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"Fuller M, Mellett N, Hein LK, Brooks DA, Meikle PJ. Absence of α-galactosidase cross-correction in Fabry heterozygote cultured skin fibroblasts. Mol Genet Metab 2015; 114(2):268-73."

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Absence of α-galactosidase cross-correction in Fabry heterozygote cultured skin fibroblasts

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Abbreviations: GLA**,** α-galactosidase A; FD, Fabry disease; M6P, mannose 6-phosphate; CTH, ceramide trihexoside; MPS II, mucopolysaccharidosis type II

Abstract

Fabry disease (FD) is an X-linked lysosomal storage disorder resulting from deficiency of α-galactosidase A (GLA). Traditionally, heterozygotes were considered asymptomatic carriers of FD, but it is now apparent that the asymptomatic female carrier is the exception and most heterozygotes suffer significant multisystemic disease. To determine why the process of cross-correction does not occur effectively in FD heterozygotes, we investigated GLA production and secretion in cultured skin fibroblasts as well as GLA levels in plasma. The maturation of GLA was similar in FD heterozygotes and control fibroblasts, confirming that both produce the 46 kDa mature form; the same as that present in control plasma. However, the proportion of GLA secreted into the culture media was substantially less than eight other lysosomal proteins. Artificial generation of FD heterozygotes *in cellulo*, along with another lysosomal storage disorder, mucopolysaccharidosis type II, revealed no cross-correction in the FD system, whereas MPS II fibroblasts were able to cross-correct. In plasma, GLA was present as the 46 kDa mature form, which lacks the mannose 6-phosphorylated moiety and is not able to be efficiently endocytosed by affected cells. Our evidence shows that fibroblasts secrete minimal amounts of GLA and consequently normal fibroblasts are unable to cross-correct FD fibroblasts. We suggest that symptomatic FD heterozygotes arise due to the secretion of primarily the mature form, with only small amounts of the mannose 6-phosphorylated form of GLA from unaffected cells. This limits capacity for enzyme cross correction of affected cells, despite uptake of exogenous recombinant GLA.

Keywords: Fabry disease, α-galactosidase, lysosomal enzyme, lysosomal storage disorder, cross correction, ceramide trihexoside

INTRODUCTION

Fabry disease (FD) is an X-linked lysosomal storage disorder caused by deficiency of the lysosomal hydrolase, α-galactosidase A (GLA, EC 3.2.1.22), which is responsible for cleaving terminal α-galactosyl residues from glycosphingolipids. Consequently, partially degraded glycosphingolipids, particularly the primary substrate, ceramide trihexoside (CTH), accumulate in lysosomes of affected cells, mainly of the vasculoendothelial system [1]. Classical FD hemizygotes usually manifest in childhood with symptoms such as acroparasthesias, angiokeratoma, corneal dystrophy, and sweating abnormalities, progressing to cardiovascular, cerebrovascular and renal disease resulting in premature death in the fourth or fifth decade of life. Some patients present with a later onset phenotype; attenuated symptoms that appear to target certain organs such as the heart and/or kidneys and often do not present until later adulthood [2]. FD heterozygotes have traditionally been considered asymptomatic carriers; similar to another X-linked lysosomal storage disorder, mucopolysaccharidosis type II (MPS II) [3]. However, recent studies have demonstrated that this standpoint is no longer correct. Females with an attenuated form of FD are in the minority and most heterozygotes suffer a similar disease burden and symptoms as their male hemizygous counterparts, albeit manifesting at a later age in life [4]. Biochemically it is difficult to explain the presence of symptoms in females, because within heterozygote cell populations there would be, on average, a 50:50 distribution of affected and unaffected cells in the total cellular pool. The unaffected cells should produce sufficient amounts of GLA, which if able to be secreted and taken up by the affected cells, could correct the enzyme deficiency, prevent substrate accumulation and lead to an asymptomatic phenotype. This process of cross-correction seems to occur readily in another X-linked lysosomal storage disorder, MPS II, where asymptomatic carriers are the norm [5].

GLA is synthesised in the endoplasmic reticulum as a 50 kDa glycoprotein, which is then mannose 6-phosphorylated in the Golgi to generate a 52 kDa precursor form. Additional processing, *via* a 47–50 kDa intermediate, results in a mature 46 kDa lysosomal molecular form. Golgi post-translational modifications can include the addition of two oligomannosyl carbohydrates, which together generate a high affinity ligand for the mannose 6-phosphate (M6P) receptor [6]. The majority of GLA is targeted to the pre-lysosomal/endosomal compartments by interaction with M6P receptors [7]. Once within these compartments the acidic pH causes the enzyme to dissociate from the receptor, facilitating transfer to the lysosome where GLA functions to break down its substrate [6, 8, 9]. A portion of synthesised GLA can avoid traffic to the lysosome due to either alternate high mannose carbohydrate processing or aberrant sorting and this protein can be released from the cell. Figure 1 shows a schematic of the biosynthesis and trafficking of GLA. This secreted form of GLA, referred to as the precursor form, contains the M6P targeting signal enabling the same or adjacent cells to internalise it by M6P receptor mediated uptake. This mechanism of M6P receptor mediated uptake is utilised to deliver exogenously administered GLA into FD patient cells for enzyme replacement therapy.

FD heterozygote cells therefore respond to exogenously administered GLA, which is effective in reducing the storage burden. However, the process of cross-correction from an unaffected cell to an affected cell in a heterozygote population does not appear to be sufficient to avoid the onset of disease. Here, we have investigated why this process of cross-correction does not occur effectively in FD heterozygote fibroblasts and provide evidence that this is due to insufficient secretion of the mannose 6-phosphorylated form of GLA.

MATERIALS AND METHODS

Materials

Human plasma samples were collected from healthy adults (n = 6) and FD patients (n = 4, unknown genotype) submitted to this Hospital for diagnosis. Cell culture products were from MP Biomedicals Inc (Aurora, OH, USA) and Sigma/SAFC (St Louis, MO, USA). The 10 kDa cut-off filters and the horseradish peroxidase (HRP)-conjugated secondary antibody used in western blot analysis were from Chemicon/Millipore (Billerica, MA, USA). The Immun-Star HRP substrate kit used to develop the western blots was from Bio-Rad (Hercules, CA, USA). The sheep anti-AGA polyclonal antibody was affinity purified, as previously described for another lysosomal enzyme [10]. All other western blot materials were from Invitrogen (Carlsbad, CA, USA) as part of the X-cell electrophoresis and transfer system. M6P, monoclonal anti-β-actin-peroxidase combined primary and secondary antibody and Triton-X-100 were from Sigma (St. Louis, MO, USA). Tween-20 was from Merck (Whitehouse Station, NJ, USA). *N*-heptadecanoyl ceramide trihexoside internal standard (CTH 17:0) was from Matreya LLC (Pleasant Gap, PA). All solvents were HPLC grade, except chloroform, which was reagent grade and contained 1% ethanol.

Cell culture

De-identified human skin fibroblasts from five FD hemizygotes with the following mutations R227Q, F169S, M284T, 717delAA, 1073delGAG, two FD heterozygotes (mutations C52R and G128E) and two MPS II patients were cultured from skin biopsies submitted to the Women's and Children's Hospital, Adelaide, Australia, for diagnosis. Control fibroblasts were cultured from skin biopsies taken from five, apparently healthy, adult volunteers following informed consent. The Women's and Children's Hospital Research Ethics Committee, Adelaide, Australia, approved the use of skin fibroblasts in this study. Fibroblasts from unaffected controls, FD and

MPS II patients were maintained in basal modified Eagles (BME) media supplemented with 10 % (v/v) foetal calf serum at 37 °C in a 5 % CO_2 humidified atmosphere. Cells were harvested by disruption with trypsin, and cell extracts prepared in 0.2 ml 20 mM Tris-HCl containing 0.5 M NaCl (pH 7.2) with 0.1 % (v/v) NP-40 and sonicated for 20 seconds at 4 °C. Total cell protein was determined by the method of Lowry et al [11]. Hybrid flasks were prepared from two FD hemizygote (genotypes R227Q and 717delAA) and two MPS II cell lines by seeding T_{75} flasks with either 1 x 10^6 FD hemizygote or MPS II cells and 1 x 10^6 control cells and cells were allowed to adhere for 24 hours before media was replaced with serum-free BME.

Ceramide trihexoside (CTH) analysis

Lipids (including CTH) were extracted from fibroblast extracts (100 µg total cell protein) according to established methods, incorporating 400 pmol of CTH 17:0 as internal standard [12]. Analysis was performed by liquid chromatography/electrospray ionisation-tandem mass spectrometry (LC/ESI-MS/MS) using a Shimadzu binary pump and autosampler system combined with a PE Sciex API 4000 triple quadrupole mass spectrometer with a turbo-ionspray source (200°C) and Analyst 1.4.2 data system. LC separation of lipids was performed on an Alltima C18, 3 µm (50 x 2.1 mm) column using the following gradient conditions at a flow rate of 0.2 ml/min. The column was equilibrated in 70% mobile phase A and then linearly reduced to 0% A (100% B) over three minutes followed by five minutes at 100% B, a return to 70% A over 0.1 minute, then 1.9 minutes at 70% A prior to the next injection. Mobile phases A and B consisted of tetrahydrofuran:methanol:water in the ratios (30:20:50) and (70:20:10), respectively, both containing 10 mM ammonium formate. Quantification of individual CTH species (16:0, 22:0, 24:0 and 24:1) was performed using multiple-reaction monitoring in positive ion mode with the product ion of *m/z* 264 corresponding to the sphingosine base. Nitrogen was used as the

collision gas at a pressure of 2 x 10^{-5} Torr, and each ion pair was monitored for 100 ms with a resolution of 0.7 amu at half peak height and averaged from continuous scans over the elution period. Concentrations of each molecular species were calculated by relating the peak area of each CTH species to the peak area of the CTH 17:0 internal standard.

Total glycosaminoglycan determination

The amount of glycosaminoglycan in control, MPS II and hybrid fibroblast extracts was determined using the method of de Jong et al [13]. In brief, 20 μg of total cell protein was incubated with 11 mg/L 1,9-dimethylmethylene blue chloride in 5.5 mM formic acid, 0.5 M sodium acetate, pH 5.8 for 5 minutes at room temperature. Absorbance was measured at 520 nm and the concentration of total glycosaminoglycans in each sample was interpolated from a standard curve (0-100 mg/L chondroitin sulfate).

Western blot analysis of fibroblast extracts and plasma

Western blot analysis was performed using a sheep anti-GLA polyclonal antibody and an anti-sheep HRP-conjugated secondary antibody. Analysis of β-actin as a loading control was performed using a monoclonal anti-β-actin-peroxidase combined primary and secondary antibody. Following SDS/PAGE electrophoresis of duplicate gels containing, 10 μg/well control, FD heterozygote (C52R, G128E) and hemizygote (R227Q, 717delAA) cell extracts or 5 μL of control human plasma on an Invitrogen X-cell system, the protein was transferred onto polyvinylidene fluoride membranes using the same Invitrogen system. The membranes were incubated with block solution (5 % (w/v) skim milk powder in 0.02 M Tris-HCl (pH 7.0) containing 0.25 M NaCl and 0.1 % (v/v) Tween-20) for one hour at room temperature, then

incubated with either the anti-GLA polyclonal antibody (diluted to 1 μg/mL in 10 ml block solution) overnight at $4^{o}C$, with rocking or the monoclonal anti-β-actin-peroxidase combined primary and secondary antibody (diluted 1:3,000 in block solution) for one hour at room temperature. After washing with 0.02 M Tris-HCl (pH 7.0) containing 0.25 M NaCl and 0.1 % (v/v) Tween-20, the GLA membrane was incubated for one hour at room temperature with the HRP-conjugated secondary antibody. Following washing with 0.02 M Tris-HCl (pH 7.0) containing 0.25 M NaCl and 0.1 % (v/v) Tween-20, both membranes were treated with Bio-Rad Immun-Star ECL reagent and developed using the Fujifilm LAS-4000 for between 10 seconds and 3 minutes, using ImageReader and MultiGauge 1D software.

Simultaneous assay of nine lysosomal proteins

Immune-quantification of nine lysosomal proteins was performed using micro-bead suspension array technology, as previously described, but with the omission of LAMP-1 and saposin C [14]. Skin fibroblasts, five FD hemizygotes, two FD heterozygotes and five controls, were harvested at five, seven and 10 days and the culture medium concentrated 10-fold using a 10 mL Amicon stirred-cell concentrator with 10 kDa protein cut-off ultra filtration membrane. Cell extracts were diluted to 0.625 μg/well or 0.3125 μg/well and 4 μL of plasma diluted to 100 μL in phosphate buffered saline with 0.05 % (v/v) Tween-20, 0.5 % (w/v) bovine serum albumin, 0.05 % (w/v) γ-globulin, and 0.05 % (w/v) sodium azide, pH 7.4; 100 μL of the media samples were directly added to the wells. Concentrations of *N*-sulphamidase, acid sphingomyelinase, α-iduronidase, α-glucosidase, β-glucosidase, GLA, arylsulphatase A, iduronate 2-sulphatase and *N*-acetylgalactosamine 4-sulphatase in each sample were then determined by relationship to the corresponding calibration curve.

RESULTS AND DISCUSSION

Properties of GLA

The clinical presentation of heterozygote women with FD has historically been explained by skewed X inactivation, producing an uneven distribution of affected and unaffected cells in the body, leading to organ-specific presentation [15, 16]. However, evidence from Maier et al [5] showed that heterozygous females with FD have random X inactivation. We therefore created an artificial hybrid FD heterozygote system with a 50:50 mix of affected and unaffected fibroblasts to investigate the properties of GLA.

The GLA maturation process may influence the nature of the enzyme that is secreted from cells including its molecular form and uptake properties. En-route from the trans-Golgi network to the lysosome, GLA is processed from a 52 kDa glycosylated precursor to the active 46 kDa mature enzyme (Figure 1). Figure 2 shows that the 46 kDa mature form of GLA was present in both control fibroblasts and the artificial hybrid FD heterozygote system indicating that there was no fault in the intracellular maturation process. The high uptake mannose 6-phosphorylated form of GLA is 52 kDa [17]- the molecular size of the recombinant protein - but this was not detected in either control fibroblasts or the artificial hybrid FD heterozygote system. In control plasma circulating levels of GLA were similar to those of iduronate 2-sulphatase, with these two enzymes present in plasma at concentrations higher than *N*-sulphamidase, acid sphingomyelinase, α-iduronidase, α-glucosidase, β-glucosidase, arylsulphatase A and N-acetylgalactosamine 4-sulphatase (Table 1). However, the form of GLA present in plasma is the mature 46 kDa enzyme (Fig. 2), rather than the high uptake, mannose 6-phosphorylated 52 kDa form.

Table 2 shows that no GLA was detected in the five FD hemizygote fibroblast lines. The amount of intracellular GLA produced in control fibroblasts was similar to eight other lysosomal proteins measured, but the amount of GLA secreted into the culture medium was relatively low (Table 2, Fig. 3). Iduronate 2-sulphatase is a soluble luminal protein, which, like GLA, is trafficked to the lysosome *via* M6P receptors, and although iduronate 2-sulphatase and GLA are both present in similar concentrations within the cell (data from Table 2, 47 and 53 ng/mg of protein in control fibroblasts, respectively), 10-fold more iduronate 2-sulphatase is released into the culture medium (Fig. 3). Unlike GLA, iduronate 2-sulphatase is highly sialylated, a property presumed to maintain a circulating pool of enzyme preventing both antibody recognition and receptor mediated recapture [18].

Substrate levels in fibroblasts

The next question was to compare the level of substrate in the artificial hybrid FD heterozygote system with an MPS II cell culture system that was produced in the same way. Measurement of substrate in these fibroblasts showed that at confluence the FD hybrid cells had half the amount of CTH than that observed in the FD fibroblasts (Fig 4A). Following one week in culture both culture systems showed a significant increase in CTH compared with controls, and the amount of CTH in the hybrid cells remained proportional to that in the FD cells. These results show that even in a ‘perfect’ heterozygote system with a 50:50 distribution of FD and unaffected fibroblasts the GLA that is present in the culture media is unable to reduce the storage burden in the affected fibroblasts. In contrast, the MPS II affected and hybrid cells showed an initial increase in glycosaminoglycan storage above the controls, but following one week in culture the storage in the hybrid cell line was reduced to the amount detected in control cells (Fig. 4B). As a positive control, we also treated the Fabry affected, hybrid and control cell lines with 1 μg/mL

recombinant human GLA for one week, which normalised CTH in both the affected and the hybrid systems to the amount detected in the controls (data not shown). Of note, the artificial heterozygote system was generated by starting a culture with an even number of both cell types, and although unlikely, as the fibroblasts used had similar growth properties, we cannot rule out the possibility of a survival advantage/disadvantage of one fibroblast type.

The correction of storage in FD and hybrid fibroblasts by the 52 kDa high uptake form of recombinant GLA and the clinical use of enzyme replacement therapy, suggest that the level of M6P receptor expression on cells is not the reason for the failure of GLA to cross-correct. A more likely explanation is that the two high affinity M6P ligands reported for GLA [6] result in efficient binding to the M6P receptor [19] resulting in preferential delivery of this enzyme to the lysosome. It would appear that some of this mature GLA is able to leave the cell but lacking the M6P is ineffective in cross correction. This may also explain why GLA produced from over-expression systems, which saturate the M6P targeting system, and release the high uptake 52 kDa precursor GLA generate a form of the enzyme that is capable of correction in FD patients who are receiving enzyme replacement therapy. A limitation of our study is the use of a fibroblast system – not the primary cell type affected in FD. Ideally, these experiments should be conducted in a vascular endothelial system that truly reflects the FD phenotype.

Conclusion

These results suggest that in FD heterozygotes, the limited amount of GLA that is released from cells that ends up in intracellular spaces or in the plasma, is the mature 46 kDa form rather than the high uptake mannose 6-phosphorylated 52 kDa form. Consequently affected cells do not receive GLA to alleviate the storage burden. This is likely a consequence of high affinity binding of GLA to the M6P receptor during intracellular sorting and the subsequent efficient delivery of

nascent GLA to the lysosome. Partial or intermittent disruption of the M6P trafficking pathway may represent a therapeutic target for FD heterozygotes.

Acknowledgments

We would like to thank Justin Tucker and Debbie Lang for assistance with the multiplex analysis of lysosomal proteins and Troy Stomski for running the mass spectrometry samples. This work was supported by the Women's and Children's Hospital Research Foundation.

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Figure legends

Figure 1: Schematic of the fate of intracellular GLA

GLA is synthesised in the rough endoplasmic reticulum (RER), which is then mannose 6-phosphorylated in the Golgi. The majority of GLA is subsequently targeted to the endosomes by the interaction with mannose 6-phosphate receptors, within which the acidic pH causes GLA to dissociate from the receptor facilitating transfer to the lysosome. A portion of GLA can avoid this process and be released from the cell. GLA is denoted by a filled circle and P represents phosphorylation of mannose.

Figure 2: Western blot of GLA in cultured fibroblasts and plasma

Western blot analysis of two different control (Cont), two FD (C52R, G128E) heterozygotes (Het) and two FD (R227Q and 717delAA) hemizygotes (Hem) cultured skin fibroblast extracts. The 46 kDa mature form of GLA is depicted in the Cont and Het but no GLA is present in the Hem. As a positive control, 2 pg recombinant human GLA (rhα-gal) at 52 kDa was included. As a loading control a duplicate blot was prepared and probed using a monoclonal anti-β-Actin-Peroxidase combined primary and secondary antibody and exposed for 20 seconds.

Figure 3: Percentage of lysosomal protein secreted into the culture medium from unaffected cultured skin fibroblasts

The concentration of nine lysosomal proteins was determined in cell extracts and the culture medium of unaffected skin fibroblasts using a multiplex immune quantification assay [14]. Results were expressed as a percentage of the amount secreted (ng/mL of culture medium) and

intracellular (ng/mg of total cell protein) lysosomal protein, as shown in Table 1. Mean and SD are displayed for five unaffected cultured skin fibroblast cell lines. N-Sulph, *N*-sulphamidase; ASM, acid sphingomyelinase; IdUA, α-iduronidase; GAA, α-glucosidase; β-gluc, β-glucosidase; GLA, α-galactosidase; ASA, arylsulphatase A; I2S, iduronate 2-sulphatase; 4S, *N*-acetylgalactosamine 4-sulphatase.

Figure 4: Substrate levels in FD and MPS II artificial hybrid culture systems

Artificial hybrid heterozygote culture systems were established from two FD hemizygotes (R227Q and 717delAA) and two MPS II fibroblast cells. Cells were harvested at day zero (Confluence) and a subsequent culture harvested at one week post-confluence (Post-confluence) and assayed for the amount primary storage material. Panel A, the primary storage material in FD, ceramide trihexoside (CTH), as represented by the sum of the four most abundant species (16:0, 22:0, 24:0 and 24:1), in control, hybrid and FD affected fibroblasts. Panel B, the total glycosaminoglycan accumulation in control, hybrid and MPS II affected fibroblasts. Error bars represent the standard deviation between the duplicates of the two different cell lines harvested from each time point (n=4); * denotes a significant difference from control $p < 0.05$ (student t-test).

Table 1 Lysosomal proteins in plasma

lysosomal enzyme[a]	plasma sample[b]	
	Fabry	control
N-Sulph	0.5 (0.1)	0.6 (0.2)
ASM	10 (1.6)	9 (2.9)
IdUA	20 (1.2)	13 (3.1)
GAA	6.7 (2.4)	8.2 (3.1)
β-gluc	4.2 (0.7)	4.1 (0.8)
GLA	3.3 (0.5)	25 (8)
ASA	9 (2.2)	14 (4.3)
I2S	30 (3.8)	32 (11)
4S	0.3 (0.1)	0.5 (0.2)

[a]expressed as ng/ml of plasma
[b]mean (standard deviation), n=6 for control and n=4 for Fabry hemizygote samples
N-Sulph, *N*-sulphamidase; ASM, acid sphingomyelinase; IdUA, α-iduronidase; GAA, α-glucosidase; β-gluc, β-glucosidase; GLA, α-galactosidase; ASA, arylsulphatase A; I2S, iduronate 2-sulphatase; 4S, *N*-acetylgalactosamine 4-sulphatase

Table 2 Lysosomal proteins in skin fibroblast extracts and culture media

cell	N-Sulph		ASM		IdUA		GAA		β-gluc		GLA		ASA		I2S		4S	
line[a]	cells[b]	media[c]	cells	media	cells	media	cells	media	cells	media	cells	media	cells	media	cells	media	cells	media
cont day5	67[d]	1.6	37	1.1	240	2.5	223	2.0	113	0.8	53	0.3	92	1.9	47	3.3	18	0.4
	30-118[e]	1-3	17-64	0-2	141-434	1-7	90-422	1-3	70-180	0-1	35-75	0-0.5	28-160	1-2	27-84	1-6	9-22	0-1
d7	63	1.8	35	1.3	235	4.7	226	2.2	114	1.0	52	0.4	94	2.0	51	4.4	17	0.4
	41-117	1-4	20-63	1-2	143-463	2-14	103-441	1-3	76-192	0-2	36-75	0-0.5	33-165	2-3	26-97	3-7	9-24	0-1
d10	98	1.8	35	2.0	256	3.2	241	3.0	130	1.4	63	0.5	123	2.5	66	6.7	20	0.4
	65-149	1-2	16-67	1-3	134-524	2-4	108-461	2-5	75-207	0-3	34-93	0-1	57-188	2-3	29-138	4-13	12-23	0-1
het day5	122	3.3	37	0.5	350	2.5	157	1.7	147	0.5	32	0.2	163	1.9	43	1.7	19	0.5
	96-148	1-6	16-59	0-1	224-477	2-3	117-197	1-2	74-219	0-1	32-33	0-0	154-171	1-2	26-60	1-3	11-26	0-1
d7	98	1.5	33	0.7	344	3.6	175	2.0	127	1.0	84	0.3	138	2.0	42	3.4	16	0.3
	82-115	1-2	26-39	0-1	321-367	2-5	144-205	2-2	94-161	1-1	82-84	0-0.5	115-161	2-2	41-44	2-5	12-21	0-0
d10	132	1.7	41	1.0	420	5.2	204	2.3	178	1.0	70	0.3	167	2.2	55	4.7	21	0.3
	95-169	1-2	28-54	1-1	331-509	3-8	199-209	2-3	110-247	1-1	39-101	0-0.5	166-169	2-3	46-65	4-5	12-30	0-0.5
hem day5	77	1.0	52	1.5	263	3.0	241	2.6	162	1.5	0	0	129	1.8	52	3.7	24	0.4
	40-174	0-1	32-70	1-3	146-469	1-5	188-340	1-4	134-234	1-2	n/a	n/a	92-149	1-3	33-77	2-5	17-30	0-1
day 7	77	1.5	47	2.5	244	5.4	227	3.6	145	2.2	0	0	122	2.0	51	6.1	22	0.5
	40-183	1-2	28-72	1-5	125-476	2-9	170-359	2-5	111-229	2-3	n/a	n/a	82-157	1-3	31-83	3-10	14-27	0-1
day 10	79	1.6	44	2.9	263	6.4	234	4.1	153	2.2	0	0	126	2.2	47	7.5	23	0.5
	39-191	1-2	29-63	1-7	136-443	3-14	164-359	3-4	107-222	2-3	n/a	n/a	97-160	2-3	29-77	5-12	16-29	0-1

[a]cont day5, day7, day10, control cells harvested at 5, 7 and 10 days; het day5, day7, day10, Fabry heterozygote cells harvested at 5, 7 and 10 days; hem day5, day7, day10, Fabry hemizygote cells harvested at 5, 7 and 10 days

[b]expressed as ng/mg of total cell protein

[c]expressed as ng/ml of culture media

[d] mean and [e]range, n=5 for control and Fabry hemizygote samples, n=2 for Fabry heterozygote samples

N-Sulph, *N*-sulphamidase; ASM, acid sphingomyelinase; IdUA, α-iduronidase; GAA, α-glucosidase; β-gluc, β-glucosidase; GLA, α-galactosidase; ASA, arylsulphatase A; I2S, iduronate 2-sulphatase; 4S, *N*-acetylgalactosamine 4-sulphatase

Figure 1

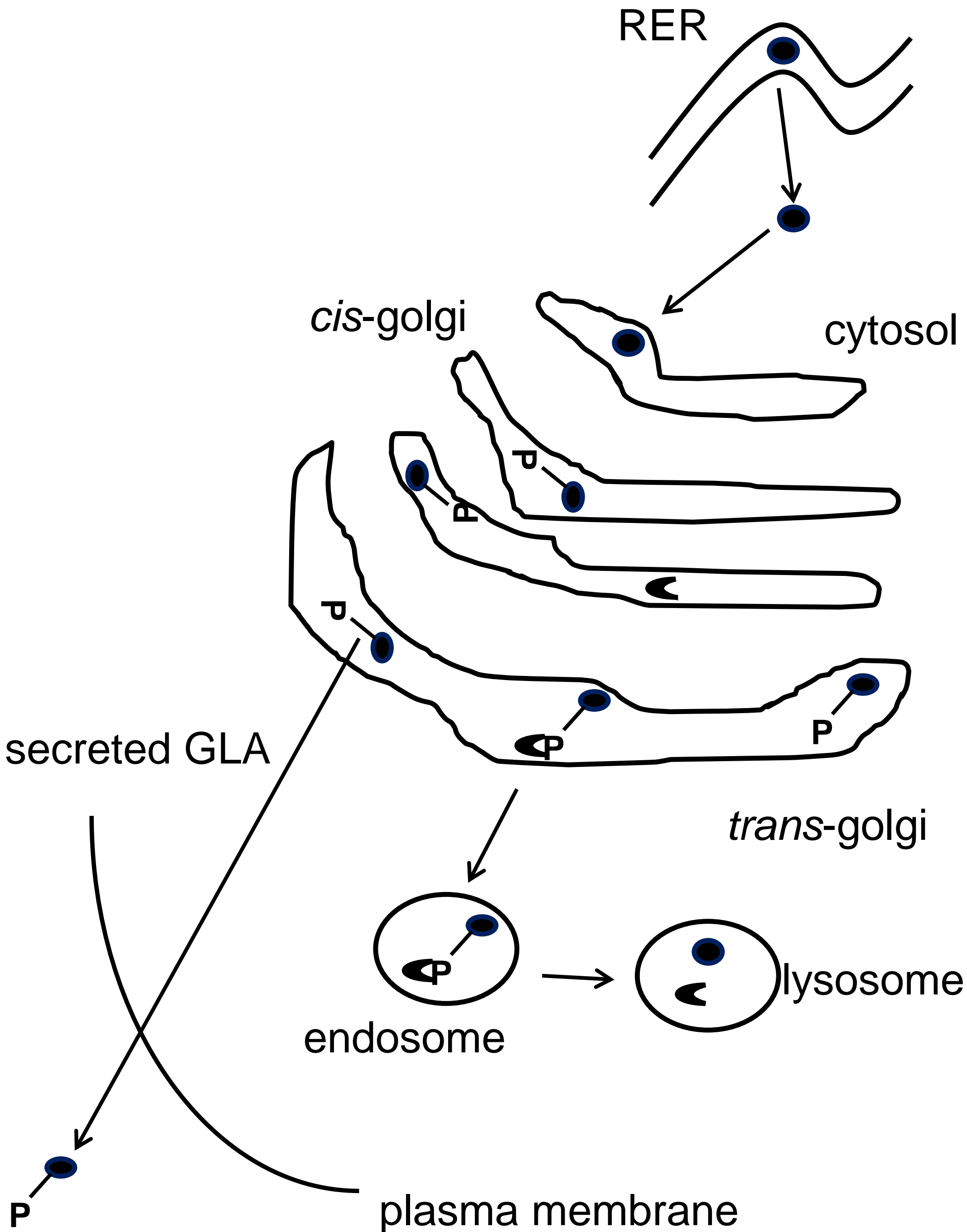

Figure 2

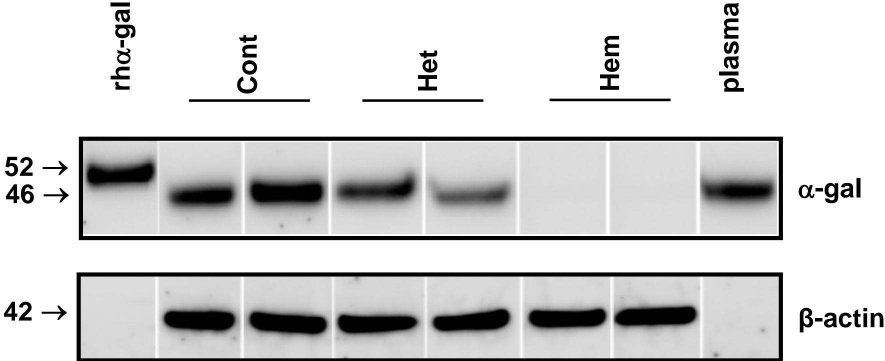

Figure 3

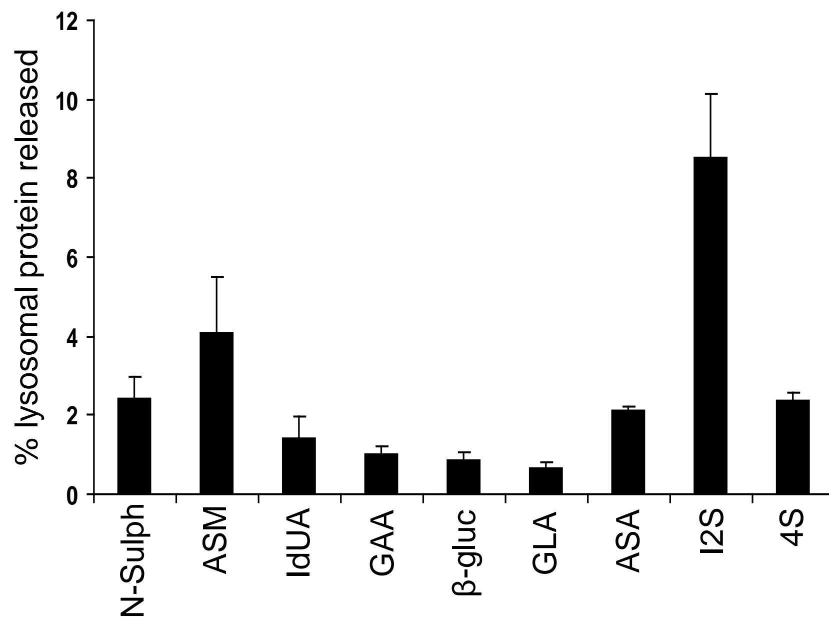

Figure 4

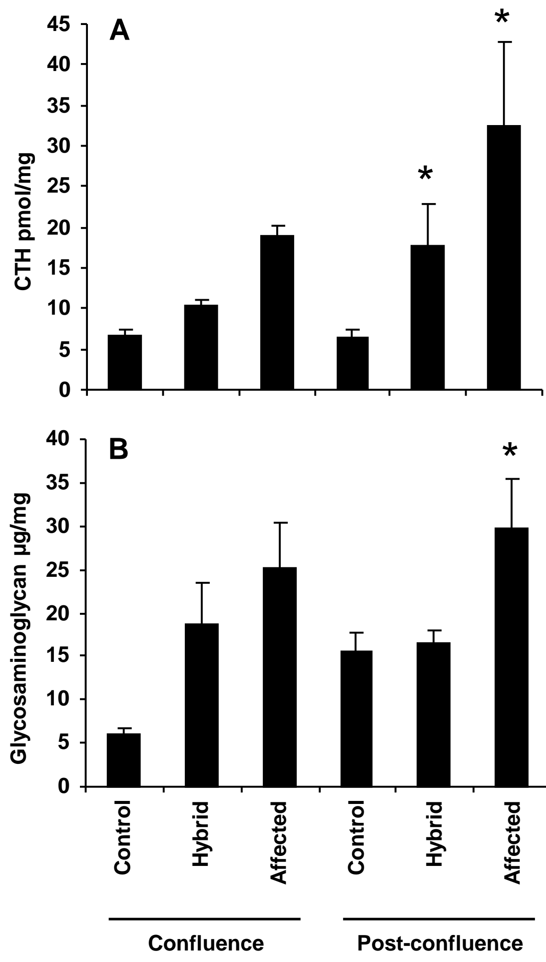

Figure 1

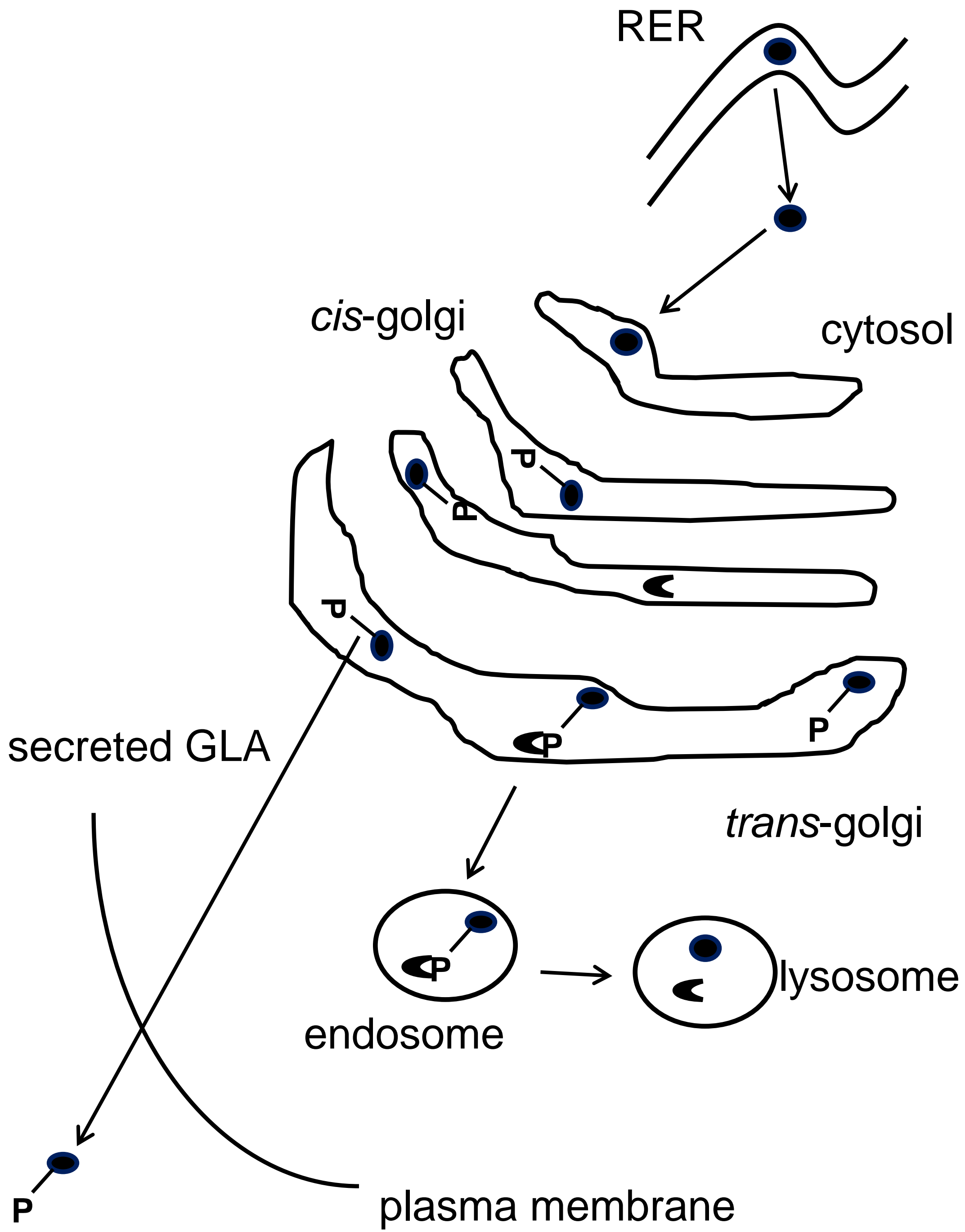

Figure 2

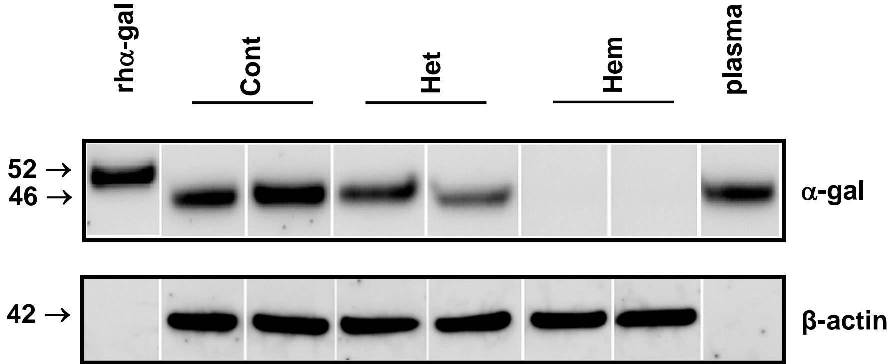

Figure 3

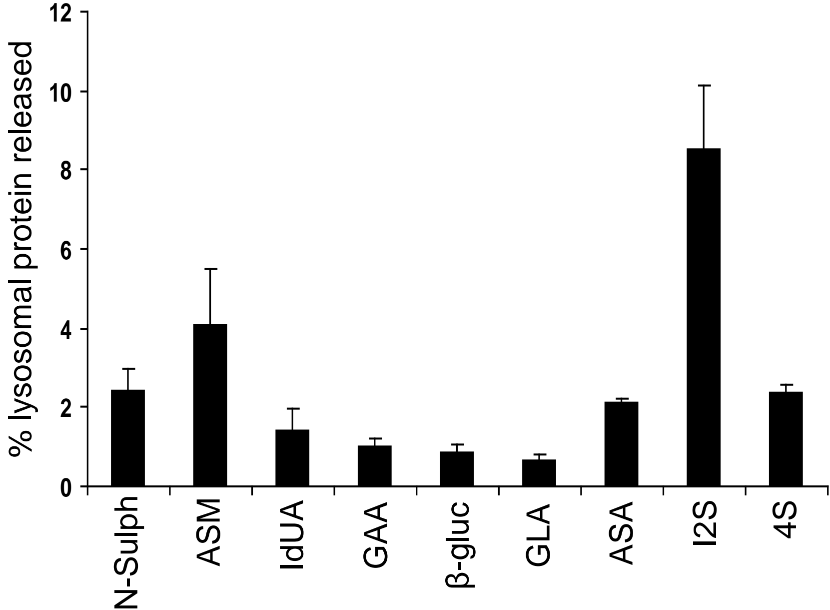

Figure 4