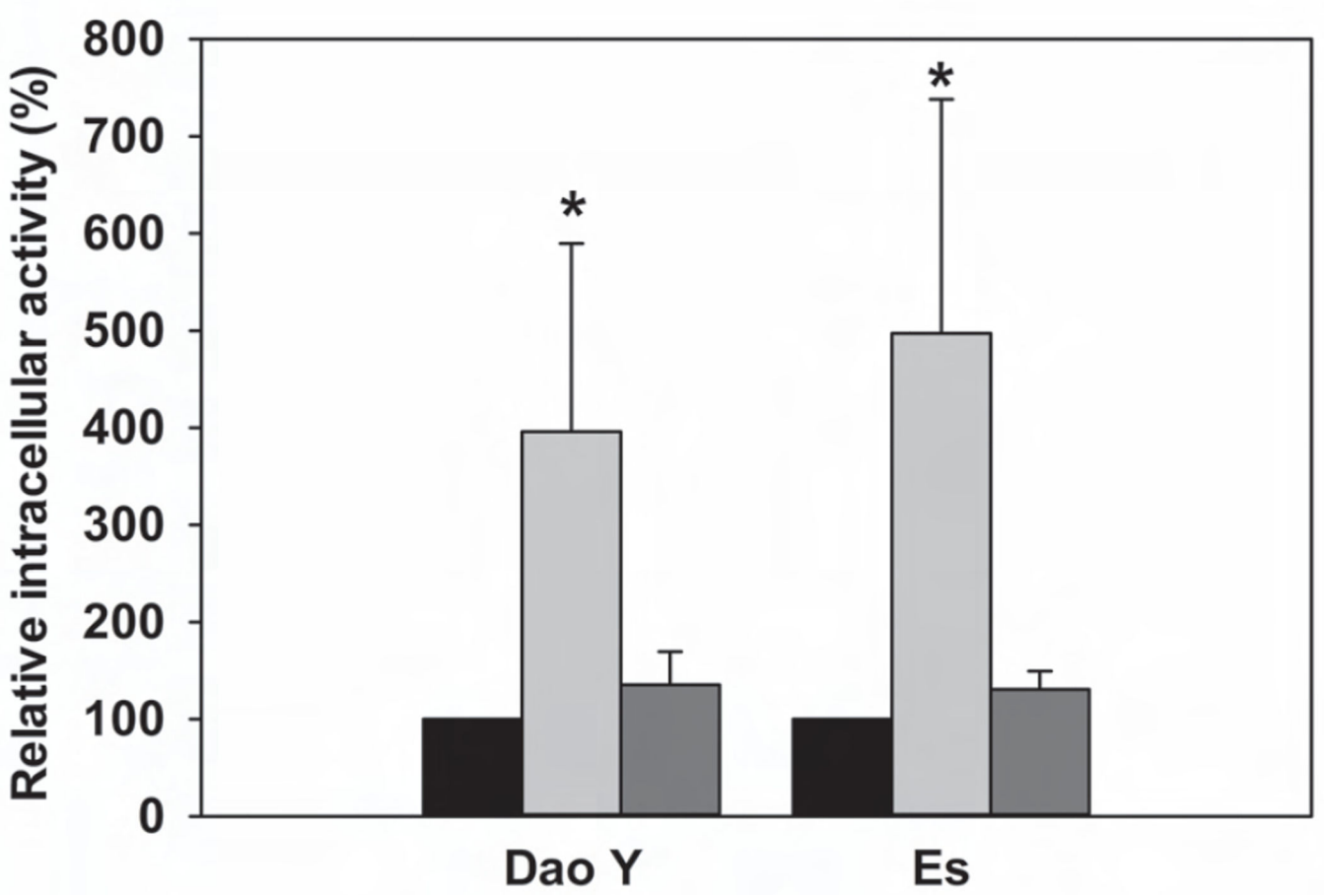

Fig. 7. rhNAGLU-IGF-II uptake in brain tumor-derived cell lines

Intracellular delivery of exogenous rhNAGLU-IGF-II was performed in Dao Y (cerebellar medulloblastoma) and Es (glioblastoma) cell lines in the presence of 160 units/ml purified rhNAGLU-IGF-II with or without 5 μg/ml recombinant human IGF-II as an inhibitor. The endogenous intracellular enzyme activity was defined as 100% (black bars) and the intracellular enzyme activities observed in cells treated with rhNAGLU-IGF-II (light gray bars) and with both rhNAGLU-IGF-II and rhIGF-II peptides (dark gray bars) were normalized to this value. The mean of triplicate experiments is shown. Asterisks (*) indicate $p<0.05$ vs. control and rhNAGLU-IGF-II + rhIGF-II groups.

Table 1

Purification of rhNAGLU-IGF-II from CHO culture medium

Steps	Volume (ml)	Total NAGLU (units)	Total Protein (mg)	Specific activity (units/mg)	Purification (fold)	Yield (%)
Conditioned medium	560	55,000	103.00	533	1.00	100
Concanavalin A	170	24,500	11.9	2,060	3.8	44.5
α-c-myc affinity	2.0	10,700	0.084	127,000	240	19.4

Abbreviations: NAGLU, α-*N*-acetylglucosaminidase; IGF-II, insulin-like growth factor II; CHO, Chinese hamster ovary cells. One unit of NAGLU activity is defined as 1 nmol of converted 4-methylumbelliferyl substrate per hour.