# Human α-galactosidase A: glycosylation site 3 is essential for enzyme solubility

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Human α-galactosidase A (EC 3.2.1.22; α-Gal A) is the homodimeric glycoprotein that hydrolyses the terminal α-galactosyl moieties from glycolipids and glycoproteins. The type, site occupancy and function of the N-linked oligosaccharide chains on this lysosomal hydrolase were determined. Endoglycosidase treatment of the purified recombinant enzyme and mutagenesis studies indicated that three (Asn-139, Asn-192 and Asn-215) of the four potential N-glycosylation consensus sequences were occupied by complex, high-mannose and hybrid-type oligosaccharides respectively. When expressed in COS-1 cells, glycoforms with glycosylation site 1 or 2 obliterated had more than 70 % of wild-type activity, and both glycoforms were secreted. In contrast, the glycoform with only site 3 eliminated had decreased activity (less than 40 %); little, if any, was secreted. Expressed mutant glycoforms in which site 3 and site 1 or 2 were obliterated had little, if any, intracellular or secreted enzymic activity, and immunofluorescence microscopy revealed that the expressed mutant glycoforms were retained in the endoplasmic reticulum, presumably where they were degraded. Thus glycosylation at site 3 was crucial to the formation of soluble, active enzyme, as well as transport to the lysosome. Absence of the site 3 hybrid-type oligosaccharide exposed an adjacent, normally protected, hydrophobic region, resulting in aggregation of the enzyme polypeptide in the endoplasmic reticulum. In support of this concept, endoglycosidase H-treated enzyme or mannose-terminated enzyme expressed in *Autographa californica* cells also aggregated when concentrated, emphasizing that site 3 occupancy by a hybrid-type oligosaccharide was required for enzyme solubility.

## INTRODUCTION

Human α-galactosidase A (EC 3.2.1.22; α-Gal A) is the lysosomal exoglycosidase responsible for the hydrolysis of terminal α-galactosyl moieties from various glycoconjugates [1]. Biosynthetic studies indicated that the enzyme precursor was an approx. 55 kDa glycopeptide that was processed by cleavage of the signal peptide and by oligosaccharide modifications in the Golgi and lysosomes to form the mature approx. 100 kDa active homodimeric enzyme [2,3]. The subunits of the glycosylated precursor and mature enzyme each contained two to four oligosaccharide chains. The human enzyme has been purified from a variety of sources and their physical and kinetic properties have been characterized [4,5]. Notably, the enzyme isolated from plasma was more highly sialylated than, and had slightly different physical and kinetic properties from, the mature enzyme isolated from tissue lysosomes [4].

In the late 1980s, the full-length cDNA and the entire genomic sequence encoding human α-Gal A were isolated and characterized [6–8]. The 1.4 kb full-length cDNA encodes a peptide of 429 residues including a 31-residue signal peptide. The coding sequence contained four putative N-glycosylation consensus sites at positions Asn-139, Asn-192, Asn-215 and Asn-408. The stable overexpression of human α-Gal A in Chinese hamster ovary (CHO) cells provided the ability to produce and purify large quantities of the cellular and secreted forms of the human enzyme for physical and kinetic studies, including the analysis of the enzyme's oligosaccharide chains [3]. Treatment of the purified recombinant secreted enzyme with N-glycanase, O-glycanase or endoglycosidase F (endo F) revealed that the enzyme was N-glycosylated and did not contain O-linked oligosaccharide chains (Y. A. Ioannou and R. J. Desnick, unpublished work).

The deficient activity of α-Gal A is the enzymic defect in Fabry disease, an X-linked inborn error of glycolipid catabolism [1]. Of the more than 100 different mutations identified in the α-Gal A gene in unrelated families with Fabry disease [9–12], only one disease-causing mutation occurred at an N-glycosylation site, a point mutation at a CpG dinucleotide that predicted an Asn → Ser substitution at residue 215 (designated N215S). This allele encoded a mutant enzyme with 3–10 % of normal activity in various tissues, and essentially no detectable enzymic activity secreted into the plasma [13]. Affected males with this lesion have a mild phenotype characterized primarily by cardiac involvement with onset of symptoms at 50–60 years of age, whereas patients with the classical phenotype, little or no enzymic activity, usually expire in the fourth or fifth decade of life owing to vascular disease of the kidney, heart and/or brain [1,14].

The importance of N-glycosylation for the structural integrity, subcellular transport and/or catalytic function of lysosomal hydrolases has been investigated for several enzymes [15–20]. These studies determined the glycosylation site occupancy for each enzyme glycopeptide and the effect of eliminating individual occupied sites and/or combinations of the occupied sites on enzyme activity and stability, and/or transport to lysosomes or secretion. For example, when the single site in cathepsin L containing a phosphomannose residue was obliterated, active enzyme was synthesized and secreted rather than targeted to the lysosome [15]. Similarly, when either of the two occupied sites in human arylsulphatase A was eliminated, active enzyme was synthesized; however, phosphorylation at site 3 was required for lysosomal sorting [16]. For human acid β-glucosidase, which is transported to the lysosome by a mannose 6-phosphate-independent pathway [17], individual elimination of occupied site 2, 3 or 4 did not affect activity or transport; however, elimination

Abbreviations used: α-Gal A, α-galactosidase A; CHO, Chinese hamster ovary; DMEM, Dulbecco's modified Eagle's medium; endo H, endoglycosidase H; ER, endoplasmic reticulum; PNGase F, protein N-glycosidase F.

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of site 1 resulted in normal amounts of an inactive protein that was presumably transported to the lysosome [17].

More recently, N-glycosylation has been recognized as important for the proper polypeptide folding of various glycoproteins (reviewed in [18]). Among lysosomal enzymes, elimination of individual occupied sites or combinations of the four occupied sites in human $\beta$-glucuronidase decreased enzymic activity and/or increased secretion; however, only alteration of all four sites rendered the enzyme inactive and not secreted, presumably owing to misfolding. Analogously, when any one of the three occupied sites in the human $\beta$-hexosaminidase $\alpha$-chain was obliterated, the mutant enzyme subunits were properly folded and participated in subunit assembly [19]. However, when all three occupied sites were eliminated, the enzyme polypeptide did not fold properly and was detected in the endoplasmic reticulum (ER) bound to binding protein. Only in acid $\alpha$-glucosidase was a single N-glycosylation site (Asn-233) crucial to enzyme stability and transport [20]. Elimination of this site caused enzyme retention in the ER; however, the mechanism for this retention was not investigated.

Here we report the site occupancy, oligosaccharide type, phosphorylation and functional importance of the four N-glycosylation consensus sites in recombinant human $\alpha$-Gal A. Studies using digestion with exoglycosidase, mutagenesis and expression indicated that the first three glycosylation consensus sites in the enzyme subunits were occupied by complex, high-mannose and hybrid-type oligosaccharide chains respectively. Of note, N-glycosylation site 3 (Asn-215) was crucial to the retention of enzymic activity and stability. Immunofluorescence studies revealed that obliteration of site 3 site in combination with either site 1 (Asn-139) or site 2 (Asn-192) resulted in polypeptides that were primarily localized to the ER, where they were presumably degraded. Analysis of the amino acid sequence adjacent to site 3 revealed a hydrophobic patch that was present in mammalian glycosylated forms of $\alpha$-Gal A, but was absent from the enzyme polypeptide from plants and other non-glycosylated homologues. Therefore it is suggested that the site 3 oligosaccharide prevented the aggregation of this hydrophobic region and was essential for enzyme solubility.

## EXPERIMENTAL

### Materials

Restriction endonucleases, Vent DNA polymerase and T4 ligase were obtained from New England Biolabs (Beverly, MA, U.S.A.). COS-1 cells were obtained from the American Type Culture Collection (Rockville, MD, U.S.A.). Endoglycosidases and *Vibrio cholera* $\alpha$-neuraminidase were from Boehringer Mannheim (Indianapolis, IN, U.S.A.). QAE-Sephadex was from Pharmacia Biotech (Uppsala, Sweden). Oligonucleotides were synthesized with an Applied Biosystems DNA synthesizer model 380B.

### Production and purification of recombinant $\alpha$-Gal A

Recombinant human $\alpha$-Gal A was overexpressed in stably transfected CHO cells and the cellular and secreted forms were individually purified as described previously [3] to characterize the nature and phosphorylation of the enzyme's oligosaccharide chains. To overexpress human $\alpha$-Gal A in the baculovirus system, the full-length cDNA was cloned into the baculovirus expression vector, pAc1392. Recombinant baculovirus containing the $\alpha$-Gal A cDNA was produced in cloned *Spodoptera frugiperda* (Sf9) cells by calcium phosphate-mediated transfection and homologous recombination between the expression plasmid and the baculovirus genomic DNA (Baculogold®) as described [21]. Pure recombinant baculovirus clones containing the $\alpha$-Gal A cDNA were isolated by plaque hybridization, amplified, titred and used at a multiplicity of infection greater than 10 to infect Sf9 cells. The expressed enzyme was collected and purified as described previously [3]. Purified Sf9-produced $\alpha$-Gal A was concentrated to approx. 5 mg/ml with a Microcon concentrator (Amicon, Beverly, MA) with a molecular-mass cut-off of 30 kDa.

The terminal oligosaccharides on various recombinant forms of $\alpha$-Gal A were characterized with a lectin glycan differentiation kit (Boehringer Mannheim), in accordance with the manufacturer's instructions. In brief, 1 $\mu$g of each glycoform and positive and negative control proteins were blotted on a nitrocellulose membrane. The blot was cut into strips containing the appropriate positive and negative controls, and the three $\alpha$-Gal A glycoforms. Strips were probed with *Galanthus nivalis* agglutinin to detect terminal mannose moieties, *Sambucus nigra* agglutinin to detect sialic acid linked $\alpha$(2-6) to galactose, *Maackia amurensis* agglutinin to detect sialic acid linked $\alpha$(2-3) to galactose, peanut agglutinin to detect terminal galactose linked $\beta$(1-3) to *N*-acetylgalactosamine, and *Datura stramonium* agglutinin to detect terminal galactose linked $\beta$(1-4) to *N*-acetylgalactosamine.

### Endoglycosidase digestions

Endoglycosidase H (endo H), D or F1 and protein N-glycosidase F (PNGase F) digestions were performed essentially as described [22]. In brief, digestions with endo H (5 milliunits per reaction) were performed at 37 °C overnight in 5 mM sodium citrate, pH 5.5, containing 0.2 mM PMSF. Digestions with endo D (10 milliunits per reaction) were performed at 37 °C overnight in 0.2 M citrate phosphate buffer, pH 6.0, containing 0.2 mM PMSF. Endo F1 digestions (50 milliunits per reaction) were performed overnight at 30 °C in 0.17 M sodium acetate, pH 6.0, containing 1.6 % (v/v) Nonidet P40 and 0.2 mM PMSF. PNGase F digestions (100 milliunits per reaction) were performed overnight at 30 °C in 0.17 M potassium phosphate, pH 8.6, containing 1.6 % (v/v) Nonidet P40 and 0.2 mM PMSF. Samples were diluted to 0.2–0.5 % SDS before digestion. All reaction volumes were 50 $\mu$l. A drop of toluene was added to each reaction tube to prevent bacterial growth. For PNGase F digestion after urea denaturation, $\alpha$-Gal A was added to the denaturation buffer [5 M urea/40 mM Tris/HCl (pH 8.0)/0.1 M 2-mercaptoethanol] and boiled for 5 min. PNGase F (100 milliunits) and n-octylglucoside (1.25 %, w/v) were added and digestion was performed overnight at 30 °C, as described above.

### QAE-Sephadex chromatography

QAE-Sephadex chromatography in a 3.0 cm × 0.8 cm column was performed as described [23,24]. In brief, after digestion with endo H, the released [$^3$H]mannose-labelled oligosaccharides were isolated and desalted on an 18.0 cm × 0.8 cm Sephadex G-25 column. Samples were applied to the QAE-Sephadex column and were eluted with successive 5 ml aliquots of 2 mM Tris/HCl, pH 8.0, containing 0, 20, 40, 80, 100, 120, 140, 160, 200, 400 and 1000 mM NaCl. Oligosaccharides were eluted according to their number of negative charges [23,24].

### Cell culture and DNA transfections

COS-1 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % (v/v) fetal bovine serum, 0.292 mg/ml glutamine and 0.25 mg/ml gentamycin (growth medium) in a humidified incubator at 37 °C, under air/$CO_2$ (19:1). Exponentially growing COS-1 cells ($5 \times 10^6$ cells per

75 cm² flask) were treated with 1 μg/ml colcemid for 16 h, detached from the plastic flasks by trypsin treatment, collected by centrifugation at 3000 *g* for 10 min and then washed once in growth medium. After centrifugation at 3000 *g* for 10 min the cells were resuspended in 0.7 ml of growth medium and placed in a 0.4 cm gap electroporation cuvette. Plasmid DNA (10–15 μg) was added and the cell-containing cuvette was placed in a Gene Pulser electroporation apparatus (Bio-Rad, Hercules, CA, U.S.A.), and pulsed once at 250 V, 500 μF. The cells were plated immediately into a 100 mm culture dish containing 10 ml of growth medium.

### PCR and DNA sequencing

For site-directed mutagenesis of the four putative N-glycosylation consensus sites, mutant cDNA constructs were synthesized with the overlap PCR technique [25,26]. In brief, the full-length α-Gal A cDNA was amplified with primers designed to replace the Asn codon of the N-glycosylation consensus sequence, singly or in combinations, with Gln codons. Four constructs were designed to eliminate a single consensus sequence at Asn-139 (designated ΔGS-1), Asn-192 (ΔGS-2), Asn-215 (ΔGS-3) and Asn-408 (ΔGS-4). Three constructs obliterated two of the first three sites (ΔGS-1,2, ΔGS-1,3 and ΔGS-2,3) and one construct eliminated the first three sites (ΔGS-1,2,3). Each of the eight constructs was sequence-confirmed, digested with *Eco*R1 and subcloned into the eukaryotic expression vector p91023(B) for expression studies [3].

### SDS/PAGE and autoradiography

PAGE was performed under reducing conditions in a slab 1.5 mm thick containing 10 % (w/v) polyacrylamide as described [27]. The gel was fixed in 10 % (v/v) acetic acid and 20 % (v/v) methanol for 30 min and then soaked in Amplify (Amersham, Arlington Heights, IL, U.S.A.) for 30 min with agitation. Gels were vacuum-dried for 90 min and exposed to Kodak X-Omat AR film for 4–72 h.

### Enzyme and protein assays

For enzyme assays the cells in a 100 mm culture dish were washed twice with 5 ml of PBS and incubated with 1 ml of lysis buffer [50 mM sodium phosphate buffer, pH 6.5, containing 150 mM NaCl, 1 mM EDTA, 1 % (v/v) NP40 and 0.2 mM PMSF] at 4 °C for 10 min. The lysates were collected and transferred to 1.5 ml test tubes and clarified by centrifugation at 16000 *g*.

The α-Gal A activities in the cell lysates and medium were determined with 50 mM 4-methylumbelliferyl α-D-galactopyranoside as described previously [3]. In brief, a stock solution of 5 mM 4-methylumbelliferyl α-D-galactopyranoside was prepared in 0.1 M citrate/0.2 M phosphate buffer (pH 4.6) in an ultrasonic bath. The reaction mixture, containing 10–50 μl of cell extract and 150 μl of the stock substrate solution, was incubated at 37 °C for 10–30 min. The reaction was terminated with the addition of 2.3 ml of 0.1 M ethylenediamine. The fluorescence was determined with a Ratio-2 System Fluorometer (Optical Technologies Devices, Elmsford, NY, U.S.A.). Protein concentrations were determined by the fluorescamine method [28].

### Labelling of cells with [$^{35}$S]methionine and [$^{3}$H]mannose

For [$^{35}$S]methionine labelling, confluent cultures in 100 mm dishes were washed once with 5 ml of methionine-free DMEM and incubated at 37 °C for 30 min with 5 ml of methionine-free DMEM. The medium was removed and a fresh aliquot (1 ml) of methionine-free DMEM, supplemented with 10 % (v/v) dialysed fetal calf serum and 200 μCi of [$^{35}$S]methionine, was added. Cells were incubated at 37 °C for 16 h, washed and lysed as above. For [$^{3}$H]mannose labelling, cultures were grown as above in supplemented DMEM. Cells were washed with 5 ml of low-glucose DMEM and a fresh aliquot of medium was added that contained 250 mCi [$^{3}$H]mannose (dried under nitrogen and resuspended in DMEM). Cells were incubated at 37 °C for 24 h.

### Immunofluorescence microscopy

COS-1 cells were plated in two-well chamber slides (Nunc, Naperville, IL, U.S.A.) after electroporation. After a 48 h incubation the transfected cells were washed with PBS and then fixed in PBS containing 4 % (w/v) paraformaldehyde for 10 min at room temperature, washed and permeabilized in PBS containing 0.1 % (v/v) Triton X-100 for 10 min at room temperature. After incubation in PBS containing 3 % (w/v) BSA (PBS/BSA) for 30 min at room temperature, the cells were incubated with affinity-purified rabbit anti-(α-Gal A) antibody in 3 % (w/v) BSA for 2 h at 4 °C. A secondary fluorescein-labelled goat anti-(rabbit IgG) (Boehringer Mannheim) was added in PBS/BSA for 1 h at 4 °C. Cells were washed, placed on coverslips with Fluoromount-G (Southern Biotechnology Associates, Birmingham, AL, U.S.A.), and photographed with the ×40 objective of a Zeiss Axiophot fluorescence microscope (Zeiss, Jena, Germany).

## RESULTS

### Oligosaccharide analysis of recombinant human secreted α-Gal A

Purified recombinant human α-Gal A secreted by CHO cells [3] was digested with endo H, F1 and D or PNGase F to determine the number and type of oligosaccharides on the enzyme subunit. Digestion with PNGase F caused the generation of two bands that had increased mobility on SDS/PAGE analysis (Figure 1A), consistent with the cleavage of three N-linked oligosaccharide chains. Digestion of the recombinant enzyme with a mixture of endo H, endo F1 and PNGase F eliminated the slower-migrating

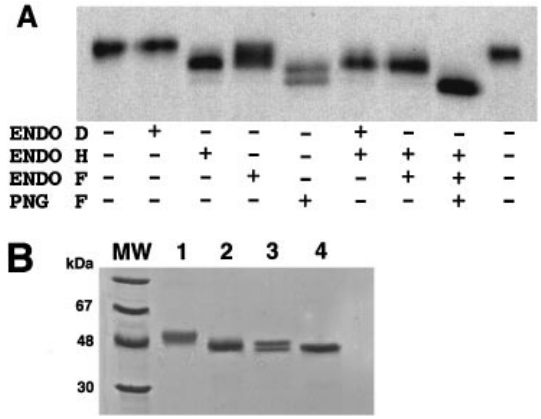


**Figure 1 Endoglycosidase analysis of recombinant α-Gal A**

(**A**) Digestion of human recombinant α-Gal A with endoglycosidase. Purified recombinant α-Gal A was treated with various endoglycosidases and analysed by SDS/PAGE as described in the Experimental section. (**B**) Digestion of human recombinant α-Gal A with PNGase F. Purified recombinant α-Gal A was treated with endo H (lane 2), and PNGase F before (lane 3) or after (lane 4) denaturation of α-Gal A with urea. Lane 1, undigested α-Gal A; lane MW, molecular-mass markers (molecular masses indicated at the left).

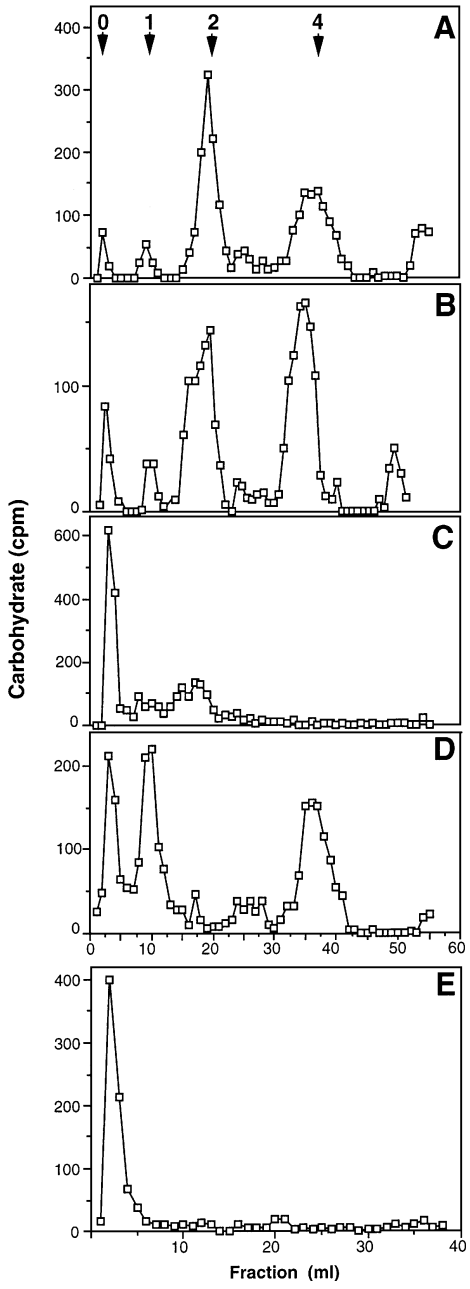


**Figure 2 QAE chromatographic profiles of endo H-sensitive oligosaccharides from α-Gal A**

CHO cells overexpressing α-Gal A were metabolically labelled with [$^3$H]mannose. Recombinant α-Gal A was immunoprecipitated and treated with endo H to remove the high-mannose oligosaccharides. The oligosaccharides released were purified and desalted on a Sephadex G-25 column. They were subsequently chromatographed on QAE-Sephadex to determine the various ionic species released by endo H. Profiles of secreted α-Gal A oligosaccharides: untreated (**A**), and treated with dilute HCl (**B**), α-neuraminidase (**C**) or alkaline phosphatase (**D**), and untreated cellular oligosaccharides (**E**). Species with 0, 1, 2, or 4 ionic charges were eluted with a NaCl gradient as indicated by the arrows.

band but did not decrease further the subunit molecular mass of the faster-migrating band, indicating that the generation of the slower-migrating band after PNGase F treatment might be due to α-GalA's sterically hindering this endoglycosydase. To determine whether this was so, α-Gal A was digested with PNGase

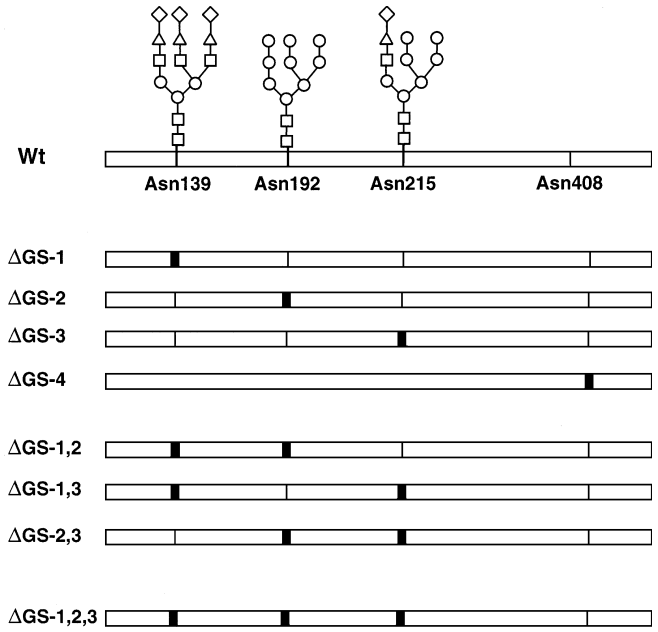


**Figure 3 Schematic representation of the mutant α-Gal A cDNA species**

The consensus glycosylation sites are shown on the wild-type (WT) α-Gal A subunit at Asn-139, Asn-192, Asn-215 and Asn-408. The deleted consensus sites in each mutant glycoform are indicated. Each site was eliminated by an Asn → Gln substitution, indicated by a solid bar.

F before and after complete denaturation in 5 M urea. As can be clearly seen in Figure 1(B), treatment with PNGase F, in the absence of urea denaturation of α-Gal A, generates the two bands seen in Figure 1, whereas denaturation of α-Gal A with urea before treatment with PNGase F eliminated the slower-migrating band without altering the mobility of the faster-migrating band, indicating that the enzyme did not contain any PNGase F-resistant N-linked oligosaccharide moieties. Endo D, a glycosidase with a strict specificity for the lower Manα1,3-(Manα1,6) branch of the high-mannose core pentasaccharide [23], did not effect the mobility of α-Gal A, indicating that the recombinant enzyme did not contain this oligosaccharide structure (Figure 1A). In contrast, digestion with endo H and endo F resulted in an approx. 4 kDa shift, indicating that two of the three oligosaccharide chains were of the high-mannose or hybrid type [24].

### Analysis of endo H-sensitive oligosaccharides

To characterize the phosphate and sialic acid moieties on the endo H-sensitive oligosaccharides, CHO cells expressing α-Gal A were metabolically radiolabelled with [$^3$H]mannose. The high-mannose oligosaccharides were removed by treating the immunoprecipitated [$^3$H]mannose-labelled enzyme with endo H. Aliquots of the released oligosaccharides were analysed by chromatography on QAE-Sephadex [23,24]. Two major ionic forms were detected, one with two negative charges and one with four negative charges (Figure 2A). These negative charges were contributed by a phosphodiester bond (1−), a phosphomonoester bond (2−) or sialic acid (1−). Treatment of an aliquot of endo H-released oligosaccharides with dilute HCl did not shift the profile of any of the peaks, indicating that there were

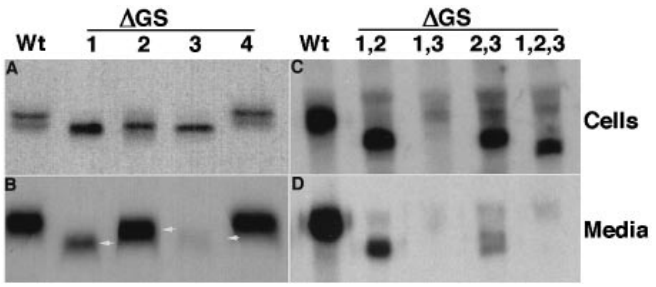


**Figure 4 Expression of various α-Gal A glycosylation mutants in COS-1 cells**

COS-1 cells transfected with various mutant constructs were metabolically labelled with [$^{35}$S]methionine. At 48 h after transfection, α-Gal A was immunoprecipitated from cell lysates (Cells) and growth medium (Media). The immunoprecipitates were analysed by SDS/PAGE and autoradiography as described in the Experimental section. Abbreviation: Wt, wild type.

no phosphodiester groups on the liberated oligosaccharides (Figure 2B) [24]. In contrast, treatment with α-neuraminidase caused a shift of the 2− peak resulting in two new peaks with negative charges at 0 and 1− (Figure 2C), indicating that the 2− peak represented one, or more probably two, sialic acid moieties. Presumably the 1− peak was due to partial digestion of the 2− peak by α-neuraminidase. Treatment of an aliquot of these oligosaccharides with alkaline phosphatase caused a shift in the 4− peak to a 0 negative charge (Figure 2D). There was no effect on the 2− peak, indicating that the charge of the 4− peak was due to two phosphomonoester bonds, whereas the 2− peak did not contain alkaline phosphatase-cleavable linkages. These findings indicate that endo H released two types of high-mannose oligosaccharides from recombinant secreted α-Gal A, one containing sialic acid (presumably a hybrid-type oligosaccharide) and one containing two phosphomonoester bonds (presumably a high-mannose type oligosaccharide with two mannose 6-phosphate moieties). Similarly, the endo H-sensitive oligosaccharides from the purified cellular (lysosomal) glycoform of recombinant α-Gal A were analysed (Figure 2E). No charged oligosaccharides were observed, indicating that the mannose 6-phosphate and/or sialic acid residues were removed on the arrival of α-Gal A at the endosome or lysosome. These results were consistent with secreted α-Gal A glycoform's having a complex, a high-mannose and a hybrid-type oligosaccharide per subunit and are in agreement with the results of the complete structural analysis of the α-Gal A oligosaccharides [29].

### Human α-Gal A subunits contain three N-linked oligosaccharides

Site-directed mutagenesis of the α-Gal A cDNA was used to eliminate the four putative N-glycosylation sites, singly and in combinations, by altering the asparagine residues to glutamine (Figure 3). Each mutagenized α-Gal A cDNA construct was expressed in COS-1 cells that were metabolically radiolabelled with [$^{35}$S]methionine, and the expressed α-Gal A glycoforms were immunoprecipitated, resolved by SDS/PAGE and detected by radiography as shown in Figures 4(A) and 4(B). Single-site mutant constructs, ΔGS-1, ΔGS-2 or ΔGS-3, expressed enzyme subunits with increased mobility compared with the wild-type enzyme, consistent with the loss of a single N-linked oligosaccharide chain. In contrast, the ΔGS-4 cDNA construct expressed a glycoform whose subunit had the same mobility as that of the wild type, indicating that Asn-408 was not occupied. Immunoprecipitates of the ΔGS-3 construct had markedly

**Table 1 α-Gal A activities of control and glycosylation mutants expressed in COS-1 cells**

Enzyme activities are averages for at least three independent determinations. Abbreviations: pAGA, expression vector containing wild-type α-Gal A cDNA; n.d., not determined.

| | α-Gal A activity | | | | |
|---|---|---|---|---|---|
| | Intracellular | | Secreted | | |
| Construct | (units/mg) | (% of pAGA) | (units/mg) | (% of pAGA) | pH optimum |
| COS 1 | 125 | – | < 5 | – | 4.6 |
| pAGA | 2215 | 100 | 355 | 100 | 4.6 |
| ΔGS-1 | 1630 | 72.0 | 325 | 91.5 | 4.8 |
| ΔGS-2 | 1820 | 81.0 | 158 | 44.5 | 5.0 |
| ΔGS-3 | 930 | 38.5 | 14 | 3.9 | 5.0 |
| ΔGS-4 | 1495 | 65.6 | 340 | 95.8 | n.d. |
| ΔGS-1,2 | 885 | 36.4 | < 5 | < 1.4 | n.d. |
| ΔGS-1,3 | 110 | – | < 5 | < 1.4 | n.d. |
| ΔGS-2,3 | 160 | – | < 5 | < 1.4 | n.d. |
| ΔGS-1,2,3 | 108 | – | < 5 | < 1.4 | n.d. |

smaller amounts of secreted α-Gal A protein (Figure 4B), whereas the ΔGS-1, ΔGS-2 and ΔGS-4 constructs expressed essentially normal amounts of secreted enzyme protein (Figure 4B). Analysis of the gel mobilities of the secreted mutant glycoforms revealed that ΔGS-1 had the most increased mobility, whereas ΔGS-2 had the least (Figure 4, arrows). Taken together with the results of the glycosidase treatments (Figure 1A), these findings are consistent with the presence of complex, high-mannose and hybrid type oligosaccharide chains on sites 1, 2 and 3 respectively (Figure 2).

The intracellular and secreted enzyme activities expressed by each mutant construct were determined at 72 h after transfection (Table 1). The ΔGS-1, ΔGS-2 and ΔGS-4 glycoforms had intracellular activities that ranged from 65 % to 81 % and secreted activities that ranged from 45 % to 96 % of the intracellular or secreted wild-type activities respectively. Thus individual elimination of the oligosaccharide at site 1 or site 2 did not significantly impair enzyme stability, transport or activity. The ΔGS-4 glycoform was fully glycosylated but had decreased intracellular (but not secreted) activity, presumably due to the Asn → Gln substitution that made the enzyme slightly less active and/or less stable at lysosomal pH. In contrast, the ΔGS-3 glycoform had only approx. 40 % of the wild-type intracellular levels, and had little, if any, secreted activity (Table 1) or immunologically detected enzyme protein, indicating that the ΔGS-3 glycoform was synthesized but not transported to the lysosome or secreted. The pH optima of the expressed mutant glycoforms were shifted slightly from pH 4.6 to 4.8 (ΔGS-1) or 5.0 (ΔGS-2 and ΔGS-3), as indicated in Table 1. The pH optima could not be determined for the highly unstable mutants with multiple glycosylation sites.

### Effect of multiple glycosylation mutations on the stability and activity of α-Gal A

Constructs were generated to determine the effect of deleting combinations of two or all three occupied glycosylation sites (ΔGS-1,2, ΔGS-1,3, ΔGS-2,3 and ΔGS-1,2,3). These constructs were expressed in COS-1 cells, and the cellular and secreted enzymic activities expressed by each mutant construct were determined (Table 1). Mutant glycoforms expressed from ΔGS-1,3, ΔGS-2,3 and ΔGS-1,2,3 had 0–2 % of wild-type intracellular

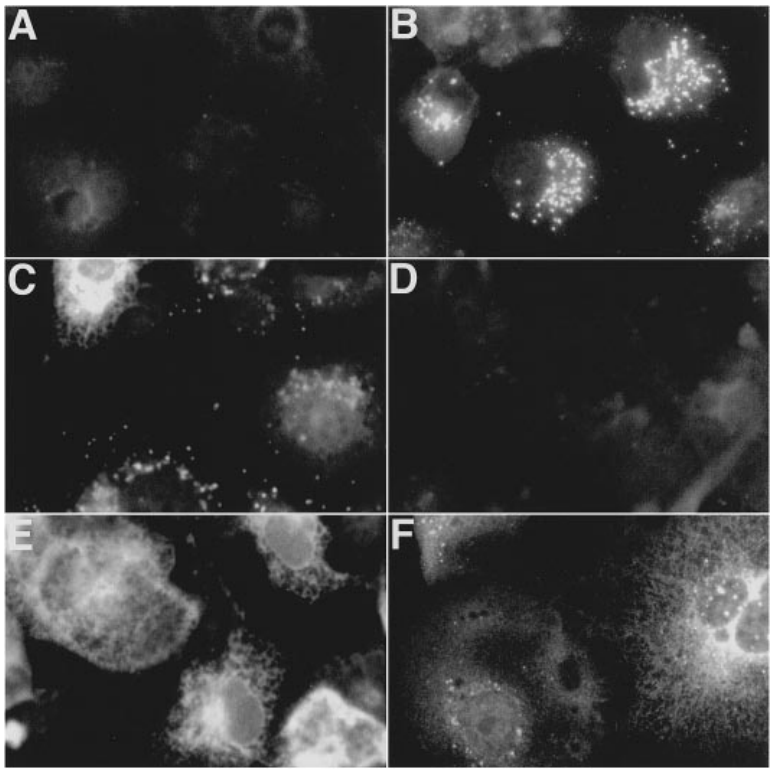


**Figure 5 Immunofluoresence microscopy of various α-Gal A N-glycosylation mutants expressed in COS-1 cells**

Transfected cells were grown for 48 h and then subjected to immunofluorescence microscopy. Cells were fixed, washed and incubated with a monospecific antibody against human α-Gal A. The enzyme was detected by incubation with a fluorescein-conjugated anti-rabbit antibody. (**A**) no DNA; (**B**) wild-type α-Gal A; (**C**) ΔGS-1,2; (**D**) ΔGS-1,3; (**E**) ΔGS-2,3; (**F**) ΔGS-1,2,3. See the text for details.

activity and no detectable activity in the culture medium. In contrast, the glycoform expressed by construct ΔGS-1,2 retained approx. 35% of intracellular wild-type activity but had no detectable activity in the medium.

Biosynthetic studies of the glycoforms expressed by mutant constructs ΔGS-1,2, ΔGS-1,3, ΔGS-2,3 and ΔGS-1,2,3 demonstrated that these constructs expressed detectable intracellular enzyme protein, with the exception of ΔGS-1,3 (Figure 4C). In contrast, secreted enzyme protein was detected only for the ΔGS-1,2 glycoform (Figure 4D), although this mutant enzyme was inactive (Table 1).

### Subcellular localization of the multiple glycosylation mutants

The fact that all the single glycosylation site deletions produced detectable intracellular and secreted enzymic activity and protein (alhough ΔGS-3 had low, but detectable, secreted enzyme) indicated that the synthesis and sorting of these mutant glycoforms were essentially normal (Figure 4 and Table 1). In contrast, expression of constructs with multiple glycosylation site deletions suggested that they were either highly unstable or improperly sorted. Therefore, to determine the subcellular fate of the multiple glycosylation mutants, immunofluorescence microscopy was performed on COS-1 cells that had been transfected with these constructs and then stained with a monospecific polyclonal antibody against human α-Gal A. As shown in Figure 5(B), wild-type α-Gal A was targeted to small vesicular structures consistent with lysosomes. Similarly, the mutant glycoform expressed by the double mutant ΔGS-1,2 was distributed in small vesicular structures; however, a staining pattern characteristic of the ER was also observed (Figure 5C) (see, for example, [30]). The glycoform expressed by ΔGS-2,3 was localized solely to the ER with no observable vesicular lysosomal staining (Figure 5E). In contrast, cells expressing the ΔGS-1,3 glycoform did not exhibit any staining, suggesting that the enzyme was rapidly degraded (Figure 5D). These results were consistent with the low intracellular and secreted enzymic activity levels expressed by the ΔGS-1,3 construct (Table 1) and the biosynthetic studies, reflecting the instability of the polypeptides in which site 3 was deleted (Figure 4). Interestingly, the enzyme polypeptide expressed by the ΔGS-1,2,3 construct, which had no detectable intracellular activity (Table 1), was observed primarily in the ER, but a small amount of the mutant enzyme was localized in small vesicular structures (Figure 5F). Note that no α-Gal A staining was detected in control COS-1 cells (Figure 5A).

### Site 3 is required for enzyme solubility

Obliteration of site 3, particularly when site 1 or site 2 also was eliminated, resulted in markedly decreased enzyme stability and transport, presumably owing to the inability of the mutant glycoform to exit from the ER. Analysis of the amino acid sequence surrounding site 3 revealed a highly hydrophobic amino acid patch, as shown in Figure 6(A). Secondary-structure analysis of this region with the Chou–Fasman algorithm [31] from the MACVECTOR sequence analysis software package (Oxford Molecular Group) revealed a loop containing site 3 and the adjacent hydrophobic patch with β-pleated sheet structures on

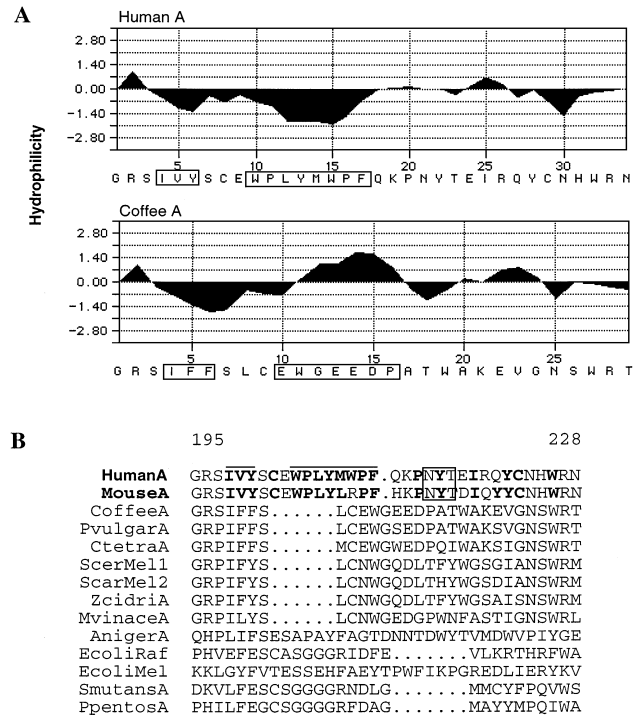


**Figure 6 Hydropathy and homology comparisons of the human α-Gal A Asn-215 region**

(**A**) Hydrophilicity plots for human α-Gal A (upper panel) and unglycosylated coffee bean α-Gal (lower panel). (**B**) Sequence homologies surrounding the Asn-215 glycosylation consensus site in human and murine α-Gal A (boxed sequences). Hydrophobic amino acids are highlighted in bold type. Two hydrophobic patches in the human and murine enzymes are overlined. Note the absence of this region from the enzyme in other species. For comparison, the homologous unglycosylated α-galactosidase sequences of *Coffee arabica* bean (coffeeA), *Phaseolus vulgaris* (PvulgarA), *Cyamopsis tetragonoloba* (CtetraA), *Saccharomyces cerevisiae* (ScerMel1), *Saccharomyces carlbergensis* (ScarMel2), *Zygosaccharomyces cidri* (ZcidriA), *Mortierella vinacea* (MvinaceA), *Aspergillus niger* (AnigerA), *Escherichia coli* raffinose operon (EcoliRaf), *Escherichia coli* mellibiose operon (EcoliMel), *Streptococcus mutans* (SmutansA), and *Pediococcus pentosaceus* (PpentosA) are indicated.

**Table 2 Effect of glycosidase treatment on human recombinant α-Gal A solubility**

Enzyme activities are averages for duplicate determinations. Abbreviation: β-Hex, β-hexosaminidase.

| Treatment | $10^{-6}$ × Activity (units) | | Activity remaining (%) |
|---|---|---|---|
| | Before treatment | After treatment | |
| None | 4.4 | 4.4 | 100 |
| 37 °C | 4.0 | 3.9 | 97.5 |
| Endo H | 3.9 | 2.3 | 58.9 |
| β-Gal | 4.3 | 3.3 | 76.7 |
| β-Gal + β-Hex | 4.1 | 2.9 | 70.7 |
| β-Gal + β-Hex + Endo H | 4.1 | 2.1 | 51.2 |

each side. Analysis of this region in α-Gal sequences from other species revealed that only the mammalian sequences encoded glycosylation site 3 and the adjacent hydrophobic patch (Figure 6B). To determine whether site 3 was important for enzyme solubility, recombinant α-Gal A was treated with endo H to remove the putative endo H-sensitive oligosaccharides from sites

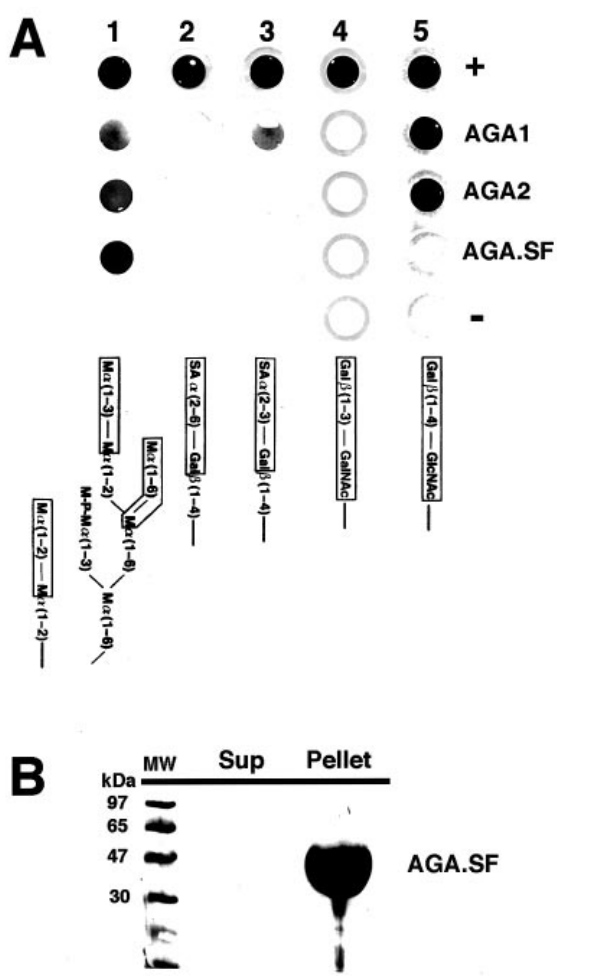


**Figure 7 Characterization of the terminal oligosaccharides on the purified human recombinant α-Gal A produced by Sf9 cells**

(**A**) Recombinant α-Gal A was dot-blotted on nitrocellulose. Abbreviations: +, positive control; AGA1, human recombinant sialylated α-Gal A from CHO cells; AGA2, human recombinant non-sialylated α-Gal A from CHO cells; AGA.SF, Sf9-cell-produced α-Gal A; −, negative control. The blot was probed with various lectins that recognize the indicated terminal sugars: 1, terminal mannose residues in α(1-2)-, α(1-3)- or α(1-6)-linked mannose; 2, sialic acid α(2-6)-Gal; 3, sialic acid α(2-3)-Gal; 4, galactose β(1-3)-GalNAc; 5, galactose β(1-4)-GalNAc. (**B**) SDS/PAGE of purified recombinant human α-Gal A from Sf9 cells. After centrifugation of concentrated (5 mg/ml) enzyme, the supernatant (Sup) and pellet were subjected to electrophoresis. Note that the enzyme aggregated and was detected in the pellet only. Lane MW, molecular mass markers (molecular masses indicated at the left).

2 and 3. After digestion, the partly deglycosylated enzyme protein aggregated and precipitated when concentrated to approx. 5 mg/ml (Table 2). Similarly, sequential treatment of the recombinant secreted enzyme with α-neuraminidase, β-galactosidase and then β-*N*-acetylglucosaminidase to trim the hybrid oligosaccharide to the mannose core, also induced aggregation and precipitation of the concentrated enzyme (Table 2). These findings demonstrate that partial trimming of the hybrid oligosaccharide at site 3 was detrimental to enzyme solubility. Although this treatment also trimmed the complex oligosaccharide at site 1, it is unlikely that it caused enzyme aggregation because the complete obliteration of this site had minimal, if any, effect on enzyme activity and subcellular transport (Table 1 and Figure 4). Moreover, it is unlikely that the mutant glycoforms with site 3 obliterated were retained in the ER owing to

misfolding, particularly because the ΔGS-3 glycoform had approx. 40% of normal activity and was glycosylated at sites 1 and 2, which required folded domains.

Further, the importance of the hybrid-type oligosaccharide at site 3 was investigated by using the purified recombinant α-Gal A enzyme produced by a baculovirus expression system in *Autographa californica* cells, which are known to produce protein glycoforms with predominanlty high-mannose-type oligosaccharides [32]. To determine the terminal oligosaccharides on recombinant α-Gal A produced by Sf9 cells, the purified enzyme was analysed by various lectins, as shown in Figure 7(A). No terminal *N*-acetylglucosamine, galactose or sialic acid oligosaccharides were detected (Figure 7A, AGA.SF), indicating that recombinant α-Gal A produced by Sf9 cells contains only high-mannose oligosaccharides. In constrast, CHO cell-produced recombinant α-Gal A had oligosaccharides with terminal galactose and sialic acid moieties (Figure 7A, AGA1 and AGA2). When purified recombinant human α-Gal A produced in this system was concentrated to approx. 5 mg/ml, the enzyme also formed aggregates and precipitated (Figure 7B). These results indicate that occupation of site 3 by a high-mannose oligosaccharide did not prevent precipitation, further supporting the requirement for a hybrid-type (or complex) oligosaccharide at position 3 to maintain enzyme solubility.

## DISCUSSION

In the present study, the type, site occupancy and functional importance of the N-linked oligosaccharide chains on recombinant human α-Gal A were determined. Initial characterization of the carbohydrate chains by endoglycosidase digestion revealed the presence of high-mannose, hybrid and complex-type oligosaccharides (Figure 1). Further analysis by QAE-Sephadex chromatography of the endo H-released oligosaccharide chains indicated that the high-mannose oligosaccharides contained two phosphomonoester bonds, presumably as mannose 6-phosphate residues, whereas sialic acid was detected on the hybrid and complex-type oligosaccharides. To determine the effect, if any, of these oligosaccharides on enzyme activity, stability and intracellular transport, site-directed mutagenesis was used systematically to eliminate the N-glycosylation consensus sequences singly and in combinations. Of the four putative N-glycosylation sites, the first three were used, whereas the fourth sequence (Asn-Pro-Thr) was not. This was consistent with previous studies indicating that consensus sequences with proline were not occupied [33].

Of the single-site α-Gal A mutants analysed, only the ΔGS-3 glycoform showed a markedly decreased intracellular activity and essentially no secreted activity or immunologically detected enzyme protein, presumably owing to its retention, aggregation and subsequent degradation in the ER. Consistent with this notion, the residual intracellular activity was presumably from aggregated enzyme in the ER that was reactivated when assayed in dilute homogenized cell extracts at pH 4.6. The importance of site 3 for the solubility and export of human α-Gal A from the ER was further demonstrated by expression of the multiple N-glycosylation mutants. All of the multiple deletion constructs in which site 3 was eliminated had little (ΔGS-2,3), if any (ΔGS-1,3, ΔGS-1,2,3), detectable intracellular or secreted activity. Only the ΔGS-1,2 glycoform retained partial (approx. 35%) intracellular activity and was secreted, consistent with the importance of site 3 for enzyme solubility and function. Immunoprecipitation studies indicated that 20–30% of the ΔGS-1,2 glycoprotein was secreted into the culture medium; however, the lack of detectable activity in the medium presumably reflected the instability of the ΔGS-1,2 glycoform at neutral pH.

Analysis of the multiple glycosylation mutants revealed that elimination of sites 1 and 3 (ΔGS-1,3) and all three sites (ΔGS-1,2,3) were most detrimental to enzyme activity and stability. Of note, ΔGS-1,3 essentially did not express any immunoprecipitable protein, either intracellularly or extracellularly. Mutants ΔGS-2,3 and ΔGS-1,2,3 expressed detectable amounts of intracellular protein; of these, ΔGS-2,3 had cellular activity detectable above the endogenous COS-1 α-Gal A activity. Interestingly, these multiple glycosylation mutants secreted more enzyme protein than the construct with only site 3 eliminated, indicating that they were transported from the Golgi to the lysosome or secreted. The subcellular transport of the multiple glycosylation mutants of α-Gal A was further analysed by immunofluorescence microscopy. It was apparent by the immunofluorescence results that a single oligosaccharide at site 3 was sufficient to permit the proper folding and transport of α-Gal A. However, elimination of this site, either alone or in combination with other sites, resulted in a protein that is trapped and degraded in the ER, presumably because it is not soluble. Thus, site 3 is necessary for enzyme solubility and, as a result, enzyme transport and activity.

In the multiple glycosylation mutants the position of the mutated sites in relation to glycosylation site 3 determined the magnitude of their effects on enzyme activity and stability. In fact, elimination of sequential sites (e.g. ΔGS-1,2, ΔGS-2,3 or ΔGS-1,2,3) resulted in expressed enzyme proteins that were inactive but immunologically detectable. Glycosylation only at site 2 (i.e. ΔGS-1,3) produced little, if any, intracellular or extracellular immunologically detectable protein. This finding suggested that glycosylation of only site 2 resulted in abnormal folding and subsequent polypeptide degradation. Thus the glycosylation of both sites 2 and 3 is presumably required for the proper folding and solubility of human α-Gal A.

Additional support for the importance of site 3 in enzyme solubility was based on the finding of a hydrophobic amino acid sequence adjacent to the site. It is hypothesized that when the highly hydrophobic patch is not covered by the hybrid-type oligosaccharide, the enzyme aggregates and is trapped in the ER. Digestion with endoglycosidase and subsequent concentration of the purified recombinant enzyme secreted by CHO cells was consistent with this concept. In addition, human α-Gal A produced in the baculovirus system also aggregated when concentrated, presumably because the high-mannose-type chain at site 3 did not adequately prevent the association of this hydrophobic patch. In contrast, the normally secreted enzyme produced by CHO cells was soluble at more than 15 mg/ml, a concentration used to crystallize this enzyme [34]. Acid α-glucosidase was the only other lysosomal enzyme in which the obliteration of a single N-glycosylation site resulted in an inactive enzyme that was localized to the ER [20]. Inspection of the amino acid sequence around N-glycosylation site 2 (Asn-233) of acid α-glucosidase revealed approx. 17 residues that formed a hydrophobic patch. On the basis of our studies of the α-Gal A mutant glycoforms, it is presumed that this leads to acid α-glucosidase aggregation when site 2 is not occupied. Presumably, the N-glycosylation sites and adjacent hydrophobic sequences in α-Gal A and acid α-glucosidase evolved for more efficient enzyme function. These glycosylation sites ensured the masking of the respective hydrophobic patches and prevented enzyme polypeptide aggregation. This hypothesis could be tested by characterizing the enzyme expressed by a construct from which the hydrophobic patch and adjacent glycosylation sites had been eliminated. An active and stable enzyme glycoprotein that hydrolysed the synthetic substrate but not certain natural glycoconjugates would demonstrate

the importance of the deleted region. Thus the results of these studies indicate that α-Gal A glycosylation at site 3 is crucial for enzyme solubility and its subsequent exit from the ER.

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