Sialic Acid Storage Diseases

A Multiple Lysosomal Transport Defect for Acidic Monosaccharides

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Abstract

A defective efflux of free sialic acid from the lysosomal compartment has been found in the clinically heterogeneous group of sialic acid storage disorders. Using radiolabeled sialic acid (NeuAc) as a substrate, we have recently detected and characterized a proton-driven carrier for sialic acid in the lysosomal membrane from rat liver. This carrier also recognizes and transports other acidic monosaccharides, among which are uronic acids. If no alternative routes of glucuronic acid transport exist, the disposal of uronic acids can be affected in the sialic acid storage disorders. In this study we excluded the existence of more than one acidic monosaccharide carrier by measuring uptake kinetics of labeled glucuronic acid ([^{3}H]GlcAc) in rat lysosomal membrane vesicles. [^{3}H]GlcAc uptake was carrier-mediated with an affinity constant of transport (K_t) of 0.3 mM and the transport could be *cis*-inhibited or *trans*-stimulated to the same extent by sialic acid or glucuronic acid. Human lysosomal membrane vesicles isolated from cultured fibroblasts showed the existence of a similar proton-driven transporter with the same properties as the rat liver system (K_t of [^{3}H]GlcAc uptake 0.28 mM). Uptake studies with [^{3}H]NeuAc and [^{3}H]GlcAc in resealed lysosome membrane vesicles from cultured fibroblasts of patients with different clinical presentation of sialic acid storage showed defective carrier-mediated transport for both sugars. Further evidence that the defective transport of acidic sugars represents the primary genetic defect in sialic acid storage diseases was provided by the observation of reduced, half-normal transport rates in lymphoblast-derived lysosomal membrane vesicles from five unrelated obligate heterozygotes. This study reports the first observation of a human lysosomal transport defect for multiple physiological compounds. (*J. Clin. Invest.* 1991. 87:1329–1335.) Key words: glucuronic acid • carrier • proton cotransport • heterozygotes • Salla disease

Introduction

Sialic acid storage diseases represent a clinically heterogeneous group of inherited disorders, characterized by abnormal accumulation of free (unbound) sialic acid in urine and in lysosomes of different tissues. They must be distinguished from other forms of sialuria without lysosomal storage (1). The two main phenotypes of lysosomal sialic acid storage are the "Finnish" Salla disease, presenting with mental retardation of early onset, ataxia, and near-normal life span (2, 3), and an infantile form without any ethnical prevalence, presenting severe visceral involvement, dysostosis multiplex, psychomotor retardation, and early death (4–10). In addition, some patients have been described with intermediate phenotypes between these two extremes (11–15). Impaired efflux of sialic acid (*N*-acetylneuraminic acid, NeuAc)[1] from lysosome-rich subcellular fractions of fibroblasts from patients with all the different clinical forms of the disease has suggested a defect of a putative sialic acid transport system (16–20). Recently, we have detected and characterized a proton-driven carrier specific for sialic acid and many other acidic monosaccharides, including glucuronic acid, in rat liver lysosomal membrane vesicles (21). Glucuronic acid is normally present in the lysosomes as a degradation product of glycosaminoglycans. Sialic acid storage diseases therefore may represent a genetic transport defect of a carrier with wide substrate specificity. Consequently, storage of different compounds may be involved in the pathogenesis of the disorder, unless it is demonstrated that more than one carrier exists for the lysosomal disposal of different acidic sugars. Biochemically distinct lysosomal carriers with partially overlapping substrate specificity have been described for amino acids (22, 23). Evaluation of the number and properties of the human lysosomal acidic monosaccharide transport mechanisms is mandatory for understanding the metabolic defect in sialic acid storage diseases. In this study, we have further investigated the transport kinetics of D-glucuronic acid (GlcAc) and NeuAc in rat liver lysosomal membranes and extended the studies to resealed lysosomal vesicles from cultured human fibroblasts and lymphoblasts. Transport activity was also determined in cell lines from patients and obligate heterozygotes of various types of sialic acid storage disorders. The results provide strong evidence that the impaired transport mechanism for different acidic monosaccharides is the primary genetic defect of these diseases.

Methods

Cell cultures. Fibroblasts were grown in Ham's F10 medium supplemented with 10% FCS and antibiotics. Fibroblast and lymphoblast cell lines from patients with Salla disease and their relatives were provided by one of the authors (P. Aula). Fibroblast cell line 9015 is from a fetal skin biopsy of an interrupted pregnancy after a positive prenatal diagnosis for Salla disease (Renlund, Dr. M., personal communication).

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1. *Abbreviations used in this paper:* GlcAc, D-glucuronic acid; K_t, affinity constant of transport; MES, 2-(*N*-morpholino)ethanesulfonic acid; NeuAc, sialic acid (*N*-acetylneuraminic acid).

Cells from patient A. Z. (5) are from Dr. E. Vamos, Dept. of Medical Genetics, University of Brussels; cells of patient E. P. (14) are from Prof. A. Fois, Dept. of Pediatrics, University of Siena; cell line D. R. is from a patient observed by Prof. F. van Hoof, I. C. P. University of Louvain; and cell line E. B. is from a patient observed by Dr. F. G. I. Jennekens, Dept. of Neurology, University of Utrecht (manuscript in preparation). Lymphoblast strains were established by EBV infection of peripheral blood lymphocytes and grown in CO_2 atmosphere with RPMI medium supplemented with 20% FCS and glutamine. The transformation procedure does not seem to interfere with later isolation of lysosomes and integrity of the lysosomal membrane (24).

Lysosomal membrane vesicles. Rat liver was used as a source of lysosomal membrane vesicles as described earlier (21). Proton-driven sialic acid uptake was only observed in fractions highly enriched for lysosomal membrane markers and not in less pure fractions obtained during the purification procedure. For fibroblast lysosomal membrane vesicles, cells from 12 to 15 850-cm^2 roller bottles were harvested by trypsinization and homogenized with 30 strokes of a Potter Elvejem glass-Teflon type of homogenizer in 0.25 M sucrose, 0.2 M KCl, 10 mM NaHepes pH 7.4 plus a cocktail of protease inhibitors as used for rat liver isolations. Differential centrifugation steps (650 *g* and 11,000 *g*) produced a lysosomal/mitochondrial pellet representing about 20% of the lysosomes in the total homogenate, 80% of which were intact, as judged by the distribution and latency of the soluble lysosomal marker enzyme β-hexosaminidase. No difference in lysosome recovery was observed between preparations from controls and patients. Afterwards, lysosomal membrane vesicles were prepared by selective hypotonic shock of this pellet according to the procedure of Ohsumi et al. (25) applied to human fibroblasts (26). Because of the hypotonic shock (2 × 30 min at 0°C in 0.02 M sucrose) and the following extensive washings, lysosomes lost their content and patient membranes obviously lost their storage products. In the final preparation, the specific activity of the lysosomal membrane marker enzyme β-glucocerebrosidase was comparable to the rat liver lysosomal membrane vesicles (about 2 μmol/h per milligram protein). The total enrichment of this marker was lower for fibroblast than for rat liver membranes (20-fold against 90-fold), the difference being caused by the high specific enzyme activity in the original fibroblast homogenate compared with the total liver tissue homogenate. The final recovery of membrane markers in fibroblast preparations was comparable to rat liver (4–8%). No difference in purity and recovery was observed between patient and control preparations, but usually better yields were obtained from fetal fibroblasts (cell lines 9015, 53, 698). Lysosomal membranes were also prepared from 1–2 g (wet wt) transformed lymphoblasts following the same procedure used for fibroblasts. Although the specific activity of β-glucocerebrosidase in lymphoblasts is much lower than in fibroblasts (~ 5%), the enrichment of this marker in the final preparation was similar to fibroblasts. The membrane vesicles were suspended in 20 mM NaHepes pH 7.4 plus 0.01 mM EDTA at a protein concentration of 3–5 mg/ml for both fibroblast and lymphoblast preparations and were kept frozen at −70°C before use. Frozen lysosomal membranes from any source retained normal transport activity for at least 4 mo. Each preparation was sufficient to perform duplicate determinations of marker enzymes, of GlcAc/NeuAc transport and glucose *trans*-stimulation. For each cell line transport parameters were assayed in at least two separate membrane preparations.

Transport studies. Uptake of the acidic monosaccharides GlcAc and NeuAc was assayed in the presence and absence of an inward directed proton gradient (pH_{out} 5.5 < pH_{in} 7.4, or pH_{in} = pH_{out} = 7.4) generated with impermeant buffers as described (21), except that for fibroblast and lymphoblast lysosomal membranes about 20–40 μg protein was used per each assay and incubations were performed at 37°C. Briefly, 20-μl vesicles, prepared in 20 mM Na- or K-Hepes buffer pH 7.4 plus 0.01 mM valinomycin, were incubated with 10 μl radiolabeled sugar plus 100 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) free acid, to give a final concentration of 33 mM MES, 13 mM Hepes, and an extravesicular pH of 5.5. Reactions were stopped by diluting the sample with 70 μl ice-cold incubation buffer, and vesicles were filtered and eluted with 1 ml buffer on a small Sephadex G50 (fine) column (Pharmacia LKB Biotechnology Inc., Uppsala, Sweden) run at 0.3 ml/min at 4°C as we described earlier. Vesicle-associated radioactivity was counted by liquid scintillation. Uptake at pH_{in} = pH_{out} = 7.4 (no proton gradient) was assayed in 20 mM Na- or K-Hepes pH 7.4 present inside and outside the vesicles. *Trans*-stimulation of [^{3}H]GlcAc uptake was studied at pH_{in} = pH_{out} = 5.5 in the presence of the ionophore monensin, as previously described (21). *Trans*-stimulation of [^{3}H]D-glucose uptake was followed at pH 5.5 after preloading the vesicles with 100 mM unlabeled D-glucose, essentially as described for the rat liver system (27), except that the assay was performed at 37°C using about 30 μg membrane protein. Details for different assay conditions are indicated in the figure legends. Experiments were performed under controlled osmotic conditions and uptake of NeuAc and GlcAc was usually done in the presence of equimolar intra- and extravesicular K^+ and 0.01 mM valinomycin, to eliminate membrane potentials that could artificially affect movements of the charged sugars. As blanks, vesicles exposed to ice-cold buffer containing radiolabeled substrate were filtered, without incubation, through a Sephadex column. This blank was subtracted from all the determinations (about 200 dpm). Each assay was performed as duplicate or triplicate determination and the accepted variation from the mean was 10%. Kinetic parameters, obtained from the experimental data, were calculated using the computer program Enzfitter, from Elsevier-Biosoft, Cambridge, UK.

Miscellaneous. Activities of β-hexosaminidase and latency measurements were performed as described (16). β-glucocerebrosidase activity in the presence of Triton X-100 and taurocholate was assayed with a synthetic substrate at pH 5.3 as described (28). Radiolabeled D[1-^{3}H]GlcAc (sp act 1.8 Ci/mmol) and D[2-^{3}H]glucose (sp act 23 Ci/mmol) were prepared by Amersham International, Amersham, UK, and [9-^{3}H]NeuAc (sp act 10 Ci/mmol) was purchased from American Radiolabeled Chemicals, St. Louis, MO. Osmolarity measurements were done with a freezing point-depression osmometer (Advanced Instruments, Inc., Needham Heights, MA). The unlabeled sugars NeuAc, GlcAc, and D-glucose, the ionophore monensin, the buffers Hepes and MES were obtained from Sigma Chemical Co., St. Louis, MO. Valinomycin was from Boehringer Mannheim GmbH, Mannheim, Germany.

Results

Glucuronic acid transport in rat liver lysosomal membranes. In previous studies we demonstrated that, in the rat liver, the lysosomal carrier for sialic acid also recognizes and transports glucuronic acid (21). However, using radiolabeled NeuAc as a substrate, we could not exclude the existence of alternative routes of transport for uronic acids. We therefore investigated the kinetics of [^{3}H]GlcAc transport in rat liver membranes in the presence and absence of an inward-directed proton gradient, by manipulating the intra- and extravesicular pH with impermeant buffers. As shown in Fig. 1, similarly to what we observed for sialic acid, initial uptake rates for glucuronic acid were strongly stimulated above equilibrium level (overshoot) in the presence of the proton gradient (pH_{out} 5.5 < pH_{in} 7.4), which is the driving force of this cotransport mechanism. Saturability of the process at increasing substrate concentrations, typical of carrier-mediated transport, was observed. The affinity constant of transport (K_t) of this process, calculated by a Michaelis-Menten equation, was 0.3 mM (n = 2), close to that previously measured for sialic acid (0.24 mM). When the data from a representative experiment were applied to the Lineweaver-Burk and Scatchard analysis, the process linearized (Fig. 2). This indicates that, in this concentration range, only one mechanism of transport is responsible for the uptake. To confirm this observation, we could demonstrate that the *cis*-in-

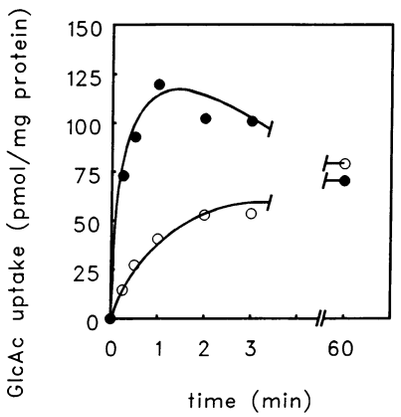

Figure 1. Time curve of glucuronic acid uptake in rat liver lysosomal membrane vesicles. 20-μl membrane vesicles in 7 mM Na^+, 13 mM K^+, 20 mM Hepes pH 7.4 plus 0.01 mM valinomycin were incubated at 20°C with 10 μl containing 2 μCi [^{3}H]GlcAc (0.1 mM final total concentration) in 100 mM MES free acid, when uptake was followed in the presence of an inward-directed proton gradient (pH_{out} 5.5 < pH_{in} 7.4) (● — ●); when assays were performed without the pH gradient (pH_{out} = pH_{in} = 7.4), vesicles were incubated with 10 μl radiolabeled substrate in 20 mM NaHepes pH 7.4 (○ — ○). Reactions were stopped with 70 μl ice-cold buffer and vesicles were filtered at 4°C as described in Methods.

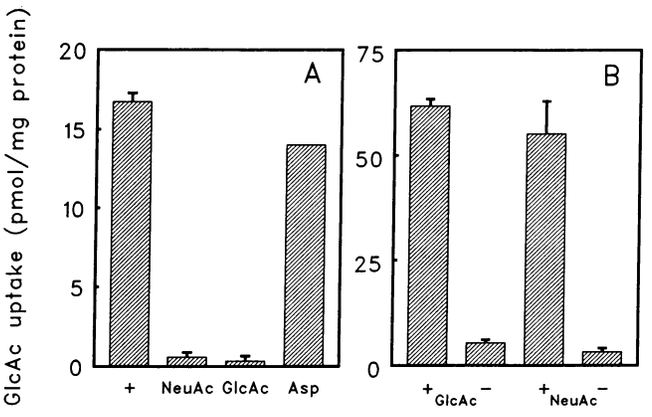

Figure 3. Substrate specificity of the acidic monosaccharide carrier in rat liver lysosomal membranes. (*A*) *Cis*-inhibition. Uptake of 0.02 mM [^{3}H]GlcAc was assayed for 30 s at 20°C in the presence of an inward-directed proton gradient (pH_{out} 5.5 < pH_{in} 7.4) plus K^+ and valinomycin as described in Fig. 1 without further addition (+), or upon addition of 7 mM unlabeled sialic acid (*NeuAc*), or glucuronic acid (*GlcAc*), or aspartic acid (*Asp*). All the acidic compounds were titrated with NaOH. Results are expressed as picomoles of radiolabeled GlcAc/30 s per milligram protein. (*B*) *Trans*-stimulation. 15 μl lysosomal membrane vesicles in 7 mM Na^+, 13 mM K^+, 20 mM Hepes, 33 mM MES pH 5.5 plus 0.01 mM monensin, and 0.01 mM valinomycin were preincubated 60 min at 20°C in the presence (+) or absence (−) of 1 mM unlabeled sialic acid (*NeuAc*) or glucuronic acid (*GlcAc*). The samples were then diluted 6.7-fold in the same prewarmed buffer containing 2 μCi [^{3}H]GlcAc. In the samples that were preincubated without unlabeled sugar (−), 0.15 mM unlabeled NeuAc or GlcAc was added together with radiolabeled substrate at the start of the assay to give the same extravesicular substrate concentration in both experiments.

hibition of [^{3}H]GlcAc uptake achieved in the presence of 7 mM unlabeled GlcAc was identical to that obtained with unlabeled NeuAc. In contrast, other anionic compounds not recognized by the sialic acid carrier, like aspartic acid, did not produce significant inhibition (Fig. 3 *A*). Moreover, the same extent of *trans*-stimulation of [^{3}H]GlcAc transport was achieved by preloading the vesicles either with 1 mM NeuAc or GlcAc (Fig. 3 *B*). *Trans*-stimulation commonly proves the symmetrical function of the carrier, and that the compounds are actually transported across the membrane. Our present data in the rat liver lysosomal membranes therefore prove there is only one lysosomal carrier for the investigated acidic monosaccharides.

Glucuronic acid and sialic acid transport across the human lysosomal membrane. To investigate if human cells possess a similar transport mechanism for acidic sugars as so far demonstrated in rat liver, we measured transport of [^{3}H]GlcAc in resealed lysosomal membrane vesicles obtained from human cultured fibroblasts. When the uptake of [^{3}H]GlcAc was studied in the presence and absence of a proton gradient we found that the human lysosomal membrane indeed possesses a proton-dependent transport mechanism for [^{3}H]GlcAc. The transport could be inhibited to the same extent by the addition of either unlabeled GlcAc or NeuAc, but not by neutral sugars like glucose (Fig. 4). Similar experiments using [^{3}H]NeuAc showed comparable rates of proton-dependent transport and substrate inhibition pattern (data not shown). Therefore, we conclude that in human lysosomal membranes also no residual routes of glucuronic acid transport exist alternative to the sialic acid-inhibitable one. In another set of experiments, we observed that the proton-driven uptake disappears upon addition of monensin. This ionophore, in the presence of Na^+, shunts the proton gradient across the membrane (21, 29), confirming that the pH effect is strictly linked to the presence of a cross-membrane gradient (data not shown). Since, similarly to rat liver membranes, 30-s incubations showed clear proton-dependent stimulation, we considered these conditions as representative for zero-*trans* uptake. Proton-driven [^{3}H]GlcAc transport, assayed for 30 s as a function of the substrate concentration, showed saturability: the K_t of this process was about 0.28 mM (Fig. 5). Uptake of 0.017 mM [^{3}H]GlcAc could be twofold *trans*-stimulated when fibroblast lysosomal vesicles were pre-

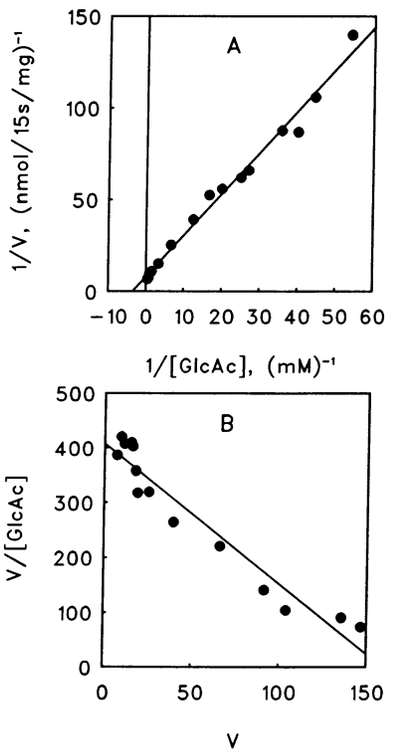

Figure 2. Kinetics of glucuronic acid transport in rat liver lysosomal membrane vesicles. 20 μl lysosomal membrane vesicles (80 μg protein) in 7 mM Na^+, 13 mM K^+, 20 mM Hepes pH 7.4 plus 0.01 mM valinomycin were incubated with 10 μl 100 mM MES free acid plus 1.5 μCi [^{3}H]GlcAc and increasing concentrations of unlabeled sodium glucuronate. Duplicate incubations were performed for 30 s at 20°C. The results were fitted to a Michaelis-Menten equation for calculation of the kinetic parameters. Lineweaver-Burk (*A*) and Scatchard (*B*) transformations of the experimental data are shown.

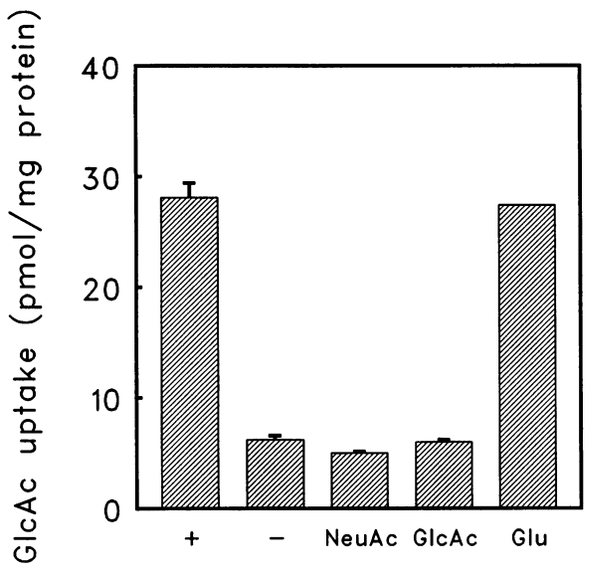

Figure 4. Substrate specificity of glucuronic acid transport in lysosomal membrane vesicles from human fibroblasts. Uptake of 0.046 mM [^{3}H]GlcAc (2.5 μCi) at 37°C for 30 s was studied in lysosomal membrane vesicles from control fibroblasts (20 μg membrane protein) in the presence of a proton gradient (pH_{out} 5.5 < pH_{in} 7.4) (+) or at pH_{out} = pH_{in} = 7.4 (−), as described in Fig. 1. Proton-driven uptake (pH_{out} 5.5 < pH_{in} 7.4) was also followed upon addition of 7 mM unlabeled sialic acid (*NeuAc*), or glucuronic acid (*GlcAc*), or D-glucose (*Glu*) to the incubation medium. In all the assays K^+ and valinomycin were present. Results are presented as average of separate determinations with the same vesicle preparation, plus standard deviations.

loaded with either 2 mM unlabeled GlcAc or NeuAc at pH 5.5 (in = out) (uptake without preloading 4.3 pmol/30 s per milligram protein; after preloading with GlcAc 10.5, and after preloading with NeuAc 9.1). Altogether, these data reveal that an acidic monosaccharide carrier, with identical properties to the rat liver system, is present in the human lysosomal membrane.

Transport of acidic sugars in lysosomal membrane vesicles from cultured cells of patients. The standardized assay system that we developed for fibroblast lysosomal membrane vesicles was used to investigate the transport of acidic sugars in cell lines from various types of sialic acid storage disorders. To check the integrity of the lysosomal membrane from fibroblasts of the patients, we measured carrier-mediated transport of [^{3}H]D-glucose. Glucose and other neutral monosaccharides are not recognized by the sialic acid carrier, but are instead transported by a distinct lysosomal transporter for neutral monosaccharides (27). We found conditions under which human lysosomal membrane vesicles show carrier-mediated *trans*-stimulation of [^{3}H]D-glucose uptake, upon preloading with unlabeled D-glucose (Fig. 6). As shown in Table I, lysosomal membranes from patients with sialic acid storage diseases demonstrate normal rates of [^{3}H]D-glucose *trans*-stimulation, proving the integrity of the lysosomal vesicles in patient preparations. The following assay system was chosen to study essential parameters of acidic monosaccharide transport in patient cell lines. The uptake of [^{3}H]NeuAc was measured after 30 s incubation in the presence of an inward-directed proton gradient (pH_{out} 5.5 < pH_{in} 7.4) and compared with the inhibitory effect of unlabeled NeuAc on such proton-driven uptake and with the uptake without a proton gradient (pH_{out} = pH_{in} = 7.4) (Fig. 7, *A* and *B*). The cell line from the patient E. P. (14) was initially used in this experiment and showed clear deficiency of proton-driven sialic acid transport. In the control cell line, the proton-mediated uptake could be inhibited by addition of 7 mM sialic acid. Since the residual uptake upon inhibition paralleled the non–proton-driven uptake, we assumed that this residual component is apparently not carrier-mediated. Accordingly, the residual uptake in the patient could not be further inhibited. The same assay was applied to the study of [^{3}H]GlcAc transport. As shown in Fig. 7, *C* and *D*, lysosomal membranes from the same patient showed comparable deficient carrier-mediated transport of glucuronic acid. We extended our study to a larger number of patient cell lines, using the same experimental conditions. The results are shown in Table II. Carrier-mediated transport activity is here expressed by the difference between the uptake in the presence and absence of the proton gradient. Each patient's cell line showed a total deficiency of lysosomal transport for both NeuAc and GlcAc. As separately tested, in no case was the residual uptake further inhibitable by 7 mM GlcAc or NeuAc, nor did it differ significantly in patient and control preparations.

Transport of acidic sugars in cell lines from obligate heterozygotes. To confirm that the transport defect directly reflects the genetic mutation, we measured transport activity in cell lines from parents of Salla disease patients. Extended pedigree studies indicated that the disease is inherited as an autosomal recessive trait (2, 30), and therefore half maximal activities are expected for obligate heterozygotes. We used transformed lym-

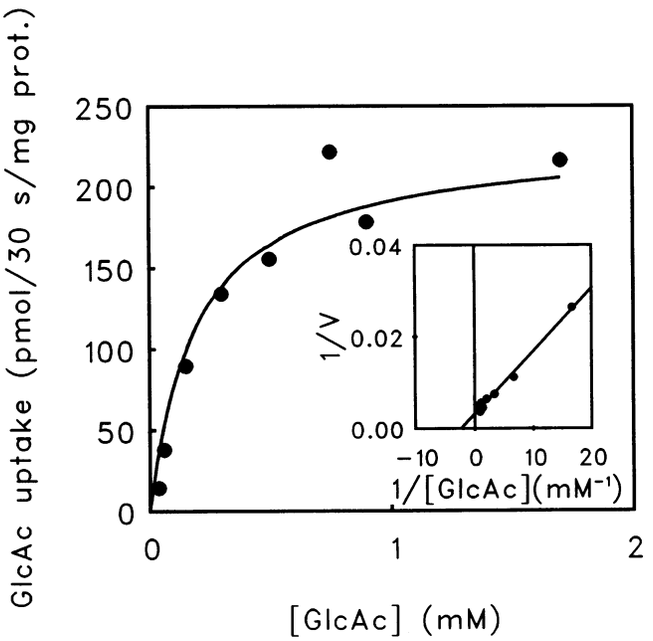

Figure 5. Concentration dependence of proton-driven glucuronic acid transport in human lysosomal membrane vesicles. Lysosomal membrane vesicles from control fibroblasts (27 μg protein per assay) were incubated with 2.5 μCi [^{3}H]GlcAc plus increasing concentrations of unlabeled sodium glucuronate at 37°C for 30 s in the presence of an inward-directed proton gradient (pH_{out} 5.5 < pH_{in} 7.4), and in the absence of a proton gradient (pH_{out} = pH_{in} = 7.4). After subtraction of the residual uptake at pH 7.4, the data relative to the proton-driven uptake were fitted to a Michaelis-Menten equation. (*Inset*) Lineweaver-Burk transformation of the data.

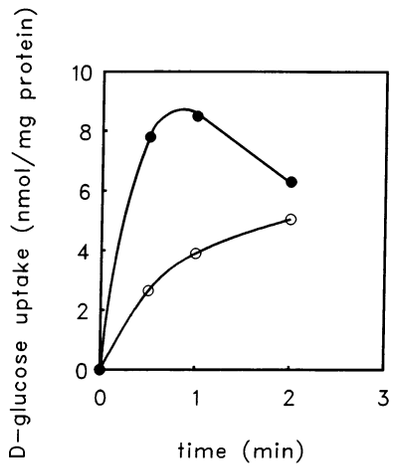

Figure 6. Trans-stimulation of [^{3}H]D-glucose uptake in fibroblast lysosomal membranes. Vesicles from control fibroblasts (32 μg protein) in 20 mM NaMES pH 5.5 were preincubated with 100 mM D-glucose for 45 min at 20°C, and the uptake of 6 μCi [^{3}H]D-glucose was followed at 37°C after diluting the sample 6.7-fold in 70 mM NaMES pH 5.5 (●—●). In control experiments (○—○), membranes were preincubated with 20 mM NaMES plus 50 mM NaCl instead of glucose and the uptake of radiolabeled glucose was studied after diluting the sample in MES buffer containing 15 mM D-glucose, to obtain the same final external substrate concentration in both experiments.

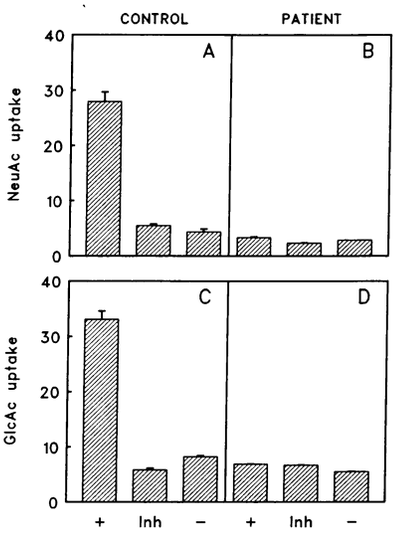

Figure 7. Determination of sialic acid and glucuronic acid transport rates in patient and control lysosomal membranes. Uptake of 0.046 mM [^{3}H]NeuAc or [^{3}H]GlcAc (2.5 μCi) was assayed in the presence of an inward-directed pH gradient generated with impermeant buffers (+), or in the presence of a proton gradient plus 7 mM unlabeled NeuAc or, respectively, GlcAc (*Inh*), or in the absence of a pH gradient (pH 7.4, in = out) (−), as specified for the experiments of

Fig. 4. Lysosomal membrane vesicles from the control and the patient contained, respectively, 20 μg and 28 μg protein per assay. Results are averages of triplicate determinations plus standard deviations and are expressed as picomoles acidic sugar/30 s per milligram protein. (*A*) and (*C*): control; (*B*) and (*D*): patient E.P.

phoblasts as a source of lysosomal membrane vesicles because of the ease of growing the large quantities of material needed for the membrane isolations. Lysosomal membrane vesicles from lymphoblasts were obtained according to the same procedure as for rat liver and human fibroblasts. In control material, proton-driven transport of glucuronic acid was present, comparable to fibroblast membranes, and defective uptake was found in vesicles of Salla disease patients (Fig. 8 *A*). When transport studies were performed on lysosomal vesicles from parents of Salla disease patients, we found that cells from five unrelated obligate heterozygotes exhibited reduced rates of [^{3}H]GlcAc lysosomal transport, compared with controls (Fig. 8 *A*); mean of control cell lines 42 (n = 4), heterozygotes 26 (n = 5), and patients 8 pmol/30 s per milligram protein (n = 2). The residual activity could still be inhibited by unlabeled substrate, confirming that it represents carrier-mediated transport (Fig. 8 *B*). Similar results were obtained from two heterozygote cell lines when [^{3}H]NeuAc uptake rates were measured (control:21 pmol/30 s per milligram protein; Salla disease patient:2.0; heterozygote 900015:7.8; heterozygote 891070:7.8; n = 2).

Table I. Trans-stimulation of [^{3}H]D-glucose Transport in Fibroblast Lysosomal Membrane Vesicles from Controls and Patients with Sialic Acid Storage Diseases

Cell line	Percentage of basal uptake
Control 698	378
Control 540	288
Control 53	231
Patient A.Z.	314
Patient D.R.	265
Patient S.P.	282
Patient 9015	283
Patient E.P.	271
Patient E.B.	224

Uptake of 6 μCi [^{3}H]D-glucose was followed for 30 s at 37°C after preloading with 100 mM unlabeled D-glucose, as described in the legend to Fig. 6. Results are reported as percentage of increased uptake after preloading, compared with the basal transport rates without glucose preloading (100%). Results are from duplicate determinations in two or three different membrane vesicle preparations per each cell line.

Discussion

Evidence for saturability, cotransport with ions, *cis*-inhibition and *trans*-stimulation are general parameters of carrier-mediated transport in biological systems (31, 32). The advantage of using resealed lysosomal membrane vesicles for transport studies lies in the absence of the intralysosomal content and the ease of performing uptake studies under close-to-normal physiological conditions. Although many lysosomal transport defects can be expected (33), only cystinosis has so far been proven to be caused by a defective carrier-mediated transport mechanism (34). The transport defect involves a single amino-acid, cystine. Significant biochemical and clinical improvement of the disease can be achieved upon treatment with cysteamine (35, 36). This compound specifically depletes lysosomes of cystine (37, 38), making cystinosis the only pharmacologically treatable lysosomal storage disease.

Various studies have previously suggested a transport defect for sialic acid in patients with Salla disease (16–18) and other forms of sialic acid storage disease (16, 19, 20), but direct evidence for saturability or other parameters has not been provided to support the involvement of a lysosomal carrier-mediated transport mechanism. The detection of a transporter for different acidic monosaccharides in rat liver lysosomal membrane vesicles pointed out the possible physiological mechanism of sialic acid transport in mammalian lysosomes (21). This carrier apparently performs a secondary active transport

Table II. Proton-driven Transport of Sialic Acid and Glucuronic Acid in Fibroblast Lysosomal Membrane Vesicles from Patients with Different Clinical Forms of a Sialic Acid Storage Disease

Cell line	Uptake rate	
	pmol/30 s/mg protein	
	[^{3}H]NeuAc	[^{3}H]GlcAc
Control 698	20.9	24.6
Control 540	12.6	17.4
Control 53	23.6	35.0
Infantile sialic acid storage disease		
Patient A.Z.	1.1	0
Patient D.R.	0.4	0
Salla disease		
Patient S.P.	0	0
Patient 9015	0	0
Intermediate non-Finnish phenotype		
Patient E.P.	0.5	1.4
Patient E.B.	0.7	0

Uptake of 0.046 mM [^{3}H]GlcAc and [^{3}H]NeuAc was assayed in the presence and absence of an inward-directed proton gradient, as described in Fig. 7. The difference between the two rates was considered as representative for carrier-mediated transport and is reported. In the control cell lines, the ratio between proton-stimulated and proton-independent uptake was about 5:1 for NeuAc and 4:1 for GlcAc. The residual uptake rates at pH 7.4 (in = out) did not significantly differ between controls and patients.

with protons, linked to the physiological pH gradient present across the membrane (32). Here we have employed resealed lysosomal membrane preparations to the clarification of a putative human lysosomal transport defect. Experiments on GlcAc transport kinetics enabled us to demonstrate that only one lysosomal carrier disposes polysaccharides-derived acidic sugars. Evidence is provided by the linear kinetics found at the Lineweaver-Burk and Scatchard analysis for [^{3}H]GlcAc transport and also by the total interchangeable possibility to use either GlcAc or NeuAc to *cis*-inhibit or *trans*-stimulate [^{3}H]GlcAc uptake. Since these properties were observed in human fibroblasts as well, we could theoretically exclude the possibility that a sialic acid transport defect could coexist with a normal glucuronic acid transport activity, unless a mutation in the same transport protein would affect the recognition site for only one sugar (39). The data from patients' material demonstrate that a lysosomal carrier function is impaired in sialic acid storage disorders. The transport defect involves multiple physiological substrates. Defective transport for sialic acid and glucuronic acid, but not for glucose, was found both in patients with classic Salla disease and in patients with a severe infantile form or with a milder intermediate form of the disease.

Strong evidence for the idea that the transport defect is indeed the primary genetic mutation is the finding that lymphoblast lysosomal membrane vesicles from obligate heterozygotes revealed intermediate transport rates. In this respect, lymphoblast lysosomal membrane vesicles proved to be a suitable and sensitive system to discover the deficient function of a membrane transport system. The use of other techniques did not allow earlier demonstration of half-maximal lysosomal sialic acid efflux in heterozygotes (19). Our findings suggest that the wide clinical heterogeneity of this disease is not based on differences between defective transport or disposal of one substrate instead of another. Nevertheless, our assay did not allow us to discriminate for residual transport activities, which would explain lower levels of storage in the milder clinical forms. Blom et al. (40) have recently found accumulation of glucuronic acid in fibroblast homogenates from infantile sialic acid storage disease, which accounted for only 5% of the cellular sialic acid storage. In the same study, glucuronic acid storage in Salla disease was even lower (sometimes undetectable) than in the infantile form, resembling the difference in sialic acid storage usually observed between these two forms of the disease. The similarity of the K_t for GlcAc and NeuAc makes it unlikely that a difference in substrate affinity determines the threshold for the storage of one or the other compound. Rather, the physiological rate of intralysosomal production of these sugars might play a role and should therefore be investigated. Urinary excretion or accumulation in tissues of glucuronic acid in patients with lysosomal sialic acid storage has not been reported. This may reflect methodological difficulties in glucuronic acid determination. The role of glucuronic acid (and perhaps of other acidic sugars as well) in the pathogenesis of these diseases needs to be reanalyzed, not only for full understanding of the disease mechanisms, but also for possible therapeutical approaches (41). The substrate affinity of the affected transporter indicates that a drug must be designed that can deplete lysosomes of acidic monosaccharides in general and not only of sialic acid.

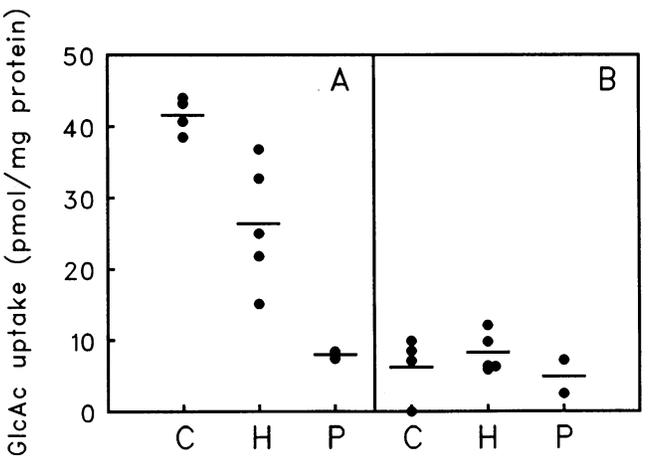

Figure 8. Transport rates of glucuronic acid in lysosomal membrane vesicles from human lymphoblasts. (*A*) Lysosomal membrane vesicles from controls, from patients with Salla disease, and from five unrelated parents of Salla disease patients were assayed for transport capacity of 0.055 mM [^{3}H]GlcAc (3 μCi) for 30 s at 37°C at pH_{out} 5.5 < pH_{in} 7.4 or at pH_{out} = pH_{in} = 7.4, as already described for fibroblast lysosomal vesicles. Proton-driven transport is reported after subtraction of the residual proton-independent uptake. (*B*) The same experiment was performed in the presence of 7 mM unlabeled sodium glucuronate and the difference between proton-driven and proton-independent uptake is presented. *C*, controls; *H*, heterozygotes; *P*, patients.

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