Lysosomal Acid Alpha-Glucosidase Consists of Four Different Peptides Processed from a Single-Chain Precursor

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Running Title: Acid α-Glucosidase Consists of Four Peptides

Summary

Pompe disease is caused by a deficiency of the lysosomal enzyme acid α-glucosidase (GAA). GAA is synthesized as a 110-kDa precursor containing N-linked carbohydrates modified with mannose 6-phosphate groups. Following trafficking to the lysosome, presumably via the mannose 6-phosphate receptor, the 110-kDa precursor undergoes a series of complex proteolytic and N-glycan processing events yielding major species of 76- and 70-kDa. During a detailed characterization of human placental and recombinant human GAA, we found that the peptides released during proteolytic processing remain tightly associated with the major species. The 76-kDa form (a.a. 122-782) of GAA is associated with peptides of 3.9-kDa (a.a. 78-113) and 19.4-kDa (a.a. 792-952). The 70-kDa form (a.a. 204-782) contains the 3.9- and 19.4-kDa peptide species as well as a 10.3-kDa (a.a. 122-199) species. A similar set of proteolytic
fragments has been identified in hamster GAA, suggesting the multi-component character is a
general phenomenon. Rabbit anti-peptide antibodies have been generated to sequences in the
proteolytic fragments and used to demonstrate the time course of uptake and processing of
recombinant GAA precursor in Pompe fibroblasts. The results indicate the observed fragments
are produced intracellularly in the lysosome and not as a result of non-specific proteolysis during
purification. These data demonstrate the mature forms of GAA characterized by polypeptides of
76- or 70-kDa are in fact larger molecular weight multi-component enzyme complexes.
INTRODUCTION

Lysosomal acid α-glucosidase (GAA; EC 3.2.1.3) is an exo-1,4 and 1,6-α–glucosidase that hydrolyzes glycogen to glucose. The cDNA for GAA encodes a protein of 952 amino acids with a predicted molecular mass of 105-kDa (1). The newly synthesized precursor has an amino-terminal signal peptide for cotranslational transport into the lumen of the endoplasmic reticulum where it is N-glycosylated at seven glycosylation sites, resulting in a glycosylated precursor with an apparent molecular weight of 110-kDa.

The intracellular processing of GAA has previously been investigated (2) (3). These authors proposed after transport through the Golgi complex and targeting to the endosome/lysosome, the 110-kDa precursor is proteolytically processed at the amino-terminus, resulting in a 95-kDa intermediate with a sequence beginning at amino acid 122. Prior to this study, the 95-kDa intermediate was proposed to be proteolytically processed to a 76-kDa form, which was believed to occur between amino acids 816 and 881 (3). The 76-kDa form is then proteolytically processed at the amino-terminus at amino acid 204 to give the 70-kDa mature form (3). The nomenclature used for the processed forms of GAA is based on apparent molecular mass as determined by SDS-PAGE.

The identities of the proteases involved in the maturation of GAA have never been established. GAA has been purified from many different tissues such as bovine testis (4), rat liver (5), pig liver (6), human liver (7), rabbit muscle (8), human heart (9), human urine (10), and human placenta (2), (11). The predominant species observed are the 76- and 70-kDa mature forms. The fate of the cleaved fragments is unknown. Several reports have noted the presence of small polypeptides in highly purified preparations of the 76- and 70-kDa forms (12-14). It
was suggested the small polypeptides might be contaminants, degradation products, or a previously described GAA activation protein (15).

Proteolytic processing appears to be required for optimal activity towards the natural substrate glycogen. There is a sevenfold to tenfold increase in the affinity of the 76-/70-kDa species for glycogen as compared with the 110-kDa precursor (16,17). In addition to the proteolytic maturation of the GAA peptide backbone, there is extensive processing of the carbohydrate chains. GAA is targeted to the lysosomes by the mannose-6-phosphate receptor, but analysis of the carbohydrate chains from purified 76-/70-kDa GAA from human placenta revealed the absence of mannose-6-phosphate and additional carbohydrate processing (18).

A deficiency of acid $\alpha$-glucosidase causes Pompe disease, which results in the accumulation of glycogen in lysosomes. Pompe disease is an autosomal recessive disorder that varies from a fatal infantile form to a more slowly debilitating adult onset form, reviewed in (19). Efforts are underway to develop enzyme replacement therapy for Pompe disease. Several similar but distinct transgenic (13, 16) or recombinant (17, 18) GAA preparations have been developed for this purpose. Several reports have demonstrated that enzyme replacement therapy using the 110-kDa precursor form of GAA degrades lysosomal glycogen in cultured Pompe fibroblasts and GAA knockout mice (13, 16, 18). Although the 110-kDa form of GAA has been administered therapeutically, it is the mature forms that are found in the lysosome and it is the mature forms which are most active towards the glycogen substrate.

In this report, we demonstrate that proteolytically processed GAA purified from human placenta consists of four different peptides processed from a single chain precursor. We demonstrate that recombinant 110-kDa precursor GAA is internalized by Pompe fibroblasts via
the mannose-6-phosphate receptor and undergoes proteolytic cleavage where several of the cleaved fragments remain associated.
EXPERIMENTAL PROCEDURES

Materials

Concanavalin A, DEAE-Sepharose FF, and Superdex 200 prep grade were obtained from Amersham Pharmacia Biotech. α-methylglucoside, Benzamidine, MSX, and 4-methylumbelliferyl α-D-glucoside were obtained from Sigma. Other chemicals were reagent grade or better and were from standard suppliers. SDS-PAGE gels were obtained from Invitrogen. Antibodies were made by Washington Biotechnology (Maryland). Roller bottles were obtained from Corning. DMEM and FBS were obtained from JRH. DPBS was obtained from Hyclone.

Methods

Acid α-glucosidase Activity and Protein Assay

Acid α-glucosidase was assayed fluorimetrically in a microtiter plate using 4-methylumbelliferyl α-D-glucoside as a substrate in 25 mM sodium acetate pH 4.8 (12). Protein concentration was estimated by absorbance at 280 nm assuming $E_{1%}^1 = 10$ or using the Micro-BCA assay standardized with bovine serum albumin (Pierce)(20).

SDS-Polyacrylamide Gel Electrophoresis

Reduced and non-reduced samples and molecular weight markers (Amersham Pharmacia Biotech) were applied to a 4-20% or 10% Tris-Glycine SDS-PAGE gel (Invitrogen). Electrophoresis was performed at 150 volts for 1.5 hours and proteins were visualized with either Coomassie or silver stain (21).
Buffers

Buffer A contained 25 mM Tris-HCl, pH 6.5, 2 mM PMSF, 2 mM Benzamidine, 50 mM NaCl, and 0.25% Triton. Buffer B contained 20 mM Tris-HCl, pH 6.5, 1 mM CaCl₂, 1 mM MnCl₂, and 500 mM NaCl. Buffer C contained 20 mM Bis-Tris-HCl, pH 5.5. Buffer D contained 20 mM Bis-Tris-HCl pH 5.5, 500 mM NaCl. Buffer E contained 3 M (NH₄)₂SO₄, 75 mM Tris-HCl pH 6.5. Buffer F contained 1 M (NH₄)₂SO₄, 25 mM Tris pH 6.5. Buffer G contained 50 mM NaH₂PO₄ pH 6.5, 20 mM NaCl. Buffer H contained 40 mM Tris-HCl pH 8.3, 39 mM Glycine, 20% MeOH, and 0.04% SDS. Buffer I contained 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM CaCl₂, 0.05% Tween 20. Buffer J contained 20 mM NaH₂PO₄ pH 6.5.

Cell lines

Chinese hamster ovary CHO-K1 cells were obtained from American Type Culture Collection (Manassas, VA). The Pompe fibroblast cell line GM00248 was obtained from Coriell Cell Repositories (Camden, NJ). To our knowledge, the GAA mutation in this cell line is not known.

Purification of hamster acid α-glucosidase from Chinese hamster ovary cells

Step 1: Harvest cells and cell lysis – CHO-K1 cells were expanded to five hundred 1700 cm² roller bottles in media containing DMEM/1x GS, 10% dialyzed fetal bovine serum, 25 µM MSX, 5% CO₂ at 37°C. Each bottle was rinsed with 40 ml DPBS then rolled for 30 min at 37°C in the presence of 40 ml of cell lysis buffer A. The cell lysate was clarified with a 10” 0.2 µm Opticap filter (Millipore) and stored at –20°C.

Step 2: Concanavalin A — The clarified cell lysate was adjusted to 1 mM MnCl₂ and 1 mM CaCl₂ and applied to a Concanavalin A column (5.0 x 18 cm) equilibrated with buffer B at
50 ml/min. The column was washed with ~ 7 column volumes of buffer B and step eluted with buffer B containing 0.25 M α-methylglucoside. Three 1 liter fractions were collected and pooled.

**Step 3: Affinity Chromatography on Superdex 200** — GAA is retarded because of its affinity for the dextran backbone of the Superdex matrix. Solid (NH₄)₂SO₄ (0.39 g/ml) was added to the Concanavalin A eluate with stirring at 4°C. The suspension was stirred overnight at 4°C. The precipitate was collected by centrifugation at 38,400 x g for 30 min in a JLA 16.250 rotor and then dissolved in a final volume of 30 ml in buffer C. The sample was then applied to a Superdex 200 column (5 x 90 cm) preequilibrated in buffer D at 10 ml/min. Fractions of 40 mls were collected and assayed for acid α-glucosidase activity and protein.

**Step 4: DEAE-Sepharose FF** — Two activity peaks eluted from the Superdex 200 column. The first activity peak contained 25% of the recovered activity. Because no additional purity was gained in the first activity peak, we could not isolate GAA from that peak. However, the second activity peak to elute, which contained 75% of the recovered activity, was significantly enriched for GAA. The second peak was pooled and diluted with 2 volumes of buffer C and applied to a DEAE-Sepharose FF column (1.6 x 10 cm) equilibrated with buffer C at 10 ml/min. After washing with 5 column volumes, the column was developed with a 30 column volume gradient from 0.0 to 0.3 M NaCl in buffer C. Fractions of 10 mls were collected and assayed for acid α-glucosidase activity and protein.

**Step 5: Reverse Phase Chromatography** — Reverse phase narrow bore HPLC was performed using a Michrom BioResources Ultra-fast Micro Protein Analyzer (Michrom BioResources, Auburn, CA). An aliquot (~50 µg) of Chinese hamster acid α-glucosidase (DEAE fraction) was diluted 1:1 with “magic mix” (4) denaturant (4.0 M guanidine...
hydrochloride, 4.0 M urea, 7.5% acetonitrile, 0.15% trifluoroacetic acid, and 0.2% Zwittergent Z8 (Calbiochem) and applied to a 2.1 x 50 mm PLRP-S (4000 A pore size; 8 \text{um} \text{ particle size}) reverse phase HPLC column (Michrom BioResources) preequilibrated at 54°C in 98% eluent A (2% acetonitrile, 0.1% trifluoroacetic acid, and 0.03% Zwittergent ZC-8) and 2% eluent B (90% acetonitrile, 0.09% trifluoroacetic acid, and 0.03% Zwittergent ZC-8). The column was rapidly stepped to 20% eluent B and developed at 0.18 ml/min with a 7.2 ml linear gradient from 20 to 95% eluent B. Fractions of ~0.18 ml were collected.

Purification of mature acid $\alpha$-glucosidase from human placenta

The purification of human placental acid $\alpha$-glucosidase was based on previously reported methods (14,18).

Step 1: Homogenization of Tissue – Three human placentas were diced after the connective tissue had been removed and added to a Waring blender. An equal weight of ice-cold 25 mM NaCl was added and the tissue was homogenized for 3 minutes. The homogenate was then centrifuged at 38,400 $x$ g for 30 minutes at 4°C. The supernatant was clarified with a 4” Opticap 0.22 $\mu$m filter (Millipore).

Step 2: Concanavalin A — The clarified supernatant was adjusted to 1 mM MnCl$_2$ and 1 mM CaCl$_2$ and applied to a Concanavalin A column (2.6 x 20 cm) equilibrated with buffer B at 9 ml/min. The column was washed with ~10 column volumes of buffer B and step eluted with buffer B containing 0.25 M $\alpha$-methylglucoside. Three 0.25 liter fractions were collected and pooled.

Step 3: Phenyl-Sepharose 6 FF – The Concanavalin A eluate was adjusted to 1 M (NH$_4$)$_2$SO$_4$ with buffer E and applied to a Phenyl-Sepharose 6 FF column (1.6 x 20 cm)
equilibrated with buffer F at 6.5 ml/min. After washing the column with 5 column volumes of buffer F, the column was washed with buffer F containing 0.25 M (NH₄)₂SO₄. Placental GAA was eluted with a 5 column volume gradient from 0.25 to 0 M (NH₄)₂SO₄ in buffer F. Fractions of 15 ml were collected and assayed for acid α-glucosidase activity and protein.

Step 4: Affinity Chromatography on Superdex 200 – Peak fractions from the Phenyl-Sepharose elution were concentrated to 10 mls in a 50 ml stirred ultra-filtration cell (Amicon), using a 30,000 NMWL, polyethersulfone membrane (Millipore). The sample was then applied to a Superdex 200 column (2.6 x 65 cm) preequilibrated in buffer G at 2.5 ml/min. Fractions of 6 mls were collected and assayed for acid α-glucosidase activity and protein.

Step 5: Superose 6— A peak fraction from Superdex 200 was applied to a Superose 6 column (10 x 30 cm) equilibrated with buffer G at 2 ml/min. Fractions of 2 ml were collected and assayed for acid α-glucosidase activity and protein.

Recombinant human precursor and intermediate GAA (rhGAA)

Precursor and intermediate rhGAA were produced using CHO cells transfected with a vector containing a full length human GAA cDNA, following the method previously described (22). Based on the previously described method for the purification of rhGAA (23), rhGAA was purified from the CHO-conditioned media as follows: CHO cell culture harvest is clarified by sequential direct-flow filtration using a polyethersulfone prefilter followed by a Whatman Polycap 75 TC, 0.2 µm PES membrane filter. The clarified harvest is concentrated 10-fold by ultrafiltration with a 30 kDa spiral wound ultrafiltration membrane (S10Y30 from Millipore, Inc.). The concentrate is diafiltered into 25 mM sodium phosphate, pH 7.0 buffer. The diafiltered harvest is loaded onto a Q Sepharose column equilibrated with diafiltration buffer at
2.5 mg of rhGAA per mL of resin. The column is washed with equilibration buffer followed by 50 mM sodium acetate, pH 5.0 buffer, which elutes some of the mature forms of GAA. The enriched precursor form of rhGAA is eluted with a 25 mM sodium phosphate, 0.17 M sodium chloride, pH 7.0. The Q Sepharose Eluate is adjusted to 0.75 M ammonium sulfate and loaded onto a Butyl Toyopearl 650 C column equilibrated with 25 mM sodium phosphate, 0.75 M ammonium sulfate, pH 6.5 buffer at 8 mg of GAA per mL of resin. The column is washed with equilibration buffer and the rhGAA protein is eluted with a 15 mM sodium phosphate, pH 6.5.

Preparation of Recombinant Human GlcNAc-phosphotransferase

A detailed description of the preparation GlcNAc-phosphotransferase is in preparation. Briefly, a production cell line secreting recombinant soluble human GlcNAc-phosphotransferase was generated by transfection of CHO cells with a ‘double gene’ expression vector in pEE14.1/pEE6.1 (Lonza Biologics, Portsmouth, NH) containing modified human GlcNAc-phosphotransferase α/β−subunit sequences and the native γ−subunit sequence. The α/β-subunit cDNA contained four modifications of the native sequence. First, the N-terminal signal-anchor sequence was replaced by a signal peptide derived from mouse immunoglobulin kappa light chain. Second, the C-terminal transmembrane sequence was deleted by truncation following amino acid 1209. Third, the sequence at the α/β-cleavage site, (amino acids 924-929), was replaced by the sequence RARYKR introducing a recognition site for the processing enzyme furin. Fourth, a twelve amino acid epitope sequence recognized by monoclonal antibody HPC4 was added following the signal peptide cleavage site. The γ−subunit sequence was unmodified from the native sequence.
To prepare recombinant soluble GlcNAc-phosphotransferase, the production cell line was cultured in DMEM containing 10% fetal bovine serum in roller bottles incubated at 37°C in a 5% CO₂ atmosphere. Conditioned media was chromatographed on a column of HPC4 immobilized at 5 mg/ml on NHS-Sepharose (Amersham Pharmacia, Piscataway, NJ). Soluble GlcNAc-phosphotransferase was eluted with 100 mM NaCl, 50 mM Tris-HCl, pH 7.2 containing 5 mM EGTA and 15 mM MgCl₂. Following elution and buffer exchange into 150 mM NaCl, 50 mM NaAc, pH 6.5 containing 15 mM MgCl₂ GlcNAc-Phosphotransferase was stored at –80°C until use. The purified GlcNAc-phosphotransferase had a specific activity of approximately 10 micromole/hr-mg. A detailed description has been published in US patent 6,642,038.

Purification of highly phosphorylated recombinant human GAA (HP-rhGAA)

A detailed method for the production of HP-rhGAA will be described elsewhere (manuscript in preparation). Briefly, recombinant human GAA was expressed in CHO cells grown in the presence of kifunensine at 1 mg/L. Kifunensine selectively inhibits α-mannosidase I and causes the accumulation of Man₇₋₉GlcNac₂ oligosaccharides on glycoproteins. A preparation containing the 110-, 95- and 76-kDa forms was purified from the conditioned media and the glycans containing terminal mannose were phosphorylated by treatment with UDP-N-acetylglucosamine: Lysosomal enzyme N-Acetylglucosamine-1-phosphotransferase (GlcNac-phosphotransferase). The transfer of GlcNac-phosphate to terminal mannoses was performed at a GlcNac-phosphotransferase to GAA ratio of 1:10 (w/w). UDP-GlcNac was added to the reaction at 3 mM. The reaction was followed by the addition of 1% (w/w) N-Acetylglucosamine-1-phosphodiester α-N-Acetylglucosaminidase (uncovering enzyme). Uncovering enzyme removes the GlcNac to give mannose-6-phosphate. The 95-/76-kDa
intermediates were separated from the 110-kDa precursor by Superdex 200 affinity chromatography.

**Western blot analysis**

Samples were boiled in 4x sample buffer (NuPage, Invitrogen) and applied to a 4-20% Tris-Glycine SDS-PAGE pre-cast gel (Novex, Invitrogen) and run at 180 V for ~60 min in 25 mM Tris, 0.2 M Glycine. Proteins were transferred to PVDF membrane (Pierce) with a semi-dry blot apparatus (Bio-Rad) in buffer H at 20 V for 60 min. The membrane was blocked in 3% nonfat dry milk in buffer I for 30 min and then incubated with the designated antibody at 1 ug/ml in 3% milk in buffer I for 1 hr at room temperature. The membrane was washed 5X in buffer I and then the secondary horse radish peroxidase-conjugated anti-mouse, anti-goat, or anti-rabbit antibody was applied at 1 ug/ml in 3% nonfat dry milk in buffer I for 1 hr at room temperature. The membrane was washed 4 x in buffer I and then 4 x in buffer I without Tween 20. Acid α-glucosidase was visualized with the SuperSignal detection kit (Pierce).

**Antibodies**

Goat polyclonal antibody to human GAA (gt Anti-GAA) was produced by Ferrell Farms (McLoud, OK). Goats were immunized with highly purified placental GAA that had been mixed with Freund’s adjuvant. Rabbit polyclonal antipeptide antibodies to GAA were produced by Washington Biotech (Baltimore, MD). Rabbits were immunized with synthesized peptides coupled to KLH. The sequence for each peptide was derived from sequence within each GAA fragment as follows: anti-GAA 57-74 (amino acids QQQASRPGPRDAQAHPGR), anti-GAA 78-94 (amino acids VPTQCDVPPNSRFDCA), and anti-GAA 183-200 (amino acids IKDPANRRYEVPLETPRV). Monoclonal antibody to GAA (GAA1) was prepared by
immunizing mice with highly purified placental GAA that had been mixed with Freund’s adjuvant. Isolation of the GAA1 hybridoma followed standard procedures.

*Reduction, alkylation, and tryptic digestion of proteins*

Fifty μg of protein samples were denatured and reduced with 150 μl 6 M Guanidine-HCL, 0.1 M Tris at pH 8.5 and 2.5μl of 2 M DTT. The samples were overlaid with nitrogen and incubated at 55°C for 1 hour in darkness. After cooling, 12.5 μl of 10 % 4-vinylpyridine 6 M Guanidine-HCL and 0.1 M Tris at pH 8.5 was added to the protein samples. The samples were then overlaid with nitrogen and incubated at room temperature for 2 hours in darkness. The reaction was quenched with 12.5 μl 2 M DTT. After reduction and alkylation, the samples were dialyzed overnight with a Slide-A-Lyzer cassette (10,000 MW cut-off, Pierce) against 50 mM Tris, pH 8.5. Trypsin was added to the protein sample in a ratio of 1:50 (w/w). After an 18 hr incubation at 37°C, the digestion was quenched with 0.1% TFA and stored at -20°C for future analysis.

*MALDI TOF MS analysis of reduced and non-reduced protein samples*

Protein samples were mixed in a 1:1 ratio with saturated sinapinic acid matrix solution (50% of 0.1% trifluoroacetic acid/H₂O and 50% acetonitrile) and one μl of the mixture applied to the sample target. The data were acquired in positive, linear mode on a Voyager DE PRO (Applied Biosystems) MALDI-TOF mass spectrometer. Singly- and doubly- charged molecular ions of bovine serum albumin (Sigma) were used to calibrate for the mass range of 20-140 kDa. The singly charged molecular ions of insulin and apomyoglobin from calibration mixture 3 (Applied Biosystems) were used to calibrate the mass range of 2-20 kDa.
**Capillary LC/MS analysis of reduced and non-reduced protein samples**

Capillary LC/MS was performed on a Q-STAR qq TOF mass spectrometer (Applied Biosystems) interfaced with an Ultimate capillary LC system (LC Packings). Protein separations were performed on a Vydac C4 reverse phase column (320 µm x 50 cm, Microtech Scientific) with a mobile phase of 1% formic acid in water (A) and acetonitrile (B). The flow rate was 4 µl/min. The mass spectra were acquired in the positive mode in range of m/z 800-3000. Protein reduction was performed by adding one µl of 10% β-mercaptoethanol to every 10 µl of protein sample. The samples were sonicated for 25 min and heated at 100°C for 1 min. before mass spectrometric analysis.

**Capillary LC/MS/MS peptide mapping of protein tryptic digests**

On-line capillary LC/MS/MS was performed on both Esquire LC (Bruker) and Q-STAR qq TOF systems. Proteins were separated on a 320 µm x 15 cm (Microtech Scientific) Vydac C18 column, using chromatography conditions previously described. On both instruments, the two most intensive peaks with m/z greater than 350 were chosen for on-line MS/MS.

**Amino-terminus micro-sequencing**

Samples of reduced and non-reduced rhGAA and placental GAA were applied to a 4-20% or 6% Tris–Glycine SDS-PAGE gel (Invitrogen). Electrophoresis was performed at 150 volts for 1.2 hours. The proteins were transferred to a PVDF membrane using 10 mM CAPS, pH 11.0. Transfer was performed for 1 hour at 100 volts. Protein bands were visualized with Coomassie Blue stain. Fifteen cycles of automated Edman chemistry were performed on protein bands excised from PVDF electroblots. N-terminal sequencing was performed with the Procise
Protein Sequencer Model 492 (Applied Biosystems), using the preprogrammed pulsed liquid PVDF method.

Affinity Chromatography of GAA

Twenty µg of purified human placental GAA and rhGAA were analyzed by affinity chromatography using a Superdex 75 HR 10/30 column (Amersham Pharmacia Biotech). GAA is retarded because of its affinity for the dextran backbone of the Superdex matrix. The column was equilibrated in 20 mM NaHPO₄ pH 6.0, 200 mM NaCl, at a flow rate of 0.5 mL/min. Peaks were detected at 215 and 280 nm with an 1100 HPLC system equipped with a diode array detector (Agilent). The column was calibrated with Gel Filtration Standard (Bio-Rad) prior to sample analysis. Fractions were collected at 2-minute intervals and analyzed by SDS-PAGE.

Fibroblast uptake of HP-rhGAA

For each time point, approximately 0.5 million Pompe fibroblasts (GM00248) in DMEM medium plus 10% FBS were incubated with 50 nM highly phosphorylated GAA prepared as described above. At designated time points, the cells were removed and washed 5 times with phosphate buffered saline and stored at –80°C. At 24 hours, the cells were washed and fresh media that did not contain GAA was added. After the final time point, all cell pellets were thawed and lysed simultaneously with 0.25% Triton. Cellular debris was pelleted and western blot analysis was performed on supernatants from each time point with several different antibodies to GAA. The blot was developed as described under “Western blot analysis.”
RESULTS

Placental GAA Contains Low Molecular Weight Polypeptides Derived from GAA

GAA was purified from human placenta, using Concanavalin A Sepharose, Phenyl Sepharose and Superdex 200 chromatography. The final step is affinity chromatography, which takes advantage of the retardation of GAA because of its affinity for the dextran backbone of the Superdex matrix (11, 14). Analysis of purified human placental GAA by silver stained SDS-PAGE demonstrated two consistent low molecular weight polypeptides with apparent masses of 19- and 10- kDa in addition to the expected 76- and 70-kDa bands (not shown). Amino-terminal sequence analysis of PVDF transferred protein indicated that the amino-terminus of the 19- and 10- kDa forms were APREPAIHSEGQ and MGQPXXFFPP, respectively. A database search using the BLASTP Algorithm (24) indicated the sequences of the 19- and 10- kDa polypeptides were from human GAA and corresponded to amino acids 792-803 and 122-131 respectively.

Since processed GAA is significantly retarded on Superdex 200, it was uncertain if the apparent co-elution of the 19- and 10-kDa polypeptides with the 76- and 70-kDa proteins indicated a physical association. To provide evidence that the 19- and 10-kDa polypeptides were physically associated with the 76- and 70-kDa polypeptides, human placental GAA was chromatographed on Superose 6 after Superdex 200. Unlike Superdex 200, Superose 6 does not have a dextran backbone and does not function as an affinity matrix for GAA. GAA eluted as a symmetric peak with an apparent molecular weight of 75,000 Da as previously reported, Fig. 1A (14). As shown in Fig. 1B, analysis of the Superose 6 fractions by SDS-PAGE demonstrates the 19- and 10- kDa polypeptides co-elute with the 76- and 70-kDa proteins at an apparent molecular weight of 75,000 Da, suggesting they are physically associated.
Characterization of Recombinant Human GAA Precursor and Processing Intermediates

It has been shown previously that recombinant CHO cells can be engineered to secrete the 110-kDa precursor form of human GAA into tissue culture media (25). In addition to the 110-kDa precursor form, recombinant CHO cells also release proteolytically processed forms of GAA into the media by an undetermined mechanism. In Fig. 2A, recombinant precursor (rhGAA) (lane 2), processed intermediates (lane 3), and mature GAA from placenta (lane 4) are compared by SDS-PAGE. The intermediate fraction contains bands of ~108-, 95-, and 76-kDa. The availability of these intermediates provided a unique opportunity to study the processing/activation of GAA. In Fig. 2B, the preparations shown in 2A are analyzed by affinity chromatography on Superdex 200. In each case virtually all the protein is derived from GAA. Inspection of the chromatograms demonstrated the 110-kDa precursor eluted earlier than the placenta derived 76-/70-kDa mature GAA and the intermediate preparation contained both early and late eluting material. SDS-PAGE analysis of the late eluting material revealed the 76- and 70-kDa forms were not separated by affinity chromatography (data not shown). The later elution of the processed forms suggest a higher affinity for the matrix, which is consistent with previous reports that the 76-/70-kDa species has a Km for glycogen which is sevenfold to tenfold lower than the 110-kDa precursor (16,17).

Characterization of Recombinant Human GAA Precursor and Processing Intermediates by Mass Spectroscopy and Amino-terminal Micro-sequencing

To fully characterize GAA processing, we utilized a combination of MALDI-TOF mass spectroscopy, amino-terminal micro-sequencing, capillary LC/MS, and capillary LC/MS/MS of tryptic peptide maps. This allowed us to determine the amino- and carboxyl-terminus for each
polypeptide and the structure of the \(N\)-glycans. The results of these analyses are summarized in Table I.

When recombinant GAA precursor was examined by MALDI-TOF, a molecular mass of 111,600 Da was determined. Amino terminal micro-sequencing identified amino acid 57 as the amino-terminus. Tryptic digest followed by reverse phase HPLC and peptide identification by mass spectroscopy identified amino acid 952 as the carboxyl terminus. The glycosylation is heterogeneous and is estimated at \(\sim 2,000\) Da per glycan. These results for the precursor (mass 111,600) are in reasonable agreement with the calculated molecular weight of the polypeptide and carbohydrate of 113,372 Da.

When the processing intermediates, containing predominantly 95- and 76-kDa species by reduced SDS-PAGE (Fig. 2A, lane 3) were analyzed unreduced by MALDI-TOF, major species with masses of \(\sim 103.5\) - and \(\sim 83.6\)-kDa were observed. After reduction, masses of \(\sim 99.8\) - and \(\sim 79.7\)-kDa were observed indicating a consistent mass shift of \(\sim 3.9\)-kDa, consistent with an interchain disulfide bond.

Analysis of human placental GAA by reduced SDS-PAGE (Fig. 1) identified polypeptides with apparent molecular weights of \(\sim 95\) -, 76-, 70-, 19-, and 10-kDa. MALDI-TOF MS analysis of reduced placenta GAA determined the corresponding masses were \(\sim 99.8\), \(\sim 79.7\), 69.3-, 19.4-, and 10.3. MALDI-TOF MS analysis of unreduced placenta GAA revealed polypeptides with masses of \(\sim 103.5\) -, \(\sim 83.6\) -, 69.3-, 19.4-, and 14.3-kDa. These data show the \(\sim 103.5\) -, \(\sim 83.6\) -, and 14.3-kDa polypeptides all shift by \(\sim 3.9\)-kDa after disulfide bond reduction, again suggesting the presence of an interchain disulfide bond. This experiment was also repeated by LC/MS, using a QSTAR Qq TOF mass spectrometer. The \(\sim 3.9\)-kDa mass shift seen in all reduced samples correlated with a new peak in the spectra with a mass of 3,927 Da.

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The GAA sequences found in each of the identified polypeptides was determined by a combination of micro-sequencing, tryptic digestion, capillary LC/MS and LC/MS/MS. The 3.9-kDa polypeptide with a determined mass of 3,927 corresponded to amino acids 78-113. The 10.3-kDa polypeptide was identified as amino acids 122-199. The 70-kDa polypeptide corresponded to amino acids 204-782. The 19.4-kDa polypeptide was identified as amino acids 792-952. These results are summarized in Table I.

Modification of N-glycans during GAA Maturation

The GAA precursor contains seven consensus sites for N-glycosylation which are all utilized. In recombinant GAA on average these sites are occupied by 5-6 complex type glycans and 1-2 high-mannose type glycans. In contrast GAA expressed in human placenta contains predominantly high-mannose type glycans (18). In either case, the newly synthesized glycans each contain about 9-11 hexoses/hexosamines and have an average molecular mass of ~2000 Da.

By mass spectroscopy the recombinant precursor contains ~14,000 Da of carbohydrate indicating an average of ~10 monosaccharides per glycan (Table 1). The 95-kDa intermediate contains 7,300 Da of carbohydrate at these same seven glycosylation sites indicating the glycans have been truncated to an average of ~5 monosaccharides consistent with an average core structure composed of 2 GlcNac and 3 mannose which can be derived from either a complex type or high-mannose type glycans. These results suggest the conversion of the 110-kDa precursor to the 95-kDa intermediate has occurred in a subcellular compartment containing a variety of glycosidases capable of cleaving a complex type glycan. This pattern of finding only highly truncated glycans on the processed GAA is repeated for all the components of the 76- and 70-kDa complexes (Table 1). For the fragments containing 1, 2, or 4 glycosylation sites the
composition can be directly confirmed to contain two hexnac and three hexose per site consistent with the structure \((\text{GlcNac})_2(\text{Man})_3\) which may also contain fucose. The complete absence of proteolytically processed intermediates whose glycans have not been truncated strongly suggests this processing occurs in a compartment containing both proteases and glycosidases, most likely in the lysosome or late endosome.

Identification of Subunits with Antipeptide Specific Antibodies

To independently evaluate the model for GAA maturation derived from mass spectroscopy; polyclonal, monoclonal, and antipeptide antibodies were generated to the following peptide sequences QQGASRPGPRDAQAHPGR (amino acids 57-74), VPTQCDVPPNSRFDCAP (amino acids 78-94), and IKDPANRRYEVPLETPRV (amino acids 183-200) as described in “Experimental procedures.” The resulting affinity purified antiserum are described as anti-GAA 57-74, anti-GAA 78-94, and anti-GAA 183-200; respectively. Additionally, a goat polyclonal antibody to placental GAA (gt anti-GAA) and a monoclonal antibody to the 70-kDa polypeptide (GAA 1) were generated as described in “Experimental Procedures.”

As shown in Fig. 3, western blot analysis was performed with a variety of antibodies on a blot containing recombinant 110-kDa precursor HP-rhGAA (P), an intermediate preparation (I) of 95/-76-kDa forms from the purification of HP-rhGAA, and the mature 76/-70-kDa species (M) purified from human placenta. HP-rhGAA is a highly phosphorylated form of rhGAA derived from \textit{in vitro} phosphorylation as described in “Experimental Procedures.” The monoclonal antibody GAA1 recognizes the 110-, 95-, 76-, and 70-kDa species (Fig. 3A, lanes 1-3). The blot was then probed again with the gt anti-GAA antibody, which demonstrated the
presence of the 19-kDa fragment, lanes 4-6. The 19.4-kDa fragment exists as a doublet in the recombinant intermediate preparation due to differences in glycosylation (data not shown). The blot was then probed again with anti-GAA 183-200 (Fig. 3A, lanes 7-9). This revealed the presence of a 10-kDa polypeptide in the intermediate and mature GAA samples (Fig. 3A, lane 8-9). These antibodies were helpful to understand the order of the cleavage events as shown in Fig. 3B.

Anti-GAA 57-74 identifies the 110-kDa precursor enzyme, which is consistent with the mass spectrometry and amino-terminal micro-sequence analysis of this preparation, but the antibody does not recognize the intermediate preparation, containing 95-/76-kDa, or the mature preparation containing the 76-/70-kDa mature forms (Fig. 3B, lanes 1-3). Since amino acids 57-74 are not detected in the intermediate preparation, it can be concluded the cleavage to release this sequence occurs early in the maturation process. It has not been possible to identify this small peptide by western blotting using either nitrocellulose or PVDF membranes. It is possible this small peptide does not bind tightly to the membrane, however, it is more likely lost during processing since the peptide was not detected by tryptic digestion/ms/ms.

Monoclonal antibody GAA1 (Fig. 3B, lane 4-6) detects the 110-, 95-, 76- and 70-kDa species, while anti-GAA 183-200 (Fig. 3B, lane 9) only detects the 110-, 95-, and 76-kDa polypeptides and does not detect the 70-kDa species. A 10.3-kDa fragment is detected by the anti-GAA 183-200 which is not recognized by the monoclonal antibody GAA1. The cleavage event which converts the 76-kDa enzyme to the 70-kDa form results in the formation of a 10-kDa fragment which was shown in Fig. 1 to co-chromatograph with GAA activity. Since the 10.3-kDa fragment is only faintly observed in the 95-/76-kDa intermediate
preparation, containing predominantly 95- and 76-kDa forms, we conclude the formation of the 10.3-kDa polypeptide occurs late in the maturation of GAA.

The presence of the 3.9-kDa polypeptide, which was originally identified by mass spectroscopy, is confirmed by western blot in Fig. 4. The blots contained recombinant 110-kDa precursor HP-rhGAA (P) (lanes 1 and 4), a preparation of intermediate 95-/76-kDa forms (I) from the purification of rhGAA (lanes 2 and 5), and the mature 76-/70-kDa species (M) purified from human placenta (lanes 3 and 6). The western blot shown in Fig. 4A was probed with anti-GAA 57-74. The intermediate preparation (I) from the purification of rhGAA was enriched for the 95-/76-kDa intermediates, however, the preparation contained some precursor (Fig. 4 A, lane 2) which migrated slower than the precursor in Fig. 4 A, lane 1. The difference in mobility is due to glycosylation differences between HP-rhGAA precursor and rhGAA precursor (data not shown).

The blot shown in Fig. 4A was then probed with the antipeptide specific antibody for the 3.9-kDa polypeptide (amino acids 78-94) as shown in Fig. 4B. The antibody detected the 95-, and 76-kDa polypeptides in the absence of β-mercaptoethanol (Fig. 4B lanes 5-6). The samples were run on a 4-20% SDS-PAGE as shown in Fig. 4C. We were not able to detect the 3.9-kDa fragment when run under reducing conditions, however, when the samples were not reduced an ~14-kDa polypeptide was observed (Fig. 4C lane 6). The ~14 kDa polypeptide is comprised of the 3.9-kDa polypeptide disulfide bonded to the previously observed 10-kDa polypeptide.

GAA Exists as a Complex in Other Organisms

To determine if cleaved polypeptides are associated with GAA in other tissues, we purified endogenous hamster GAA from CHO cells. To our knowledge hamster GAA had never
previously been isolated from a cultured cell line. The purification is described in detail in “Experimental Procedures.” Silver stain SDS-PAGE analysis of hamster GAA revealed a predominant protein with an apparent molecular mass of 70-kDa (Fig. 4). The 70-kDa protein was isolated by reverse phase chromatography and the amino-terminus was micro-sequenced. The determined amino-terminal sequence, APSQLYSVEFSEEPF, is highly homologous to the amino-terminus for the human 70-kDa mature enzyme from amino acid positions 204-218 (APSPLYVEFSEEPF).

Recombinant 110-kDa human precursor GAA, GAA purified from placenta, and hamster GAA purified from CHO cells were compared by SDS-PAGE (Fig. 5A, lanes 1-3). Unlike placenta GAA, only the 70-kDa species was present in the highly purified preparation of hamster GAA from CHO cells. Western blot analysis was performed on hamster GAA with anti-GAA 183-200 (Fig. 5B, lane 1,3) and gt anti-GAA (Fig. 5B, lanes 2,3). The blots reveal the 19-kDa and 10-kDa fragments are present in a highly purified preparation of hamster GAA from CHO cells. The presence of these subunits in highly purified GAA preparations from human placenta and CHO cells demonstrates that the formation and association of these subunits is not tissue or species specific.

The 19-kDa polypeptide in CHO preparations appears as a doublet. The cDNA for human GAA encodes for 2 glycosylation sites in the 19-kDa fragment. To determine if carbohydrate differences are responsible for the observed doublet, human placenta GAA and hamster GAA were denatured and digested with endoglycosidase H (Endo H) and peptide: N-glycosidase F (PNGase F). PNGase F is an amidase that cleaves between the innermost GlcNac and asparagine residues of high mannose, hybrid and complex oligosaccharides from N-linked glycoproteins (26). Endo H is a glycosidase that cleaves the chitobiose core of high mannose and
some hybrid oligosaccharides from \(N\)-linked glycoproteins \(26\) Endo H digestion did not have any affect on the 19.4-kDa polypeptide from human placenta or hamster GAA (Fig. 5C, lanes 1, 2, and 6). This suggests the carbohydrate is not high mannose-type. However PNGase F digestion decreased the apparent size of the 19.4-kDa polypeptide for both human placenta and hamster GAA (Fig. 5C, lanes 3, 4, 7, and 8). The 19-kDa doublet in the hamster preparation was reduced to a single band, which suggests glycosylation differences are responsible for the doublet.

Proteolytic Processing of 110-kDa Precursor after uptake in Fibroblasts

To directly demonstrate the proteolytic processing of GAA in the cell proceeds via the pathway described, we utilized M6P-receptor mediated endocytosis of GAA precursor and a GAA deficient fibroblast cell line (GM00248) derived from a Pompe patient. To increase the amount of GAA internalized HP-rhGAA was used. The protein sequence of HP-rhGAA is identical to rhGAA precursor but differs in that the \(N\)-glycoyslation of HP-rhGAA contains 7-11 M6P groups per molecule of GAA. This was determined by LC/MS/MS. The evidence for this will be described elsewhere (manuscript in preparation). At the designated time points, cell lysates were prepared and analyzed by western blotting, using anti-GAA 57-74 (Fig. 6A), a monoclonal antibody to the 70-kDa polypeptide (Fig. 6B), and an anti-GAA 183-200 (Fig. 6C). In Fig. 6A only the GAA precursor standard is identified by anti-GAA 57-74. Fragments containing this sequence are not identified in any of the lysate derived samples. This is consistent with rapid release of this peptide upon internalization. In Fig. 6B, the same blot probed with monoclonal antibody GAA1 shows the conversion of the 110 kDa precursor to the transient 95 kDa intermediate followed by slower processing to the 76 kDa and 70 kDa forms.
In Fig. 6C, a western blot of the lysates was probed with anti-GAA 183-200, demonstrating the appearance of the 10.3-kDa fragment correlates with the conversion of the 76- to 70-kDa form. These results suggest processing takes place by an ordered and sequential series of steps.
DISCUSSION

It is well known GAA is synthesized as a 110 kDa precursor of 952 amino acids yet exists in lysosomes as major species of 76- and 70-kDa. It is generally agreed that the conversion from the 110 kDa to the 76- and 70-kDa is a result of proteolytic processing at both the amino- and carboxyl-termini. However, the structure of the 76- and 70-kDa mature forms of the enzyme has not been determined. Furthermore, the details and sequence of the processing steps is incomplete. In this study, we demonstrate fully processed GAA, consists of four associated polypeptides all derived from the GAA precursor. The ability to determine the structure of each fragment, using sensitive mass spectroscopic techniques, allows us to propose the model for GAA maturation which is shown in Fig. 7 and supported by Table I.

The GAA predicted primary translation product of 952 amino acids containing seven N-glycans is probably present only transiently and was not detected. The first proteolytic processing step is likely cleavage between amino acids 28 and 29 by signal peptidase, again the product of this cleavage was not detected. The first precursor identified in this study, contains amino acids 57-952 apparently resulting from a proteolytic cleavage between amino acids 56 and 57 (Fig. 7 and Table I). A similar precursor beginning at amino acid 70 has been identified in human urine (10). The first intermediate identified, designated intermediate 1, consists of a 95-kDa polypeptide (a.a. 122-952) covalently linked via a disulfide bond to a 3.9-kDa polypeptide (a.a. 78-113). Multiple cleavages are required to generate these fragments. The identification of the 3.9-kDa disulfide linked polypeptide is entirely novel. The sequences 57-78 and 113-122 are not found, apparently having been lost during processing. The availability of the antipeptide antibody anti-GAA 57-74 allows confirmation that this sequence is not present in intermediate 1 (Fig. 3B, lane 2).
Interestingly almost all of the reduction in molecular weight going from the 110-kDa precursor to intermediate 1 is attributed to the loss of carbohydrate due to extensive glycan trimming. We have not observed glycans on intermediate 1 that were not trimmed. This suggests the processing that yields intermediate 1 takes place in a late endosome or lysosome, containing multiple glycosidases.

In the next step the 95-kDa intermediate is proteolytically cleaved at the carboxyl-terminus by an unknown protease, which we have designated as protease 3. The cleaved carboxyl-terminal 19.4-kDa fragment (a.a. 792-952) and the 3.9-kDa fragment remain associated with the 76-kDa polypeptide (a.a. 122-781). In the final step, the 76-kDa polypeptide is then proteolytically cleaved near the amino-terminus by an unknown protease, which we have designated as protease 4, to give the final complex consisting of the following polypeptides: 70-kDa (a.a. 204-781), 19.4-kDa (a.a. 792-952), 10.3-kDa (a.a. 122-200), and 3.9-kDa (a.a. 78-113). Interestingly at each cleavage site four to nine amino acids are not present in the final products (a.a.114-121, 200-203, and 783-791), whether this is the result of two specific proteolytic steps or a single cleavage followed by nonspecific aminopeptidase or carboxypeptidase activity has not been determined.

Interestingly there was no evidence that the three proteolytic processing steps could occur in any order except that proposed in our model. Had these cleavages occurred in a different order additional intermediates would have been formed which were not observed. The understanding that GAA maturation proceeds through a series of discrete, ordered steps may also have application as the basis for diagnostic tests for GSDII. In particular the identification of the fragment with masses of 3,927-, 10,392-, and 19,447-Da might be a sensitive indicator GAA is
present and maturation has proceeded successfully. Additionally, the detection of such polypeptides could be used to monitor enzyme replacement therapy of Pompe disease.

The association of GAA’s proteolytically cleaved fragments is not unique for lysosomal enzymes. The lysosomal alpha-mannosidase enzyme is synthesized as a single-chain precursor which is processed into three glycopeptides of 70-, 42-, and 15-kDa. The 70-kDa glycopeptide is further partially proteolysed into three more peptides that are joined by disulfide bridges (27).

The previously described GAA2 allele (D91N) (23, (28) is located in the 3.9-kDa (a.a. 78-113) fragment. This substitution resulted in a decreased affinity of the mature enzyme for starch, leading the authors to suggest a fragment containing the substitution might be associated with the mature enzyme. Our findings present direct physical evidence for both existence of this fragment and its association with mature GAA. Except for frameshifts, no disease causing mutations have been reported in the 10.3-kDa fragment. Several mutations have been described in the 19.4-kDa fragment (a.a. 792-952) which result in a severe phenotype. The in-frame deletion of one amino acid (Lys903) and the common deletion of exon 18 that results in a 55 amino acid deletion from amino acids 828-882 both result in the fatal infantile form of the disease (29). Other mutations in the 19.3 kDa fragment include R854X and a frameshift mutation occurring at P913 both resulting in severe phenotypes. Prior to this study, it was believed the common exon 18 deletion contained the carboxyl-terminal proteolytic cleavage site based on the belief that the carboxyl-terminal cleavage occurs between amino acids 816 and 881 (3). We have shown that the cleavage site is actually 36 amino acids upstream from the beginning of this common deletion.
The maturation process is not dependent on an active GAA enzyme. A patient with infantile glycogenosis type II was found to be homozygous for a E521K substitution (30). When COS cells were transfected with constructs containing the E521K mutation, the GAA was inactive and GAA maturation was prevented. However, when E521 was changed to E521Q, maturation of GAA was restored while there was still a loss of activity.

Since mature GAA consists of four polypeptides, this may have implications in treating GSDII. One current strategy for treating GSDII is to use enzyme replacement therapy. Using GAA -/- knockout mice (31,32), several groups have attempted enzyme replacement therapy with precursor and mature forms of recombinant GAA. Recombinant human 110-kDa precursor GAA purified from the milk of transgenic mice and rabbits demonstrated a therapeutic effect in knockout mice (17,33). Analysis of the tissues revealed the 110-kDa precursor from the rabbit milk and mouse milk was converted to the 76-kDa lysosomal form. Neither enzyme preparation appeared to be converted significantly to the 70-kDa form. Another study reported using the mature form of GAA (the composition was not described) to reverse muscle weakness in a knockout mouse (34). Our improved understanding of the processing pathway may help us develop more effective second generation candidates for enzyme replacement therapy.

Sequences of five human family 31 glucohydrolases; GAA, maltose isoamylase (MGAM), sucrose isomaltase (SIM), glucosidase II (G2AN), and neutral α-glucosidase C (GANC) (35); have all been aligned using Clustal W (Fig. 8) (30). Since the maltase isoamylase and sucrose isomaltase sequences have undergone duplication and contain two active sites, they have been divided into the amino- and carboxyl-terminal ends. Aligned in this fashion, the active site (WIDMNE) and many of the cysteines have been conserved. Sequences lost from GAA during processing are indicated in reverse bold. It is apparent from the alignment that
GAA, MGAM and SIM are most closely related while G2AN contains a number of insertions and deletions reducing the overall homology. GAA is the only family 31 enzyme which is found in the lysosome and is known to undergo proteolytic maturation/processing. Interestingly, two of the four sequences removed during processing are found in the only two insertions in the GAA sequence relative to the family 31 consensus. It will be interesting in the future to determine how these sequences alter GAA structure and function.

**Abbreviations**

GAA, acid α-glucosidase; rhGAA, recombinant human acid α-glucosidase; HP-rhGAA, highly phosphorylated acid α-glucosidase; MGAM, maltose isoamylase; SIM, sucrose isomaltase; G2AN, glucosidase II; ERT, enzyme replacement therapy; MSX, methionine sulfoximine; LC/MS, liquid chromatography mass spectrometry; MALDI TOF MS, matrix-assisted laser desorption ionization time of flight mass spectrometry; GS, glutamine synthetase supplement; NMWL, nominal molecular weight limit; SDS-PAGE, sodium-dodecyl-sulfate polyacrylamide gel electrophoresis; DMEM, dulbecco modified eagle medium; FBS, fetal bovine serum; DPBS, dialyzed phosphate buffered saline; CHO, Chinese hamster ovary; PVDF, polyvinyl difluoride; DTT, dithiothreitol; PNGase F, peptide: N-glycosidase F; Endo H, endoglycosidase H.

**Acknowledgments**

We thank Sheri Rayl, Mike Brem, Dennis Burian and the Genzyme Analytics Group for technical assistance. We thank Robert Mattaliano and Jean Elmendorf for their helpful discussions and advice. We would also like to thank K. Jackson of the Molecular Biology Resource Center at the Oklahoma Center for Molecular Medicine for protein sequencing.
References

Figure Legends

Fig. 1. Chromatography of human placental GAA on Superose 6 and analysis by SDS-PAGE. GAA purified as described in “Experimental Procedures was applied to Superose 6 gel-filtration column. A, each fraction was assayed for protein (A280) and GAA activity. Arrows denote elution volumes of gel-filtration standards. B, reduced silver stained SDS-PAGE (4-20% acrylamide) of load and indicated fractions.
Fig. 2. **Characterization of GAA processed forms.** Precursor recombinant human GAA (P), intermediate recombinant GAA (I) and mature human placental GAA (M) were purified as described under “Experimental Procedures”. The precursor (P) and intermediates (I) were obtained from the purification of rhGAA. A, Silver stained reduced SDS-PAGE (4-20%) of GAA precursor, intermediate and mature. B, GAA precursor and processed forms were analyzed by Superdex 75, HR10/30 affinity chromatography. Precursor recombinant human GAA eluted as a single peak (P). The intermediate recombinant GAA (I) from the purification of rhGAA eluted as 2 species, with the first peak eluting at the same time as the precursor GAA. The mature human placental GAA eluted as a much broader and later eluting peak (M).

Fig. 3. **Identification of the 19.4- and 10.3-kDa polypeptides by western blot.** Precursor recombinant human GAA (P), intermediate recombinant human GAA (I), and mature human placental GAA (M) were purified as described under “Experimental Procedures.” The precursor (P) and intermediates (I) were obtained from the purification of HP-rhGAA. All panels are reduced SDS-PAGE (4-20% acrylamide) western blots. A, precursor (P), intermediate (I), and mature GAA (M) were probed with anti-GAA 57-74 (lanes 1-3), monoclonal antibody GAA1, and anti-GAA 183-200 (lanes 7-9). B, blots of P, I, and M, were probed with monoclonal antibody GAA1. The same blot was then probed with an affinity purified goat polyclonal antibody raised against GAA purified from human placenta (lanes 4-6). The blot was then probed with anti-GAA 183-200 (lanes 7-9). All blots were developed as described under “Experimental Procedures.”
Fig. 4. **Identification of a disulfide bound 3.9-kDa polypeptide.**

All panels are reduced and nonreduced SDS-PAGE western blots loaded with the same samples as in Fig. 3. Panels A and B are from a 6% acrylamide gel and panel C is from a 4-20% acrylamide gel. A, precursor (P), intermediate (I), and mature GAA (M) were probed with anti-GAA 57-74, monoclonal antibody GAA1 (lanes 4-6). B, the blot shown in A was probed with anti-GAA 78-94 (lanes 1-6). C, the blot from a 4-20% SDS-PAGE gel was probed with anti-GAA 78-94. All blots were developed as described under “Experimental Procedures.”

Fig. 5. **Hamster acid α-glucosidase is a multisubunit complex.** Recombinant human GAA, human placental GAA, and hamster GAA were purified as described under “Experimental Procedures.” A, reduced silver stained SDS-PAGE (10% acrylamide) of recombinant human GAA (lane 1), human placental GAA (lane 2), and hamster GAA (lane 3). B, reduced SDS-PAGE (4-20% acrylamide) western blot of hamster GAA. Lane 1, blot probed anti-GAA 183-200. Lane 2, blot probed with affinity purified gt anti-GAA antibody raised against GAA purified from human placenta. Lane 3, blot probed with antibodies used in lanes 1 and 2. C, reduced SDS-PAGE (4-20% acrylamide) western blot of human placental GAA digested with Endo H (lane 2) and PNGase F (lane 4) and hamster GAA digested with Endo H (lane 6) and PNGase F (lane 8) with affinity purified gt anti-GAA antibody. All blots were developed as described under “Experimental Procedures.”

Fig. 6. **Fibroblast uptake and processing of HP-RHGAA.** As described under “Experimental Procedures” GAA deficient Pompe fibroblasts (GM00248) were incubated with 50 nM recombinant HP-rhGAA. At the designated time points the fibroblasts were collected and frozen
at -80°C. The cell lysates were probed with a panel of GAA specific antibodies. A, reduced SDS-PAGE (8% acrylamide) western blot. The blot was probed with anti-GAA 57-74. B, reduced SDS-PAGE (8% acrylamide) western blot. The blot was probed with a monoclonal antibody GAA1. C, reduced SDS-PAGE (4-20% acrylamide) western blot. The blot was probed with anti-GAA 183-200.

Fig. 7. Model for the maturation of GAA.

Fig. 8. Multiple sequence alignment of human family 31 glucohydrolases.
Alignment of human GAA, maltose isoamylase (MGAM), sucrose isomaltase (SIM), glucosidase II (G2AN). The putative active site (WIDMNE) is highlighted along with the proteolytic cleavage sites in GAA.

Table I. Summary of the polypeptides and glycans present for each species of GAA
The first column lists the apparent MW of the major polypeptide which represents the common designation for this species. In the subsequent columns, each polypeptide that is a component of the GAA complex is listed on a separate line. Theoretical, calculated and observed masses are as indicated in the column headers.
### Tables

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Table 1
Fig. 2
Fig. 3

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Monoclonal GAA1
gt Anti-GAA
Anti-GAA 183-200

- 76 kDa
- 70 kDa
- 19 kDa
- 10 kDa

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Anti-GAA 54-74
Monoclonal GAA1
Anti-GAA 183-200

- 110 kDa
- 76 kDa
- 70 kDa
- 10 kDa
Fig. 4

**A** Anti-GAA 57-74

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**B** Anti-GAA 57-74 + Anti-GAA 78-94

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150 kDa — 100 kDa — 75 kDa — 110 kDa — 95 kDa — 76 kDa

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**C** Anti-GAA 78-94

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<th>βME</th>
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25 kDa — 15 kDa — 10 kDa — 14 kDa

1 2 3

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Fig. 4
Fig. 5

Panel A:

- **rhGAA**
- **Human Placental GAA**
- **Hamster GAA**

Panel B:

- **Hamster GAA**
- Anti-GAA 183-200
  - +
  - -
  - +
  - +

Panel C:

- **Human Placental GAA**
- **Hamster GAA**

- Endo H PNGase F

- 76 kDa
- 70 kDa
- 10 kDa
Fig. 6

**A**

150 kDa

100 kDa

110 kDa

**B**

100 kDa

75 kDa

95 kDa

76 kDa

70 kDa

**C**

15 kDa

10 kDa

10 kDa
Lysosomal acid a-glucosidase consists of four different peptides processed from a single-chain precursor

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