

Evaluation of artificial signal peptides for secretion of two lysosomal enzymes in CHO cells

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Abstract

Enzyme replacement therapy (ERT) is a scientifically rational and clinically proven treatment for lysosomal storage diseases. Most enzymes used for ERT are purified from the culture supernatant of mammalian cells. However, it is challenging to purify lysosomal enzymes with sufficient quality and quantity for clinical use due to their low secretion levels in mammalian cell systems. To improve the secretion efficiency of recombinant lysosomal enzymes, we evaluated the impact of artificial signal peptides on the production of recombinant lysosomal enzymes in Chinese Hamster Ovary (CHO) cell lines. We engineered two recombinant human lysosomal enzymes, N-acetyl- α -glucosaminidase (rhNAGLU) and glucosamine (N-acetyl)-6-sulfatase (rhGNS), by replacing their native signal peptides with 9 different signal peptides derived from highly secretory proteins and expressed them in CHO K1 cells. When comparing the native signal peptides, we found that rhGNS was secreted into media at higher levels than rhNAGLU. The secretion of rhNAGLU and rhGNS can, however, be carefully controlled by altering signal peptides. The secretion of rhNAGLU was relatively higher with murine Igk light chain and human chymotrypsinogen B1 signal peptides, whereas Igk light chain signal peptide 1 and human chymotrypsinogen B1 signal peptides were more effective for rhGNS secretion, suggesting that human chymotrypsinogen B1 signal peptide is the most appropriate for increasing lysosomal enzyme secretion. Collectively, our results indicate that altering signal peptide can modulate the secretion of recombinant lysosome enzymes and will enable lysosomal enzyme production for clinical use.

Keywords: Mucopolysaccharidosis, enzyme replacement therapy, signal peptide, N-acetyl- α -glucosaminidase (NAGLU), glucosamine (N-acetyl)-6-sulfatase (GNS)

Introduction

Mucopolysaccharidosis (MPS) is an inherited disorder caused by deficiency of certain lysosomal enzymes, which are responsible for catabolizing glycosaminoglycans (GAGs) (1). Degradation of GAGs is a stepwise process that involves 11 different enzymes (2), therefore, impairment of any single one of these enzymes causes toxic accumulation of their substrate GAGs in the lysosome. This eventually results in cell damage in multiple organs including the brain, viscera, bone and connective tissues (2). At present, six distinct forms and numerous subtypes of MPS have been identified (3) and among the therapeutic options — enzyme replacement therapy (ERT) is the standard treatment for most MPS diseases (4). ERT introduces a functional enzyme to supplement the deficient or absent enzyme in the lysosome, thus restoring normal enzymatic activity (5). The Federal Drug Administration (FDA) has approved ERT for the treatment of five MPS disorders, including MPS I (6), II (7, 8), IVA (9), VI (10) and VII (11) and several others are undergoing clinical trial (4).

Most lysosomal enzymes used as ERT for MPS are purified from CHO expression system (12). However, purifying lysosomal enzymes as recombinant proteins in large scale from culture media can be challenging as the secretion of lysosomal enzymes from the producing cells is relatively low. Lysosomal enzymes, like other secreted proteins, are first synthesized in the endoplasmic reticulum (ER), but only 5 –20% of each lysosomal enzyme synthesized is secreted directly outside the cell via the secretory pathway, while the rest are trafficked to lysosomes (13). Altering the native signal peptide sequence, therefore, offers a method of heightening recognition by the signal recognition particle that facilitate ER lumen translocation, a rate-limiting step within the classical secretory pathway, thus increasing protein secretion (14). It has been shown that the secretion of lysosomal

enzymes is improved by replacing the native signal peptide with one derived from other highly secreted lysosomal enzymes (15, 16). Sorrentino *et al.* demonstrated that the inclusion of an iduronate 2-sulfatase (IDS) signal peptide strongly increases the secretion of N-sulfoglucosamine sulfohydrolase (SGSH) in mouse embryonic fibroblast cells by approximately 3-fold (15). However, Chen *et al.*, showed that the secretion of SGSH is only enhanced by ~30% when using a signal peptide derived from IDS or tripeptidyl peptidase 1 in HEK293 cells (16). In addition, they found that SGSH secretion is decreased using the β -glucuronidase signal peptide (16). It appears that distinct signal peptides can exert varied efficiency for lysosomal enzyme secretion depending on the enzyme itself or cell types. The effect of artificial signal peptides on the production of recombinant lysosomal enzymes has not been comprehensively studied.

In a previous study, we have investigated the of potential ERT for MPS IIID. MPS IIID is caused by deficiency of lysosomal enzyme glucosamine (N-acetyl)-6-sulfatase (GNS) which removes the sulfate group from 6-sulfated N-acetylglucosamine residues at nonreducing ends of heparan sulfate GAGs. Our results show that intracerebroventricular administration of recombinant human GNS (rhGNS) effectively reduces GAGs storage in lysosome in the mouse model of MPS IIID and providing a proof-of-concept ERT for MPS IIID patients (17). To further develop rhGNS as a biopharmaceutical for clinical use, it will require larger quantities of rhGNS for both efficacy and clinical safety evaluation. Thus, increasing rhGNS secretion level is important to maximize production yields and reduce the associated manufacturing costs. In addition, one of the enzymatic activity measurements of GNS is evaluated by a two-step method that requires the sequential action of GNS, followed by another lysosomal enzyme, N-acetyl- α -glucosaminidase (NAGLU), to liberate a fluorescent product (18). Large amounts of recombinant human NAGLU (rhNAGLU) are also needed to measure the activity of GNS.

In this study, we screened for potential signal peptides that could improve recombinant lysosomal enzymes production, particularly rhNAGLU and rhGNS. A panel of alternative signal peptides from various sources were chosen (**Table 1**). These included antibody signal peptides from Ig heavy chain signal peptide 7 and Ig κ light chain signal peptide 1, that exhibit the best secretion for 4 antibodies out of the 5 in CHO K1 cells, including Avastin, Remicade, Rituxan and Humira (19), and also from murine Ig κ light chain, that shows 1.5-fold as effective as the expression of recombinant human coagulation factor VII using native signal peptide in CHO K1 cells (20). We also selected signal peptides derived from human albumin, that increases antibody production in CHO K1 cells by 50-60% compared to control signal peptide (21), and from human azurocidin 1, that increases recombinant interleukin-21 production in CHO K1 cell media by 7-fold compared to native signal peptide (22). In addition, we selected signal peptides from human serine protease 2 and human chymotrypsinogen B1, that proved to be 14.2-fold and 11.8-fold as effective as that of human albumin for recombinant *Gaussia* luciferase production in CHO cells (23). Importantly, we included signal peptides from different species, such as *Gaussia* luciferase, that shows greater luciferase activity in CHO cells compared to the signal peptide derived from human interleukin-2 and human albumin, by 63% and 98%, respectively (24). Here, we elucidated the effects of these artificial signal peptides on the cellular expression and extracellular secretion of rhNAGLU and rhGNS in CHO K1 cells.

Materials and Methods

Plasmid DNA construction

The human full-length NAGLU cDNA (amino acid 1-743) in pEE12.4/NAGLU-IGFII with a C-terminal myc tag (25) was subcloned into pOptiVEC™-TOPO® shuttle vector (Invitrogen), bearing a gene coding for dihydrofolate reductase (DHFR). A human full-length GNS cDNA (amino acid 1-552) followed by a myc

tag was subcloned into pOptiVEC™-TOPO® vector as described (26). To construct rhGNS with an artificial signal peptide, the signal peptides followed by a *Xho*I site were fused at the 5' primer of the GNS coding sequence. The GNS cDNA fragment (amino acid 37-552 without the 36-residue native signal peptide) with different artificial signal peptides at N-terminal and a myc tag at C-terminal was individually PCR-amplified and cloned into pOptiVEC-TOPO vector. pOptiVEC-TOPO-rhGNS-TEV-Myc was modified by QuikChange site-directed mutagenesis (Agilent Technologies) to generate a *Xho*I site at the C-terminal of GNS native signal peptide. To construct NAGLU with an artificial signal peptide, a NAGLU cDNA fragment (amino acid 25-743 without the 24-residue native signal peptide) was PCR-amplified and subcloned to replace the GNS fragment in the corresponding plasmids at *Xho*I/*Not*I restriction sites. All the sequences were codon optimized for expression in CHO cells (GenScript). The primers used for PCR amplification and the resulting plasmid constructs are listed in **Supplementary Table S1**, and **S2**, respectively. A schematic representation of the expression constructs is shown in **Supplementary Figure 1**.

Transfection of CHO K1 cells

CHO K1 cells (ATCC: CCL-61) were maintained in DMEM/F12 (Corning) medium supplemented with 10% fetal bovine serum (FBS; Atlanta Biologicals), 100 µg/mL of streptomycin and 100 U/mL of penicillin (Lonza). Prior to transfection, 6×10^5 cells per well were grown overnight in a 6-well plate. Cells were transfected with 2 µg of the plasmid using 3 µL of BioT (Bioland Scientific LLC). After 6 h incubation, cells were washed with PBS and cultured in 1 mL of DMEM/F12 media with 10% FBS or HyClone PF Cho LS medium (GE Healthcare Life Sciences) and supplemented with 100 µg/mL of streptomycin and 100 U/mL of penicillin. Culture media and cells were harvested at 24, 48, or 72 h post transfection based on different experiments. The culture media was centrifuged for 5 min at $10,000 \times g$ (4°C). The supernatant was collected and ~15× concentrated by Amicon Ultra 0.5 mL

centrifugal filters molecular-weight cut off 100 kDa (Millipore). Cells were lysed in 100 μ l of acetate buffer pH 5.6 with 0.1 M 1,4-dithiothreitol (DTT) and 0.1% Triton X-100. After incubation for 30 min on ice, cell debris was pelleted for 10 min 10,000 \times g (4°C). Protein concentrations were estimated using the Bradford protein assay (Bio-Rad). Cells were transfected with each construct in three independent experiments.

Protein expression determined by Western Blotting

Comparable amounts of protein in cell lysate (10 μ g) or cell media (0.1 μ g) in each sample was loaded into 4%-20% SDS-PAGE gel and transferred to nitrocellulose membrane. Primary mouse anti-myc tag antibody (05-419, Millipore, 1 : 2000), mouse anti-DHFR antibody (sc-377091, Santa Cruz Biotechnology, 1 : 250), or rabbit anti-GAPDH antibody (2188S, Cell Signaling Technology, 1 : 5000), and secondary HRP-labeled goat anti-mouse or anti-rabbit antibodies (Bio-Rad) were used. Blots were developed using Immobilon Western Chemiluminescent HRP Substrate (Millipore) and visualized using ChemiDoc MP Imaging System (Bio-Rad). Densitometric analysis of the immunoreactive bands was performed using Image Lab (Bio-Rad). Cellular DHFR level in each cell lysate sample was normalized to the GAPDH level and subtracted the normalized DHFR level in cells treated with BioT alone without plasmids. Transfection efficiency was determined as relative to the normalized DHFR level in the cells with native signal peptide. The expression of rhNAGLU and rhGNS were normalized by the corresponding transfection efficiency and displayed as compared with the native signal peptide.

Enzymatic Activity Assays for rhNAGLU and rhGNS

NAGLU or GNS activity was measured in triplicate by detecting the amount of 4-methylumbelliferon (4-MU) to be liberated from the respective fluorogenic substrate for both GNS and NAGLU (25, 26).

NAGLU activity was determined using the NAGLU substrate, 4-methylumbelliferyl 2-acetamido-2-deoxy- α -D-glucopyranoside (M333800, Toronto Research Chemicals). Reaction mixtures consisted of 2.5 μ L of cell lysates, 2.5 μ L of 4 mM NAGLU substrate in 200 mM acetate buffer pH 5.6 with 0.01% Triton. After incubation for 2 h at 37 °C, the mixtures were quenched by adding 65 μ L of 500 mM glycine/sodium carbonate pH 10.6.

Catalytic activity of GNS was determined by a two-step protocol with the GNS substrate, 4-methylumbelliferyl 6-sulfo-2-acetamido-2-deoxy- α -D-glucopyranoside potassium salt (M334950, Toronto Research Chemicals). Reaction mixtures consisted of 2.5 μ L of cell lysates, 2.5 μ L of 10 mM GNS substrate in 200 mM acetate buffer pH 5.6 with 0.01% Triton X-100 and 50 mM Pb-Acetate. After incubation for 2 h at 37 °C, the mixtures were incubated with 10 μ L of citrate-phosphate buffer pH 4.7 and 5 μ L of purified rhNAGLU protein (3.5×10^5 nmol/hr/mg) for a secondary 2 h incubation. The reaction was quenched by adding 50 μ L of 500 mM glycine/sodium carbonate pH 10.6.

The resulting supernatant was collected after centrifugation at $10,000 \times g$ for 1 min and then dispensed into two well, 25 μ L per well, in a 384-well black plate (Corning). The fluorescence intensity of the released 4-MU was detected at excitation and emission wavelengths of 360 nm and 460 nm, respectively. NAGLU or GNS activity was calculated using a relevant standard curve of 4-MU. The enzyme activity was expressed as nanomoles (nmol) of 4-MU that is liberated at 37°C per hour by mg of protein content in cell lysate or by mL of media volume. For signal peptide screening, enzyme activity was further normalized by the corresponding transfection efficiency and displayed as compared with the native signal peptide. All statistical analysis was calculated with GraphPad Prism using the unpaired two-tailed Student's t-test with equal variance.

Results

rhGNS is secreted more efficiently than rhNAGLU in CHO K1 cells

To compare the cellular expression and secretory levels of rhNAGLU and rhGNS with their native signal peptide in CHO K1 cell, cells were transiently transfected with identical expression vectors expressing rhNAGLU or rhGNS with a myc tag at the C-terminal. Considering that serum in culture media might affect the subsequent enzyme activity determination, we then replaced cell media with serum-free media at 6 h post transfection. The intracellular and extracellular expression of rhNAGLU or rhGNS was examined by Western blotting analysis. As shown in **Figure 1A**, rhNAGLU was detected as immunoreactive bands at between 75 - 100 kDa in cell lysate and 100 kDa in media. On the other hand, rhGNS was detectable in cell lysate with molecular mass of approximately 75 - 100 kDa, whereas a smeared immunoreactive band with a molecular mass greater than that in cell lysate was detected at the range of 100 to 150 kDa in media. Of note, cellular and secretory rhNAGLU or rhGNS expression levels were similar in the presence or absence of serum (**Figure 1A**), indicating that media replacement had no apparent effects on the expression and secretion of rhNAGLU and rhGNS. We then monitored the recombinant lysosomal enzyme expression level and enzyme activity in cell lysate and culture media at 24, 48, and 72 hours post transfection. While the secreted rhNAGLU started to appear in the media at 48 h post transfection, the secreted rhGNS was detectable as early at 24 h post transfection, and the secretion level remained strong at 72 h post transfection (**Figure 1B**). We further quantified the bands on the Western blotting to obtain protein amount in media. **Figure 1C** shows that the expression level of rhGNS in media was relatively higher than native rhNAGLU at 24, 48, and

72 h post transfection, respectively, suggesting that there was relatively more rhGNS being secreted than rhNAGLU in CHO K1 cells.

Enzymatic activity of rhNAGLU and rhGNS in CHO K1 cells

To test whether the produced rhNAGLU and rhGNS are fully functional, their enzymatic activity in cell lysate and media was assessed by detecting the amount of 4-MU liberated from the respective fluorogenic substrates. As shown in **Figure 2A**, NAGLU activity in cell lysate increased gradually with time from 13.3 to 20.4 nmol/h/mg, an increase of 0.5-fold from 24 h to 72 h post transfection. NAGLU activity in media markedly increased by ~9.4 -fold (from 0.5 to 5.2 nmol/h/mL) during the same time period (**Figure 2B**). On the other hand, we observed a similar increase in GNS activity in cell lysates where its activity doubled from 25.1 to 50.6 nmol/h/mg from 24 h to 72 h post transfection (**Figure 2C**) but GNS activity in media showed only a modest ~0.8-fold increase from 3.4 to 6.3 nmol/h/mL (**Figure 2D**). These results confirm that the two lysosomal enzymes NAGLU and GNS are recombinantly expressed and secreted functionally in CHO K1 cells. We further calculated the active secreted protein amount for rhGNS and rhNAGLU in media at 72 hr post transfection. The specific activities for purified rhNAGLU and rhGNS are 350,000 and 39,000 nmol/h/mg, respectively. The calculated titers for secreted rhNAGLU and rhGNS are 0.015 mg/L and 0.162 mg/L, respectively. Of note, the secreted rhGNS was ~11-fold higher than rhNAGLU, indicating that native rhGNS had a higher secretion efficiency in CHO K1 cells.

Artificial signal peptides altered rhNAGLU expression and secretion in CHO K1 cells

To increase rhNAGLU secretion in media, we selected 9 different signal peptides (**Table 1**) to replace the native peptide of NAGLU. As the rhGNS yield in media was relatively high compared to rhNAGLU, we also determined the effects of GNS native signal peptide on rhNAGLU secretion. We transiently

transfected CHO K1 cells with the identical expression vector expressing rhNAGLU fused with each signal peptide. The DHFR gene in the expression vector is co-expressed as an internal control. At 72 h post transfection, we collected cell lysates and culture media to analyze rhNAGLU expression by Western blotting. **Figure 3A** showed similar molecular mass for all forms of intracellular and secreted rhNAGLU, regardless of signal peptide employed. The same was also observed for the secreted rhNAGLU (**Figure 3B**), indicating that there are no significant changes in protein modification. To exclude the effects caused by different transfection efficiencies, the secretory level of rhNAGLU was normalized to the corresponding DHFR intensity and then compared to that of the native signal peptide. **Figure 3C** shows that the relative extracellular secretion of rhNAGLU was increased significantly by signal peptide 8 (human chymotrypsinogen B1) to ~1.5-fold of that of the native signal peptide. We also compared the secretory rhNAGLU levels by determining NAGLU enzymatic activity in media, which was further normalized the corresponding transfection efficiency. As shown in **Figure 3C**, among the tested signal peptides, artificial signal peptides 4 (murine Igκ light chain) and 8 (human chymotrypsinogen B1) exhibited higher NAGLU activity in media, resulting in ~1.2-fold and ~1.3-fold compared to native signal peptide (**Figure 3D**). Altogether, these results show that altering the signal peptide affects extracellular levels of rhNAGLU.

Evaluation of various signal peptides for rhGNS expression in CHO K1 cells

We further examined the effects of the signal peptide on the expression and secretion of the relatively higher secreted rhGNS by fusing rhGNS with 9 different signal peptides (**Table 1**) within an identical expression vector, which were transfected into CHO K1 cells. Western blot confirmed that the different signal peptides did not affect the molecular mass of rhGNS in cell lysate or media (**Figure 4A-B**). **Figure 4C** shows that relatively higher rhGNS secretion in media was observed when using signal peptides 8 (human chymotrypsinogen B1), ~1.2-fold compared to native signal peptide. The

extracellular GNS enzymatic activity was determined and normalized as described above. As shown in **Figure 4C**, signal peptide 2 (Igκ light chain signal peptide 1) and 8 (human chymotrypsinogen B1) exhibited relatively greater extracellular GNS activity of ~1.2-fold and ~1.4-fold respectively compared to native signal peptide. Therefore, signal peptides 2 (Igκ light chain signal peptide 1) and 8 (human chymotrypsinogen B1) were effective in increasing the expression of rhGNS extracellularly among the tested artificial signal peptides. Collectively, replacing the native signal peptide with various artificial signal peptides caused different secretion levels of rhNAGLU and rhGNS, suggesting that signal peptide plays a role in modulating the production of recombinant lysosomal enzymes.

Discussion

Our study sought to optimize recombinant lysosomal enzyme production through a signal peptide screening campaign in CHO K1 cells. We selected signal peptides from various sources, including different types of protein (antibody, serum protein, and exocrine) and different species (*Homo sapiens*, *Mus musculus*, and *Gaussia princeps*), and demonstrated that artificial signal peptide 8 (human chymotrypsinogen B1) resulted in the highest secretion for both rhNAGLU and rhGNS of all the tested artificial signal peptides. This study shows the impact of signal peptides on the secretion of recombinant lysosomal enzymes, indicating that optimizing the signal peptide is a viable strategy to improve the productivity of recombinant lysosomal enzyme.

Signal peptides play a significant role in recombinant protein production. Several studies have suggested that using artificial signal peptides can improve the expression and secretion of a recombinant protein (19-24). Importantly, altering signal peptide sequences is designed to change binding to the signal recognition particle, which ideally should not affect the characteristics of a recombinant protein. The newly synthesized lysosomal enzymes in the Golgi apparatus undergoes

post-translational modification to install the mannose 6-phosphorylation (M6P) residues on their N-linked oligosaccharides (1). Most lysosomal enzymes used in enzyme replacement therapy requires the M6P modification for efficient uptake and lysosomal delivery through M6P receptor-mediated endocytosis (12). In our previous study, we demonstrated that rhGNS purified from CHO system is taken up by MPS IIID patient fibroblasts in a dose- and time-dependent manner (17). The intracellular uptake of rhNAGLU and rhGNS produced with different signal peptides should be the same since their signal peptides will be cleaved as described previously (27, 28). Furthermore, Sorrentino *et al.* demonstrated the N-sulfoglucosamine sulfohydrolase (SGSH) produced from a modified signal peptide is taken up by recipient cells as efficiently as that from a native signal peptide, implying that a change in signal peptide may not alter the M6P and receptor-mediated uptake of a lysosomal enzyme (15).

Also, however, altering signal peptides does not always strongly increase secretion efficiency of all proteins. In our study, we selected 9 different signal peptides that have been shown to increase recombinant protein secretion. It was unexpected to find that altering signal peptides did not show a more pronounced effect on the secretion of either rhNAGLU or rhGNS compared to their own native signal peptides, which is possibly because the native signal peptide may not function correctly out of the original sequence context. Further optimization such as adjusting the sequences of the signal peptides may be required to improve outcomes to a greater extent.

Optimizing the signal peptide sequence may improve the production of recombinant lysosomal enzymes. All signal peptides share a general structure consisting of three domains, the N-domain (the positive-charged domain), followed by the H-domain (hydrophobic core) and lastly, the C-terminal domain with containing a cleavage site (14). It was reported that the N-domain and H-domain are correlated with the efficiency of protein translocation by enabling the signal peptide to

anchor onto the ER membrane. As the hydrophobicity of signal peptide decreases, protein processing and translocation will be slower or completely quenched, inhibiting protein secretion (14). Zhang *et al.* showed that increasing both the basicity and hydrophobicity of artificial signal peptide from interleukin-2 by amino acid substitution augments the secretion of alkaline phosphatase and endostatin by approximately 2.5- and 3.5-fold, respectively in MDA-MB-435 cells (29). These results indicate that recombinant protein secretion can be enhanced by modifying the N-domain and H-domain within an artificial signal peptide. Moreover, the recombinant protein secretion also can be affected by the amino acids beyond the signal peptide. Güler-Gane *et al.* demonstrated that the level of secreted alkaline phosphatase is increased by up to 300% using the secretion signal peptide with an additional alanine at the +1 or +1 and +2 position after the cleavage site (30), suggesting that the secretion efficiency of a recombinant protein depends on the interplay between a signal peptide and its downstream amino acid sequence. Therefore, modification of artificial signal peptide 8 (human chymotrypsinogen B1), the most effective peptide for both rhNAGLU and rhGNS, by increasing its basicity and hydrophobicity or inserting additional amino acid beyond the signal peptide sequence may increase the production of recombinant lysosomal enzymes in CHO K1 cells.

As lysosomal enzymes are targeted and directed to lysosome after synthesis, altering their binding to the sorting receptors that guide them to lysosome may also increase their extracellular productivity. In fact, M6P is a key targeting signal on lysosomal enzyme precursors to be recognized by M6P receptors in the Golgi complex and ensuing transport to the lysosome (31). Overexpressing recombinant lysosomal enzymes can therefore saturate the M6P receptors and shunt lysosomal enzymes toward the secretory pathway. Indeed, we found that both rhNAGLU and rhGNS exhibited larger molecular size in media than that in the cell lysate, suggesting that they are the precursors secreted via secretory pathway as the maturation processes in the lysosome are absent or reduced.

Moreover, Pohlmann *et al.* found that the extracellular activities of five lysosomal enzymes, including β -hexosaminidase, β -glucuronidase, α -fucosidase, β -mannosidase, and β -galactosidase, are 5-33% in control fibroblasts and increase to 72-93% in M6P receptors-deficient fibroblasts (32), indicating that lysosomal enzymes escape from M6P receptor binding can be secreted. Therefore, manipulating the M6P receptors in CHO cells by using genetic engineering technique may represent a viable method to increase lysosomal enzyme secretion.

In summary, we focused on two recombinant human lysosomal enzymes, rhNAGLU and rhGNS with distinct secretory properties, and examined the impact of artificial signal peptides on their production in CHO K1 cells. Our results show that rhNAGLU and rhGNS were differently expressed in the transfected CHO K1 cells as well as secreted in culture media. Importantly, these two recombinant lysosomal enzymes are functionally active. In addition, artificial signal peptides 8 (human chymotrypsinogen B1) was the most effective for the secretion of both rhNAGLU and rhGNS in media compared to the others tested in this study. Further study focusing on substitution or insertion of amino acid for optimizing signal peptides is expected to enable efficient production. It is necessary to analyze the performance of artificial signal peptides in stably expression cell lines. We anticipate that these findings could be applied widely to large-scale production of various lysosomal enzymes and will promote development strategies for ERT.

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Author contributions statement

T.-F. Chou conceived the project. K.-W. Cheng designed and performed experiments. F. Wang constructed the pOptiVEC-TOPO-rhGNS-TEV-myc plasmid. G. A. Lopez constructed the pOptiVEC-TOPO-rhNAGLU-myc plasmid. F. Wang and G. A. Lopez assisted in eliminating problems during experiments and provided suggestions. K.-W. Cheng, and T.-F. Chou wrote the manuscript. S. Singamsetty, J. Wood, and P.I. Dickson, and T.-F. Chou provided suggestions, edited the manuscript and obtained funding for this work.

Competing interests statement

The authors declare no conflicts of interest.

Disclosure

SS, and JW are Phoenix Nest Inc. employees.

Data availability statement

Most data generated during this study are included in the article and its Supplementary Information. Uncropped images of all gels and blots can be found in Supplementary Figures 1 to 3. Additional measurements such as protein concentrations and enzymatic activities generated during the current study and relevant information are available from the corresponding authors upon request.

Table 1. Overview of the selected signal peptides in this study

No.	Signal peptide sequence	Origin	Organism	Accession No.
1	MEFGLSWVFLVALFRGVQC	Ig heavy chain signal peptide 7		
2	MDMRVPAQLLGLLLWLSGARC	Ig κ light chain signal peptide 1		
3	MGWSCILFLVATATGVHS	Ig heavy chain	<i>Mus musculus</i>	P01750
4	METDTLLLWVLLLWVPGSTGD	Ig κ light chain	<i>Mus musculus</i>	P01658
5	MKWVTFISLLFLFSSAYS	Albumin	<i>Homo sapiens</i>	P02768
6	MTRLTVLALLAGLLASSRA	Azurocidin 1	<i>Homo sapiens</i>	P20160
7	MNLLLILTFVAAAVA	Serine protease 2	<i>Homo sapiens</i>	P07478
8	MAFLWLLSCWALLGTTFG	Chymotrypsinogen B1	<i>Homo sapiens</i>	P17538
9	MGVKVLFALICIAVAEA	Luciferase	<i>Gaussia princeps</i>	Q9BLZ2

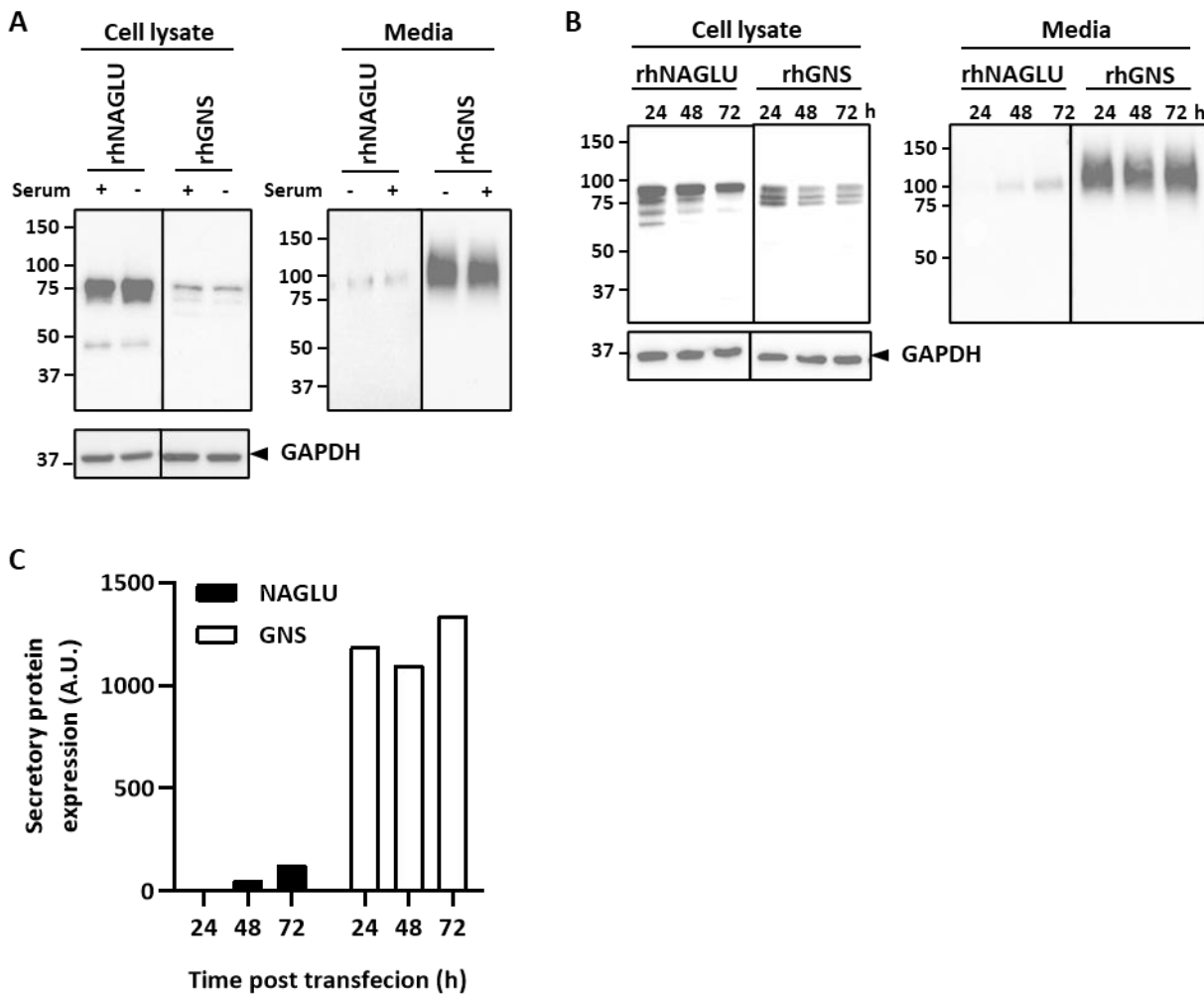


Figure 1. Expression of rhNAGLU and rhGNS with native signal peptide in CHO K1 cells. CHO K1 cells were transfected with vector expressing rhNAGLU or rhGNS. Cells and cultured media were harvested for determining protein expression by Western blotting using anti-myc antibodies to detect the myc tagged rhNAGLU or rhGNS. GAPDH, loading control. **(A)** The intracellular and extracellular expression of rhNAGLU and rhGNS expression in cells cultured in the presence or absence of serum at 48 post transfection, and **(B)** at the indicated time points in serum-free media. **(C)** The intensity of secreted rhNAGLU and rhGNS is estimated from **(B)** in arbitrary units (A.U.).

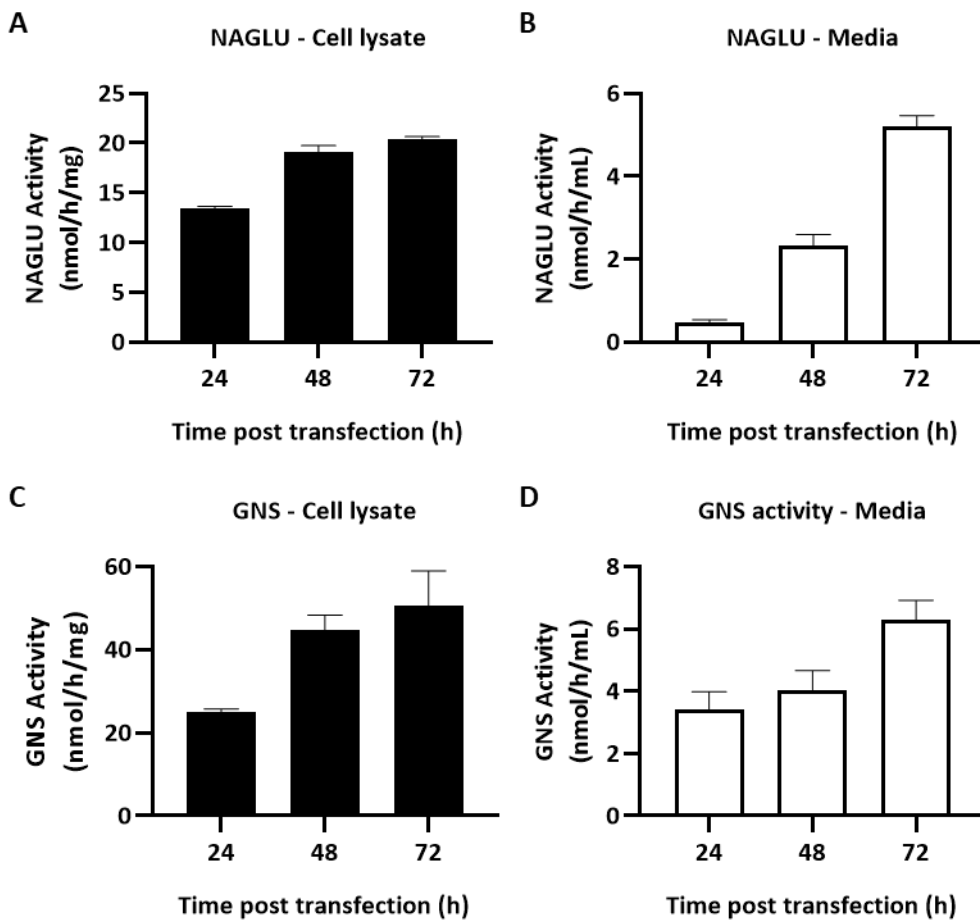


Figure 2. Enzyme activity of rhNAGLU and rhGNS with native signal peptide in CHO expression system.

CHO K1 cells were transfected with vector expressing rhNAGLU or rhGNS, the cells and culture media were harvested at the indicated time points post transfection. NAGLU (A, B) and GNS (C, D) activity in cell lysate (A, C) and media (B, D) were assayed. The enzyme activity was expressed as nanomoles (nmol) of 4-MU that is liberated at 37°C per hour by mg of protein content in cell lysate or by mL of media volume. Data are shown as Mean ± SD taken from three biological replicates.

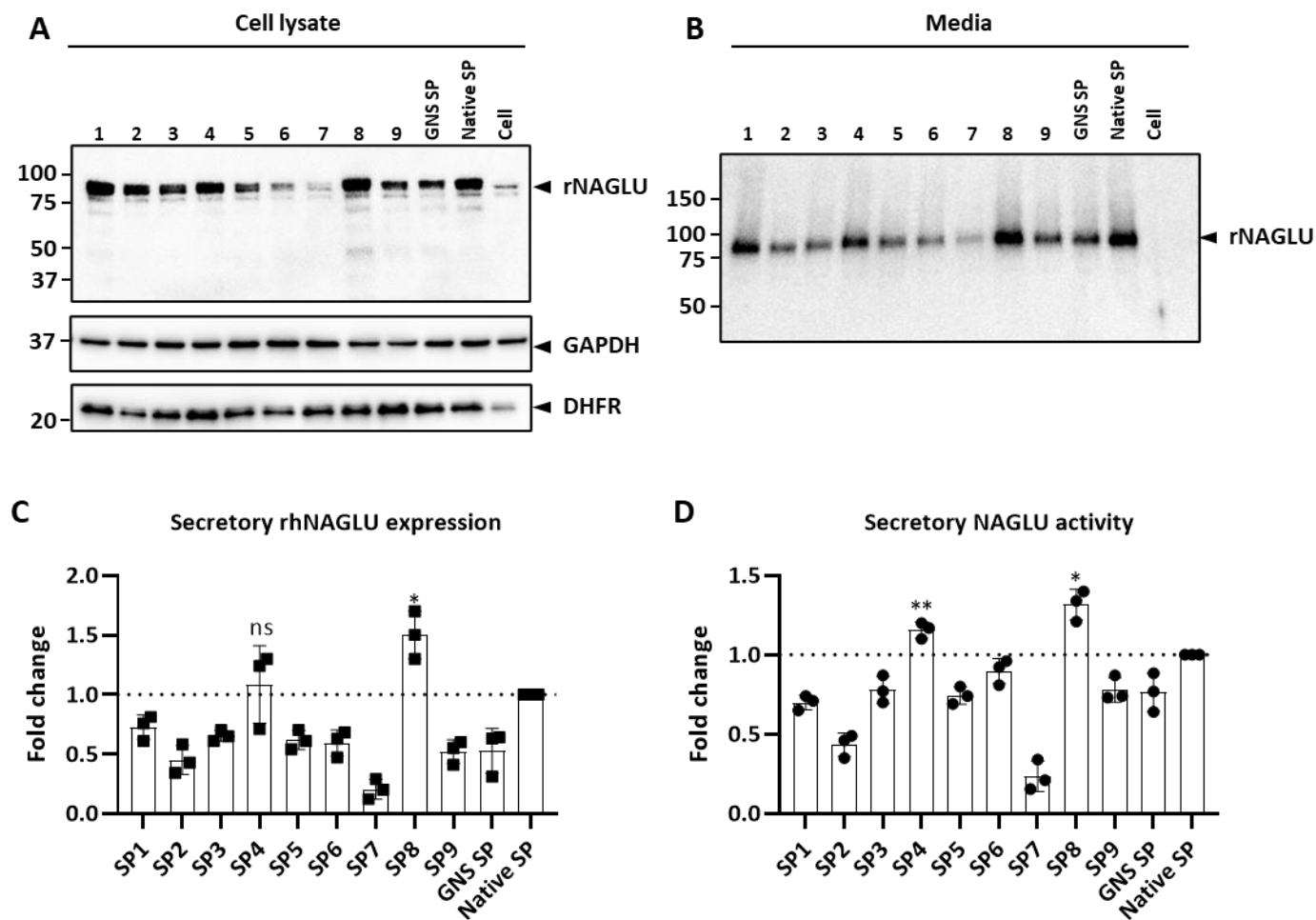


Figure 3. Secretory properties of rhNAGLU with artificial signal peptides in CHO K1 cells. NAGLU native signal peptide was switched by nine different signal peptides listed in **Table 1** and GNS signal peptide. **(A, B)** rhNAGLU expression in cell lysate and media from the transfected CHO K1 cells were determined by Western blotting using anti-myc antibodies. DHFR expression was determined for normalizing transfection efficiency. GAPDH, loading control. **(C)** rhNAGLU secretion was normalized with transfection efficiencies. **(D)** NAGLU activity in media detecting the liberated of 4-MU from the NAGLU substrate and normalized with transfection efficiencies. Value was presented as fold-change relative to native signal peptide, which is set to 1-fold. Data are shown as Mean \pm SD taken from three independent experiments. *P* values were determined by Student's *t*-test: *, *P* < 0.05; **, *P* < 0.01; ns, no significance.

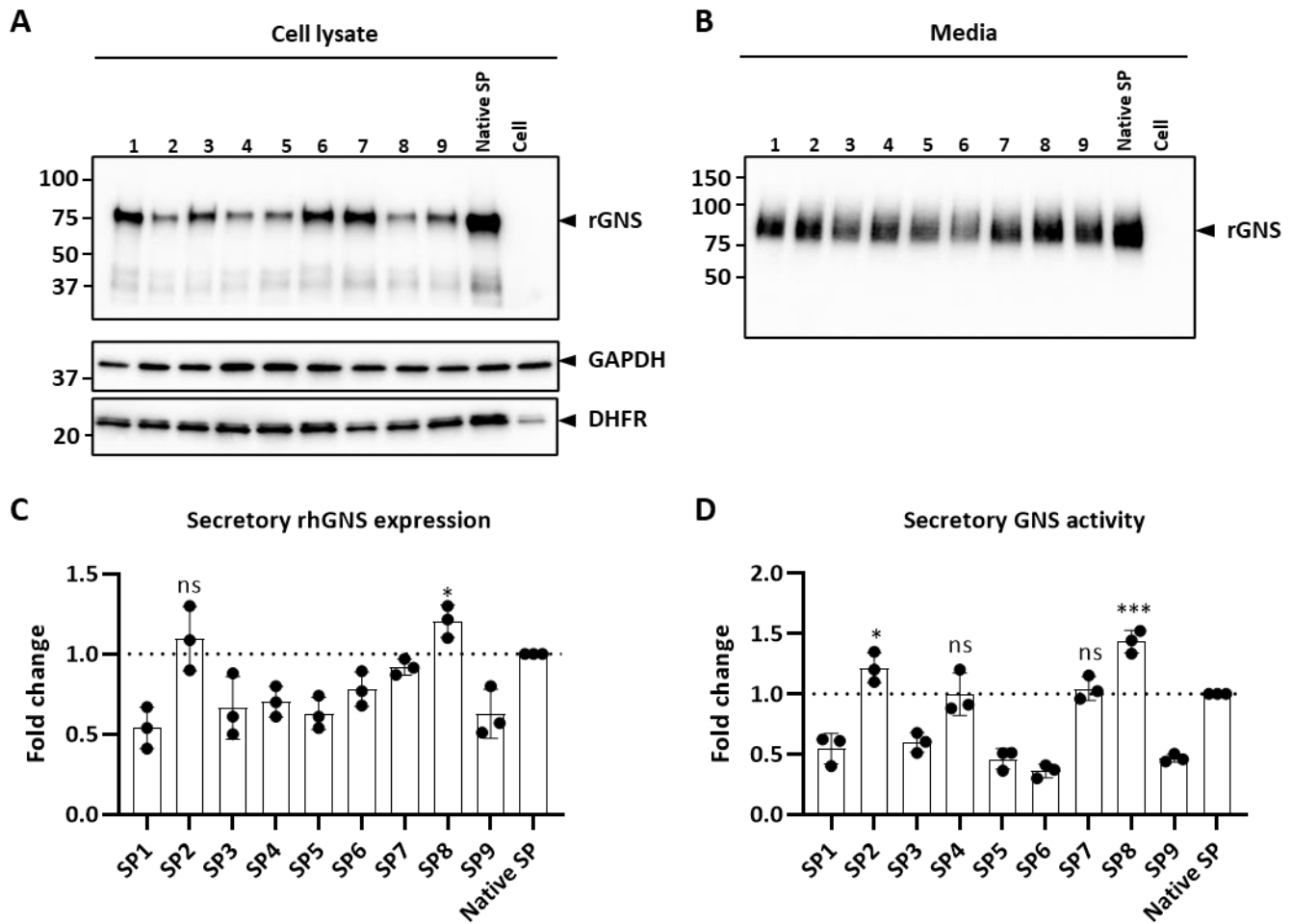


Figure 4. Screening of various signal peptides for rhGNS expression and secretion in CHO K1 cells.

(A, B) Immunoblots of cell lysate and medium from each transfected CHO K1 cells with different signal peptides. rhGNS expression was detected by anti-myc antibodies. DHFR, internal control for normalizing transfection efficiency. GAPDH, loading control. (C) rhGNS secretion was normalized respect to transfection efficiency. (D) GNS activity in media was analyzed by detecting the presence of 4-MU and normalized with DHFR expression. Value was presented as fold-change relative to native signal peptide, which is set to 1-fold. Data are shown as Mean \pm SD taken from three from three independent experiments. *P* values were determined by Student's t-test: *, *P* < 0.05; ***, *P* < 0.001; ns, no significance.

