Defective glucuronic acid transport from lysosomes of infantile free sialic acid storage disease fibroblasts

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Separation by h.p.l.c. and pulsed amperometric detection were employed to measure glucuronic acid (GlcUA) and other acidic monosaccharides in fibroblasts from patients with infantile free sialic acid storage disease (ISSD) and Salla disease. These lysosomal storage disorders result from defective carrier-mediated transport of free *N*-acetylneuraminic acid (NeuAc) out of cellular lysosomes. Three Salla disease fibroblast strains stored approx. 0.4 nmol of free GlcUA/mg of cell protein, whereas four ISSD strains stored approx. 5 nmol GlcUA/mg (normal is undetectable). The GlcUA content of the mutant cell strains, which by differential centrifugation and Percoll gradient fractionation was localized to the lysosomes, averaged 5% of the free NeuAc content of the cells. *N*-Glycolylneuraminic acid (NeuGc) also accumulated in ISSD cells, but only when they were grown in the presence of fetal calf serum, which contains abundant NeuGc. No other acidic monosaccharides were detected in any of the mutant cell strains. GlcUA egress studies revealed that 56% of the initial GlcUA content was lost from normal granular fractions after 2 min at 37 °C. For similarly loaded ISSD granular fractions, virtually no GlcUA was lost even after 6 min. The results indicate that GlcUA is recognized and transported by the lysosomal NeuAc carrier, and that GlcUA transport is impaired in the lysosomal disorders of free NeuAc storage.

INTRODUCTION

The lysosomal hydrolysis of macromolecules, e.g. proteins, mucopolysaccharides and oligosaccharides, yields small molecules which must cross the lysosomal membrane for further catabolism or re-utilization in the cytosol [1]. Lysosomal membrane transport systems have now been described for amino acids [2–8], nucleosides [9] and monosaccharides [10–13], and lysosomal storage disorders of cobalamin [14], cystine (cystinosis) [2–4] and sialic acid transport [12,15] are well recognized [16].

For sialic acid, or *N*-acetylneuraminic acid (NeuAc), there exist at least two storage disorders of different severities. Salla disease is an autosomal recessively inherited Finnish mutation characterized by truncal ataxia and psychomotor retardation [16–18]. Infantile free sialic acid storage disease (ISSD) is a rapidly fatal disorder characterized by coarse faeces, hepatosplenomegaly and impaired growth and development [16,19–22]. In both Salla disease and ISSD the egress of free (unbound) NeuAc from fibroblast lysosomes is negligible [12,15]. Although the ligand specificity of the NeuAc carrier has not been determined in any human tissue, Mancini *et al.* [23] did measure proton-driven uptake and *trans*-stimulation of NeuAc transport using purified rat liver lysosomal membrane vesicles. They demonstrated that the glycosaminoglycan catabolite glucuronic acid (GlcUA), as well as *N*-glycolylneuraminic acid (NeuGc), gluconic acid, and certain other acidic monosaccharides, competed with NeuAc for transport across rat liver lysosomal membranes. Consequently, the competing compounds were considered to be recognized by the lysosomal sialic acid carrier.

We now extend these findings to cultured human fibroblasts, demonstrating NeuGc and GlcUA storage in Salla disease and ISSD lysosomes and impaired egress of GlcUA from lysosome-rich granular fractions. The results indicate that NeuAc, GlcUA and NeuGc share a single lysosomal membrane carrier system.

EXPERIMENTAL

Materials

All carbohydrates were obtained from Sigma (St. Louis, MO, U.S.A.) or from Aldrich (Milwaukee, WI, U.S.A.), with the exception of iduronic acid (IdUA), which was kindly provided by Dr. G. Ashwell, who synthesized it according to Shafizadeh & Wolfrom [24]. Sodium acetate (h.p.l.c.-grade) and NaOH (50%, w/w) were from Fisher Scientific (Fair Lawn, NJ, U.S.A.).

Monosaccharide detection

Monosaccharides were measured on a Dionex h.p.l.c. apparatus equipped with a gradient pump and an Ionchrom/Pulsed Amperometric Detector. A Dionex Eluent Degas Model was used to sparge and pressurize the eluents. Samples were injected manually via a Rheodyne 7125 or via a Shimadzu SIL-6A autoinjector connected to a Shimadzu SCL-6A system controller. The monosaccharides were separated on a Dionex Carbopac AS6 column, equipped with an AS6 guard column, with a flow rate of 1 ml/min. The detector signal was plotted and integrated on a Spectra-Physics 4270 Integrator. In studies of normal and ISSD fibroblasts, the results of NeuAc determinations by the Dionex method closely resembled those obtained using a different h.p.l.c. technique [15].

Eluents were prepared using sodium acetate and a 50% (w/w) NaOH solution. Eluent A consisted of 0.1 M-NaOH and 0.03 M-NaAc and eluent B contained 0.1 M-NaOH and 0.24 M-NaAc. NeuAc was measured in an isocratic fashion using an 86:14 (v/v) mixture of A/B, resulting in a retention time for NeuAc of 8.5 min. For GlcUA and NeuGc measurements, the A/B mixture was 57:43 (v/v) and the retention times were 12.8 and 14.1 min respectively. All three acidic monosaccharides could also be measured in one run by a gradient starting from A/B = 9:1 at

Abbreviations used: NeuAc, *N*-acetylneuraminic acid; IdUA, iduronic acid; GlcUA, glucuronic acid; NeuGc, *N*-glycolylneuraminic acid; CMP-NeuAc, CMP-*N*-acetylneuraminic acid; ISSD, infantile free sialic acid storage disease.

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0 min to A/B = 1:9 at 20 min, and continuing at this ratio for 25 min. Thereafter, the system was equilibrated to the starting conditions by flushing for 15 min with an A/B mixture of 9:1. The retention times were 9.1 min for NeuAc, 18.6 min for GlcUA, 19.9 min for NeuGc and 23.9 min for IdUA. Baseline drift caused by the gradient was prevented either by adding extra NaOH to eluent A (up to 0.12 M-NaOH) or by adding post-column 0.3 M-NaOH with a flow rate of 0.4 ml/min using a Dionex Pump DQP-1. The detection limit for NeuAc was 40 nmol/l, for GlcUA and IdUA it was 250 nmol/l, and for NeuGc it was 50 nmol/l.

Cell culture

Normal fibroblast strains GM 1489, 1501, 3440, 3651, 5659, 5757, and ISSD cell strain GM 5520, were obtained from the Human Genetic Mutant Cell Repository (Camden, NJ, U.S.A.). Salla disease strains were courtesy of Dr. Martin Renlund, Helsinki University Central Hospital, Helsinki, Finland. ISSD cell strain W.S. [20] was provided by Drs. Mark Lubinsky and Warren G. Sanger of the University of Nebraska Medical Center, Omaha, NE, U.S.A. ISSD strains R.S. and C.C. [22] were provided by Dr. John J. Hopwood of Adelaide Children's Hospital, North Adelaide, South Australia, Australia. The cells were cultured in Dulbecco's modified Eagle's medium (Bioproducts Inc., Walkersville, MD, U.S.A.) fortified with 10 % heat-inactivated fetal calf serum (Gibco, Grand Island, NY, U.S.A.), 2 mM-glutamine and antibiotics as described previously [12,15]. In one experiment, fetal calf serum was replaced by human serum which was heat-inactivated by treating for 30 min at 58 °C. The cells (generally grown in one 175 cm² flask) were harvested by trypsin treatment and washed three times with cold phosphate-buffered saline. Whole cell extracts were prepared by sonicating twice for 20 s in 0.5–1 ml of water, followed by boiling for 2 min to deproteinize. For a cell fractionation, the fibroblasts were washed twice with cold phosphate-buffered saline and once with cold 0.25 M-sucrose; the cell pellet was taken up in 2 ml of cold 0.25 M-sucrose, dispersed gently in a homogenizer and placed in a nitrogen cavitation apparatus (Kontes, Vineland, NJ, U.S.A.) at 207 kPa for 10 min. The nuclear fraction (1000 g), the lysosome-rich granular fraction (17000 g) and the supernatant were prepared as previously described [12,15].

A Percoll gradient centrifugation was also performed as previously described [15]. Fractions (2.4 ml) were collected and analysed for enzyme activities. NeuAc and GlcUA were measured in these fractions after purification on AG-1 acetate resin (Bio-Rad X8, 100–200 mesh) followed by lyophilization [15].

GlcUA loading and egress measurements

In order to measure GlcUA egress from normal fibroblast lysosomes, these vesicles needed to be loaded with increased concentrations of GlcUA. Therefore normal fibroblasts were harvested and lysosome-rich granular fractions (0.5 ml) were exposed to 4–50 mM-GlcUA at pH 5.5 for 15 min at 37 °C. The loading procedure was stopped by diluting in 2.5 ml of cold 0.25 M-sucrose/10 mM-Hepes, pH 7.2, and the loaded granular fractions were washed with 3 × 3 ml of cold sucrose/Hepes. GlcUA egress was measured by resuspending the final pellet in sucrose/Hepes and incubating at 37 °C. After 0, 0.5, 1, 2, 6 or 18 min, a 500 µl aliquot was cooled quickly and then centrifuged at 17000 g for 10 min. After removing the supernatant, the lysosome-containing pellet was suspended in 300 µl of water and sonicated. GlcUA concentrations, measured by injecting up to 200 µl of the lysosome-containing fraction into the Dionex h.p.l.c. unit, were normalized to lysosomal content by measurements of β-hexosaminidase activity. Lysosomal rupture during the course

of egress was estimated from the activity of β-hexosaminidase in the supernatant and the lysosome-containing pellet [15].

For ISSD fibroblasts, isolated lysosome-rich granular fractions either were loaded using 50 mM-GlcUA or were not exposed to GlcUA at all. Egress measurements were performed as for normal granular fractions, except that the duration of egress measurements continued for up to 18 min.

Other assays

β-Hexosaminidase activity was determined fluorimetrically with 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside in citrate buffer containing 0.1 % Triton X-100 as described previously [15]; 1 unit of enzyme hydrolysed 1 nmol of substrate/min at 37 °C. Protein concentration was determined by the BCA method [25].

RESULTS

Intracellular free GlcUA and NeuAc concentrations were determined in normal fibroblasts and in fibroblasts from patients with disorders of lysosomal NeuAc transport (Table 1). In control fibroblasts, the mean free NeuAc concentration was 0.9 nmol/mg of protein, and GlcUA was undetectable. In three Salla disease fibroblast strains, the free NeuAc concentration was approx. 20 nmol/mg of protein, and the GlcUA content was approx. 0.4 nmol/mg of protein. Four ISSD fibroblast strains had a mean free NeuAc content of 103 nmol/mg of protein and GlcUA levels of approx. 5 nmol/mg of protein. For all mutant cell strains, the GlcUA concentration averaged 5 % of the free NeuAc concentration when the relationship was examined by linear regression analysis ($r = 0.89$, results not shown). Several other acidic monosaccharides, including IdUA, galacturonic acid, gluconic acid, mannuronic acid lactone, galactonic acid and CMP-NeuAc, could not be detected in any of the fibroblast strains studied.

In contrast, the NeuAc derivative NeuGc was present in significant amounts in all the mutant cell strains studied, although it was not detectable in normal fibroblasts. Two Salla disease fibroblast cultures, F84-71 and F-1085, contained 2.6 and 1.4 nmol of NeuGc/mg of protein and the four ISSD fibroblast strains averaged 4.8, 8.7, 7.7 and 7.4 nmol of NeuGc/mg of protein (individual determinations not shown). However, the NeuGc stored in the mutant fibroblasts appeared to be derived from fetal calf serum since culturing in human serum diminished the NeuGc content of the cells. One ISSD fibroblast strain, cultured in 10 % human serum for 15 days, retained its NeuAc and GlcUA concentrations but contained only 18 % of the NeuGc concentration of a parallel culture grown in 10 % fetal calf serum (Table 2). After 30 days in culture using 10 % human serum, NeuGc was undetectable in the ISSD cells (results not shown).

The subcellular distribution of GlcUA, determined in three ISSD fibroblast strains, closely resembled that for hexosaminidase activity and free NeuAc (Table 3). For example, for ISSD strain GM 5520, the lysosome-rich granular fraction contained 64 % of the cell's hexosaminidase activity, 58 % of the free NeuAc and 58 % of the GlcUA. Similar concordance of these three parameters in each subcellular fraction was observed for two other ISSD strains, R.S. and C.C. (Table 3). For all three ISSD strains, granular fraction GlcUA averaged 12 pmol/unit of hexosaminidase compared with 752 pmol of free NeuAc per unit of hexosaminidase. In one Salla disease strain the only compartment with measurable GlcUA was the granular fraction (results not shown); it contained 5.2 pmol of GlcUA per unit of hexosaminidase and 355 pmol of free NeuAc per unit of hexosaminidase.

Table 1. Whole-cell concentrations of free NeuAc and GlcUA in normal, Salla disease and ISSD fibroblasts

Fibroblasts were grown to confluency, harvested and assayed for free NeuAc and GlcUA as described in the Experimental section. Each result, expressed in nmol/mg of total cell protein, is for an individual culture harvested separately. ND, not detectable. The limit of detection was approximately 0.1 nmol/mg of protein.

Cell strain	Free NeuAc (nmol/mg of protein)	GlcUA (nmol/mg of protein)
Normal controls		
GM 3440	0.7, 1.2	ND
GM 3651	0.3, 0.9	ND
GM 5659	1.3, 1.3	ND
Salla disease		
F-84-71	26, 27	ND, 0.2
F-1085	21, 15	0.6, 0.3
F-975	6, 7	0.5, 0.6
ISSD		
GM 5520	86, 90, 83, 75, 90, 76, 97	0.7, 1.9, 3.3, 1.3, 2.1
W.S.	128, 83	11.2, 2.0, 3.0, 5.6
R.S.	113, 73	7.6, 10.0, 3.8
C.C.	50, 200, 130	8.5, 2.4, 4.4, 4.4

Table 2. NeuAc, GlcUA and NeuGc contents of ISSD cell strain GM 5520 grown in either 10% fetal calf serum or 10% human serum for 15 days

Two 175 cm² flasks of ISSD strain GM 5520 contained either fetal calf serum or human serum for 15 days. Cell were then harvested and assayed for NeuAc, GlcUA and NeuGc. Results are means of triplicate determinations.

Serum	Content (nmol/mg of protein)		
	Free NeuAc	GlcUA	NeuGc
Fetal calf serum	76	1.3	4.4
Human serum	65	2.1	0.8

The three ISSD cell strains, GM 5520, R.S. and C.C., grown in 10% fetal calf serum, had 63%, 36% and 31% respectively of their NeuGc in the granular fraction. The mean NeuGc concentration was 38 pmol/unit of hexosaminidase. Salla disease fibroblast strains F-84-71 and F-1085 had mean granular fraction NeuGc concentrations of 20 and 8 pmol/unit of hexosaminidase respectively.

A better localization of the GlcUA in ISSD fibroblasts was achieved by performing a Percoll gradient fractionation of the post-nuclear supernatant of fibroblast strain R.S. (Fig. 1). Both NeuAc (Fig. 1a) and GlcUA (Fig. 1b) were found in the same distribution as the lysosomal marker, β -hexosaminidase.

Since the endogenous GlcUA of ISSD fibroblasts was apparently concentrated within lysosomes, its egress from normal and mutant lysosomes was measured directly. In order to measure normal GlcUA egress, fibroblast lysosome-rich granular fractions were loaded by exposure to GlcUA at pH 5.5 (see the Experimental section). This procedure increased normal granular fraction GlcUA from undetectable levels to concentrations

Table 3. Subcellular distribution of hexosaminidase, free NeuAc and GlcUA in cultured ISSD fibroblasts

ISSD fibroblasts were grown to confluency, harvested and subjected to differential centrifugation as described in the Experimental section. Subcellular fractions were assayed for hexosaminidase activity, free NeuAc and GlcUA. R.S. and C.C. fibroblast granular fractions appeared buoyant and lysosomal hexosaminidase was increased in the soluble fractions.

Cell strain and subcellular fraction	Hexosaminidase		Free NeuAc		GlcUA	
	(units)	(% of total)	(nmol)	(% of total)	(nmol)	(% of total)
GM 5520						
Nuclear	122	17	69	21	1.5	21
Granular	451	64	187	58	4.2	58
Soluble + microsomes	133	19	65	20	1.5	21
R.S.						
Nuclear	12	7	14	8	0	0
Granular	70	43	63	39	0.7	39
Soluble + microsomes	80	49	86	53	1.1	61
C.C.						
Nuclear	31	10	51	14	0.3	8
Granular	109	35	83	23	1.3	35
Soluble + microsomes	175	56	228	63	2.1	57

Fig. 1. Percoll gradient centrifugation of the post-nuclear fraction of ISSD fibroblast strain R.S.

Fractions (2.4 ml) were collected and assayed for NeuAc, GlcUA and β -hexosaminidase activity. Density increases with fraction number. The lysosomal marker β -hexosaminidase co-fractionates with NeuAc (a) as well as GlcUA (b).

ranging from 19 to 346 pmol/hexosaminidase unit, in direct correlation with the concentration of GlcUA (4–50 mM) to which they were exposed. ISSD fibroblasts, with endogenous granular fraction GlcUA concentrations of 7–37 pmol/hexosaminidase unit, increased their GlcUA content to 24–244 pmol/hexosaminidase unit upon loading with 50 mM-GlcUA.

GlcUA egress was determined in granular fractions from four normal and three ISSD fibroblast strains. Expressed as a percentage of the initial GlcUA content in pmol/hexosaminidase unit, the normal granular fractions decreased to 56% of their initial loading by 2 min of incubation (Fig. 2). Over that time period, the mean $t_{1/2}$ for granular fraction GlcUA egress was 2.6 min. ISSD granular fractions, loaded or not, lost virtually no GlcUA after 6 min. The mean $t_{1/2}$ for GlcUA egress over this period was 53 min. Moreover, the ISSD granular fractions contained 98% of their initial GlcUA content even after 18 min at 37 °C (results not shown). For one ISSD cell strain studied (GM 5520), there was also no egress of NeuGc after 18 min at 37 °C (results not shown).

In control experiments, the velocity of GlcUA egress, determined at 2 min of incubation, was demonstrated to remain as a linear function of initial loading for normal granular fractions. This lack of saturability over the range of GlcUA loading makes legitimate the application of first-order kinetics, as well as comparisons of percentage loss of GlcUA over time (Fig. 2). In addition, the decrease in normal granular fraction GlcUA was not due to loss of non-specifically bound GlcUA, since this decrease did not occur for loaded ISSD granular fractions.

Fig. 2. Egress of GlcUA from the lysosome-rich granular fraction of normal and ISSD fibroblasts

Four normal granular fractions (●) were loaded to GlcUA levels of 19–346 pmol/unit of hexosaminidase by exposure to 4–50 mM-GlcUA at pH 5.5. Three ISSD granular fractions (○) were similarly loaded to GlcUA levels of 24–244 pmol/unit of hexosaminidase or were studied using their endogenous GlcUA contents, 7–37 pmol/unit of hexosaminidase. GlcUA egress was determined by measuring GlcUA content, in pmol/hexosaminidase unit, at various times after incubation at 37 °C. Results are expressed as percentages of the initial (zero time) GlcUA level. For each ISSD fibroblast strain, the values for loaded and unloaded granular fractions were averaged. Points and bars represent means $\pm$ S.E.M. for four normal and three ISSD fibroblast granular fractions.

Moreover, rupture of the normal, loaded granular fractions by sonication (twice for 5 s) resulted in the complete loss of both GlcUA and hexosaminidase activity into a soluble fraction.

DISCUSSION

We have demonstrated that ISSD and Salla disease fibroblasts, which have impaired lysosomal egress of NeuAc [12,15], also accumulate increased amounts of two other acidic monosaccharides, GlcUA and NeuGc. The average intracellular concentration of GlcUA in ISSD was approx. 5% that of NeuAc (Table 1), and its intralysosomal localization was supported by the co-distribution of GlcUA and hexosaminidase on subcellular fractionation (Table 3) and Percoll gradient centrifugation (Fig. 1). By both techniques, the lysosomes appeared more buoyant than normal, apparently due to the excessive concentration of stored NeuAc. This explains the anomalous location of hexosaminidase and stored sugars in the soluble + microsomal fraction on differential centrifugation (Table 3) and in the very light lysosome peak on Percoll gradient fractionation (Fig. 1). The phenomenon of very light lysosomes due to NeuAc storage has been reported previously [15].

The presence of NeuGc in any human fibroblasts was unexpected because this sialic acid derivative is not considered to be synthesized in humans [26,27]. Indeed, fetal calf serum appeared to be the source of the intralysosomal NeuGc in ISSD fibroblasts, since growth in human serum caused a gradual decline in NeuGc levels (Table 2), which became undetectable after 30 days.

These findings suggested that GlcUA (as well as NeuGc) shares the lysosomal membrane's NeuAc carrier. If so, then GlcUA and NeuGc should be poorly transported across ISSD lysosomes. Indeed, GlcUA egress from ISSD lysosome-rich granular

fractions was negligible across a broad range of initial GlcUA loadings, i.e. from 7 to 244 pmol/hexosaminidase unit (Fig. 2). Even an 18 min incubation at 37 °C resulted in only a 2% decrease in granular fraction GlcUA content. In contrast, GlcUA egress from normal lysosomes was extremely rapid, with a $t_{1/2}$ of 2.6 min across loading levels of 19 to 346 pmol/hexosaminidase unit. These 2 min egress measurements represent underestimates of the normal egress rate of GlcUA, since an initial velocity could not be measured. It can be estimated from Fig. 2 that the egress rate had already levelled off by 1 min. Impaired egress of NeuGc from ISSD granular fractions indicated that the NeuAc carrier recognizes NeuGc as well as NeuAc and GlcUA.

These findings support the work of Mancini *et al.* on NeuAc uptake using rat liver lysosomes [23]. In their studies, GlcUA and NeuGc were the most potent competitors of NeuAc transport, which was measured in the presence of a proton gradient. The knowledge that NeuAc uptake (and, presumably, GlcUA uptake) proceeds more rapidly at lower pH prompted us to load normal granular fractions with GlcUA at pH 5.5 for our egress measurements. Some GlcUA loading occurred even for ISSD granular fractions under these conditions, suggesting that the mutant lysosomal NeuAc carrier may retain some activity for GlcUA uptake at pH 5.5. This residual uptake could effectively load even ISSD granular fractions exposed to a very high extralysosomal GlcUA concentration, e.g. 50 mM. Subsequent egress of GlcUA, performed at pH 7.2, was consistently impaired (Fig. 2).

There are at least two possible explanations for the relatively low storage levels of GlcUA compared with NeuAc in ISSD fibroblasts. GlcUA may be produced at a slower rate than NeuAc in these cells, resulting in lowered lysosomal storage. Alternatively, a small amount of residual carrier may remain in the ISSD lysosomal membranes, and this may be sufficient to rid the lysosome of most GlcUA but not NeuAc. The difference could be a result of a lower K_m for GlcUA than for NeuAc. Regardless of the explanation, if NeuAc and GlcUA are transported by the same carrier, then a GlcUA storage disease would most likely also result in stored NeuAc.

The same cannot be said for IdUA. This GlcUA epimer and glycosaminoglycan constituent was not detectable in ISSD fibroblasts. Several possibilities could explain this. IdUA may be carried by the NeuAc–GlcUA system, but be stored at undetectable levels because of low production or an extremely low K_m with residual carrier activity. IdUA may normally be carried by the NeuAc–GlcUA system, but the ISSD mutation does not affect the IdUA transporting capacity. Finally, the NeuAc–GlcUA carrier may not recognize IdUA, in which case an IdUA storage disease might exist. Unfortunately, Mancini *et al.* did not test IdUA for competition with proton-driven NeuAc transport [23]. However, an IdUA storage disorder appears worthy of pursuit in patients with suggestive clinical and biochemical findings.

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