Endo-lysosomal processing of N-glycans on acid alpha-glucosidase is critical to attain the most active enzyme for hydrolyzing glycogen

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Abstract

Acid alpha-glucosidase (GAA) is a lysosomal glycogen-catabolizing enzyme, a deficiency in which leads to Pompe disease. Pompe disease can be treated with systemic recombinant GAA enzyme replacement therapy (ERT), but data suggest the existing standard of care ERT may be inefficiently delivered to lysosomes in skeletal muscles. Several next-generation GAA ERTs are focused on increasing lysosomal delivery for improved efficacy. Once delivered to lysosomes, GAA undergoes proteolytic cleavage and glycan trimming, yielding a more active enzyme towards its natural substrate glycogen. The relative contributions of each of these processing steps for increasing GAA glycogen activity, however, are unclear, though proteolytic processing has been proposed as the primary contributor. To better understand the specific role of glycan processing, we investigated the role of N-glycan trimming for increasing GAA activity towards glycogen. We generated GAA variants with chemically modified terminal sialic acids on N-glycans that were resistant to neuraminidase activity in vitro. In cellulo, following GAA uptake in Pompe patient fibroblasts, these variants underwent proteolytic cleavage, but not glycan trimming. Lack of glycan trimming resulted in only a partially activated GAA as evidenced by its lower catalytic efficiency towards glycogen compared to fully processed GAA. We also generated enzymatically 'deglycosylated' GAA that showed improved enzyme kinetics towards glycogen without proteolytic processing. These results indicate that both proteolysis and N-glycan processing are required for full GAA activation. Taken together, these data imply that the ideal ERT for treating Pompe disease should have significantly improved lysosomal targeting to all muscles, along with complete proteolytic and glycan processing in lysosomes for optimal glycogen hydrolysis.

GAA processing

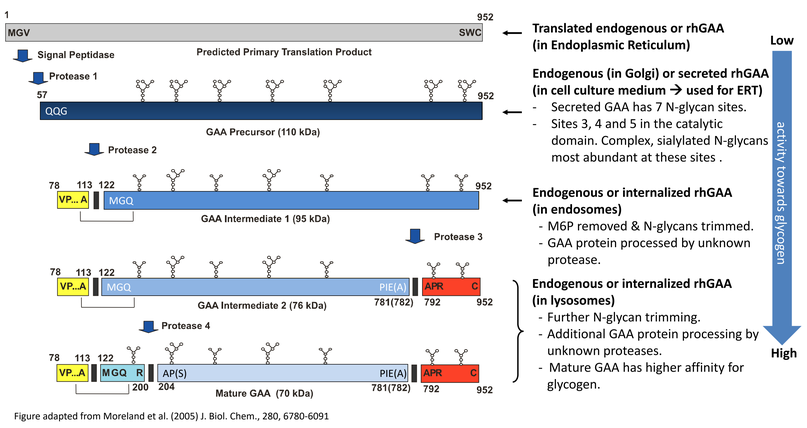


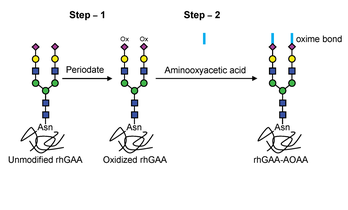
Figure adapted from Moreland et al. (2005) J. Biol. Chem., 280, 6780-6091

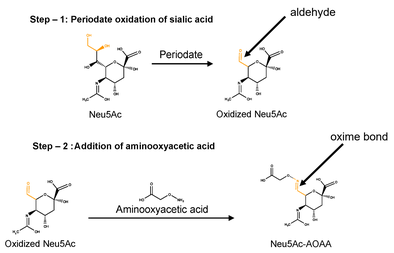
What is the relative contribution of glycan trimming and proteolytic cleavage on GAA activation? If unprocessed, would the complex N-glycan on site 5, closest to the active site of GAA, obstruct glycogen access to the active site?

Methods

- To dissect out the relative contribution of concomitantly occurring glycan trimming and proteolytic processing -> chemical modification of terminal sialic acids in complex N-glycans –> introduction of unnatural oxime bond -> obstruct sialidase activity -> block subsequent glycan processing.

Schematic of the chemical modification of sialic acids on rhGAA

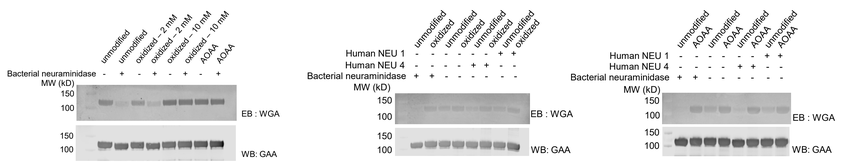




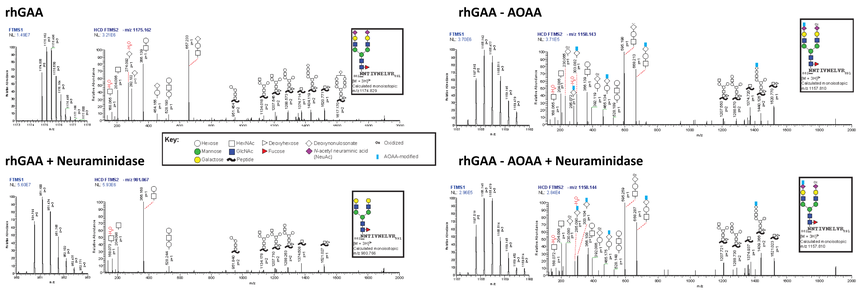
- Hypothesis -> rhGAA-AOAA -> upon uptake in to Pompe patient fibroblasts -> undergoes only proteolytic processing and no glycan trimming of complex glycans
- Variant of GAA that undergoes only glycan trimming and no proteolytic processing -> rhGAA expressed in the presence of mannosidase inhibitor kifunensine -> rhGAA-kif -> only high mannose type N-glycans -> removed *in vitro* by Endo H treatment
- Characterization of rhGAA-AOAA and rhGAA-kif -> Western/Eastern blotting and High-Resolution Tandem Mass Spectrometry (HRTMS) -> determine efficiency of chemical modification/kifunensine treatment -> understand how these variants processed *in cellulo* and *in vitro*.
- Compare glycogen hydrolytic activity of rhGAA variants above with activity of precursor unmodified rhGAA and fully processed (both glycan trimming and proteolytic processing) mature rhGAA -> normalization of relative GAA amounts in different protein preparations -> matching 4MU-α-Glc specific activity. Kinetics of hydrolysis 4MU-α-Glc not altered by GAA processing.
- Characterization of several commercial glycogen preparations -> Size Exclusion Chromatography (SEC) -> glycogen preparation with a weighted average molecular weight of ~600 kDa used for Michaelis-Menten kinetics Rate of glucose release measured by High-Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD).

Variants of rhGAA with only proteolytic processing

Sensitivity of oxidized rhGAA and rhGAA-AOAA to purified neuraminidases

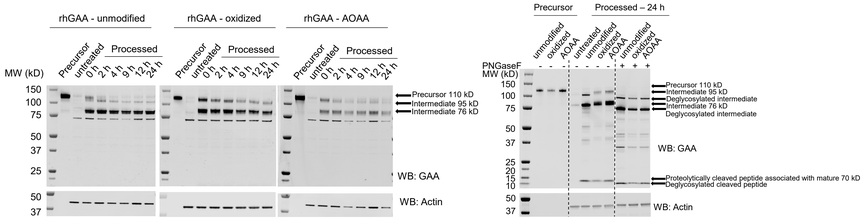


- RhGAA variants treated with purified bacterial or human lysosomal neuraminidases -> blotting analysis using WGA -> binds to sialic acid
- >90% terminal sialic acids on rhGAA oxidized with 10 mM periodate (HPAEC-PAD [data not shown]).
- RhGAA-AOAA with oxime bond -> insensitive to treatment with bacterial neuraminidase and human Neu4 and Neu1 *in vitro*.



- RhGAA variants treated with bacterial neuraminidase *in vitro* -> trypsinization -> by glycopeptide analysis using HRTMS.
- Representative Precursor MS1 and HCD MS2 spectra of highly abundant rhGAA tryptic glycopeptide, $_{882}$NNTIVNELVR$_{891}$, bearing the most abundant glycan structures at Asn882 (underlined) shown.
- Modified sialic acid on rhGAA - AOAA is insensitive to neuraminidase activity.

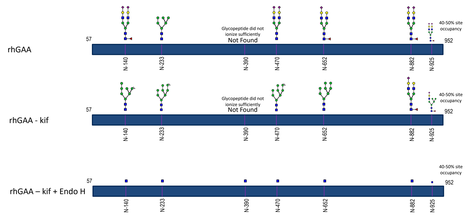
Sensitivity of oxidized rhGAA and rhGAA-AOAA to cellular neuraminidases

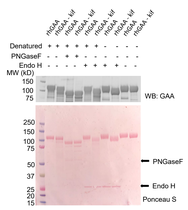


- Pompe patient fibroblasts incubated with 500 nM unmodified rhGAA, oxidized rhGAA or rhGAA-AOAA for 16 hours at 37°C -> uptake media replaced by growth media after 16 hours -> cells harvested at indicated time points -> lysed and analyzed by Western blotting.
- RhGAA and modified rhGAAs differentially processed following internalization.
- To differentiate between proteolytic and glycan processing -> PNGaseF digest performed on lysates harvested at 24-hours.
- Banding patterns suggest modification of rhGAA blocks glycan processing *in cellulo* without affecting proteolytic processing.

Oxidized rhGAA and rhGAA-AOAA are variants of rhGAA that only undergo proteolytic processing.

A variant of rhGAA with only glycan trimming



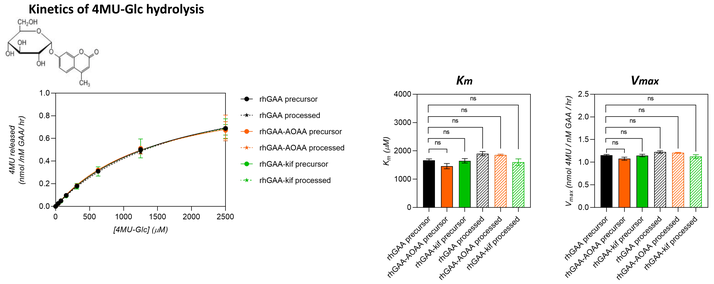


- Stably overexpressing rhGAA cell line cultured in the presence of kifunensine -> variant with mostly high mannose glycans.
- HRTMS analysis -> rhGAA variant had high mannose type glycans in highest abundance in most but not all N-glycan sites.
- Endo H treatment for 10 days at 30°C -> removal of the amenable glycan structures
- Graphical representation of MS data -> glycans observed with highest relative abundance at each site shown
- Complex structures also observed in Endo H treated samples at sites 6 and 7 - lower abundances than GlcNAc
- GlcNAc only observed structure on other sites including site 3.
- Mass shift with diffuse banding representative of partial deglycosylation in Western blotting experiments.
- Asn390, 470 and 652 are in the catalytic domain -> Asn 652 is closest to the active site of rhGAA
- Deglycosylation at this Asn 652 hypothesized to be necessary for improved access of glycogen to active site.

RhGAA-kif 'processed' by Endo H is partially deglycosylated but is proteolytically unprocessed.

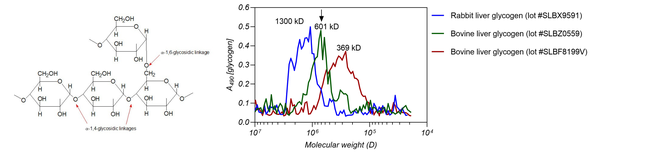
Kinetics of glycogen hydrolysis by fully or partially processed rhGAA

Kinetics of 4MU-Glc hydrolysis



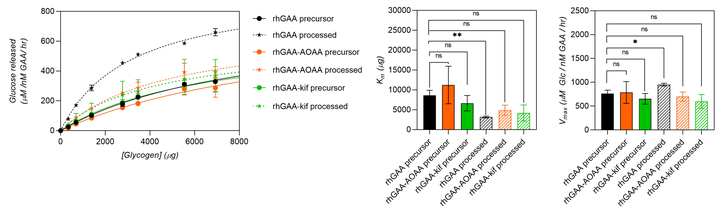
- 4MU-α-Glc - small synthetic substrate for GAA -> hydrolysis releases fluorescent product.
- 4MU-α-Glc hydrolytic activity of precursor rhGAA or fully processed rhGAA compared to the activity of rhGAA-AOAA (only proteolytically processed) or rhGAA-kif (only deglycosylated).
- Processing of rhGAA does not impact the 4MU-α-Glc hydrolytic activity
- No significant changes observed in K_m or V_{max} for partially processed rhGAA variants compared to precursor or mature rhGAA.

Characterization of glycogen size



- Glycogen is large and complex unlike 4MU-α-Glc
- Preparations of glycogen from different sources vary widely in molecular weight/chain length.
- Three commercially available glycogen preparations characterized by SEC.
- Medium sized (~601 kD) glycogen used for glycogen hydrolysis assays.

Kinetics of glycogen hydrolysis



- Glycogen hydrolytic activity of precursor rhGAA or fully processed rhGAA compared to the activity of rhGAA-AOAA (only proteolytically processed) or rhGAA-kif (only deglycosylated).
- Fully processed rhGAA has statistically significant ($p<0.05$) T-test) ~3.5-fold reduced K_m for glycogen and increased V_{max}.
- Partially processed rhGAAs both show small statistically insignificant reduction in K_m and no apparent change in V_{max} compared to precursor rhGAA.

Both proteolysis and N-glycan processing are required for full GAA activation. Only partial activation of GAA occurs if either glycan trimming or proteolytic processing are blocked.

Conclusions

- Oxidation of terminal sialic acid on N-glycans blocks glycan processing by inhibiting neuraminidase activity.
- Terminal sialic acid on rhGAA modified to contain unnatural oxime bonds is not hydrolyzed by human sialidases *in vitro or* within Pompe patient fibroblasts.
- Both proteolysis and N-glycan processing are required for full GAA activation. Only partial activation of GAA occurs if either glycan trimming or proteolytic processing are blocked.
- The ideal ERT for treating Pompe disease should not only have significantly improved lysosomal targeting to all muscles but must also undergo complete proteolytic and glycan processing in lysosomes for optimal glycogen hydrolysis.

Conflicts of interest

All authors, except JB, are current or former employees of Amicus Therapeutics Inc. and hold equity in the company.

Title: Endo-lysosomal processing of N-glycans on acid alpha-glucosidase is critical to attain the most active enzyme for hydrolyzing glycogen

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Presenting Author: Nithya Selvan

Institute: Amicus Therapeutics

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