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Purification of the Lysosomal Sialic Acid Transporter

FUNCTIONAL CHARACTERISTICS OF A MONOCARBOXYLATE TRANSPORTER*

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Adrie C. Havelaar, Grazia M. S. Mancini, Cecile E. M. T. Beerens, Ragonda M. A. Souren, and Frans W. Verheijen‡

From the Department of Clinical Genetics, Erasmus University, P. O. Box 1738, 3000 DR Rotterdam, The Netherlands

Sialic acid and glucuronic acid are monocarboxylated monosaccharides, which are normally present in sugar side chains of glycoproteins, glycolipids, and glycosaminoglycans. After degradation of these compounds in lysosomes, the free monosaccharides are released from the lysosome by a specific membrane transport system. This transport system is deficient in the human hereditary lysosomal sialic acid storage diseases (Salla disease and infantile sialic acid storage disease, OMIM 269920). The lysosomal sialic acid transporter from rat liver has now been purified to apparent homogeneity in a reconstitutively active form by a combination of hydroxyapatite, lectin, and ion exchange chromatography. A 57-kDa protein correlated with transport activity. The transporter recognized structurally different types of acidic monosaccharides, like sialic acid, glucuronic acid, and iduronic acid. Transport of glucuronic acid was inhibited by a number of aliphatic monocarboxylates (*i.e.* lactate, pyruvate, and valproate), substituted monocarboxylates, and several dicarboxylates. *cis*-Inhibition, *trans*-stimulation, and competitive inhibition experiments with radiolabeled glucuronic acid as well as radiolabeled L-lactate demonstrated that L-lactate is transported by the lysosomal sialic acid transporter. L-Lactate transport was proton gradient-dependent, saturable with a K_m of 0.4 mM, and mediated by a single mechanism. These data show striking biochemical and structural similarities of the lysosomal sialic acid transporter with the known monocarboxylate transporters of the plasma membrane (MCT1, MCT2, MCT3, and Mev).

The major function of lysosomes is the degradation of a large variety of intra- and extracellular macromolecules. The release of degradation products from the lysosome is accomplished by specific membrane transport systems. More than 20 lysosomal transporters have been characterized for specific solutes like amino acids, sugars, nucleosides, ions, and vitamins (1). Their fundamental role in biology is illustrated by the occurrence of two human inherited diseases with a defective lysosomal transport function, cystinosis and sialic acid storage diseases (2). Sialic acid storage diseases are autosomal recessive disorders that are characterized by mental retardation and a variable degree of neurodegeneration. Lysosomal accumulation and excessive urinary excretion of free sialic acid are pathognomonic findings. Previously, we have characterized a carrier in the lysosomal membrane with substrate specificity for the acidic monosaccharides sialic acid (Neu5Ac)[1], uronic acids, and aldonic acids (3). Subsequent studies in our laboratory showed that a defective transport of sialic and glucuronic acid (GlcA) is the primary defect in both clinical variants (4), Salla disease and infantile sialic acid storage disease. Recently, the gene for these disorders has been localized to the same refined chromosomal area on 6q14-q15 by linkage disequilibrium analysis (5). However, the disease gene has not been identified yet. The elucidation of the molecular structure and functional properties of the lysosomal sialic acid transporter is indispensable for further understanding of the molecular defect(s) in the clinical heterogeneous forms of sialic acid storage diseases. Previously, we have developed a functional reconstitution system for the sialic acid transporter that provided the tool to start the purification and functional characterization of the transport protein (6).

In this paper we present the purification of the sialic acid transporter from lysosomal membranes of rat liver to apparent homogeneity. Its functional properties are compared with those of other monocarboxylate transporters present in the plasma membrane of various mammalian cells (7–9).

EXPERIMENTAL PROCEDURES

Materials

Highly purified lysosomal membrane vesicles were isolated from livers of adult Wistar rats (3). The lysosomal membrane vesicles were suspended at a protein concentration of 8–10 mg/ml in 20 mM NaHepes, pH 7.4, 1 mM EDTA and were stored at −70 °C. All chemicals used were obtained from Sigma or as indicated. L-Iduronic acid, sodium salt was obtained from Toronto Research Chemicals Inc. (North York, ON, Canada). All the tested carboxylates were titrated with NaOH before use.

Reconstitution

Reconstitution of the protein eluates into liposomes was performed as described earlier (6), with the following modification: proteoliposomes were formed by incubating the protein sample, containing detergent and phospholipid (total volume, 170 μl), with 150 μl of Amberlite XAD-2 beads (Fluka) in 20 mM NaHepes, pH 7.4, 100 mM KCl. After 30 min of rotation at room temperature, beads were removed by short centrifugation, and proteoliposomes were used for transport assays.

Transport Assay

After reconstitution, the carrier activity was assayed by uptake of radiolabeled GlcA in the presence of an inwardly directed proton gradient. Because Neu5Ac and GlcA are transported by the same lysosomal transporter for acidic monosaccharides (3, 4), we have performed all studies using radiolabeled GlcA, which was more readily available. Aliquots of proteoliposomes (25 μl) were incubated at 37 °C with 5 μl of 240 mM Mes (free acid) containing 2 μCi of D-[1-^{3}H]GlcA (Amersham Pharmacia Biotech; specific activity, 6.6 Ci/mmol), resulting in an extravesicular pH of 5.5 and a final concentration of 10 μM GlcA. Blank

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‡ To whom correspondence should be addressed. Tel.: 31-10-4087350; Fax: 31-10-4362536; E-mail: verheijen@ikg.fgg.eur.nl.

[1] The abbreviations used are: Neu5Ac, *N*-acetylneuraminic acid; GlcA, glucuronic acid; Mes, 2-(*N*-morpholino)-ethanesulfonic acid; IdoA, iduronic acid; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid; PAGE, polyacrylamide gel electrophoresis.

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values were determined by incubation of proteoliposomes at 37 °C with 40 mM Mes (free acid), 7 mM unlabeled NaGlcA, pH 5.5, and 2 μCi of D-[1-^{3}H]GlcA and subtracted from all determinations. Previous experiments showed that uptake rates are linear up to 1 min. After 1 min, the reactions were stopped by diluting the sample with 70 μl of ice-cold incubation buffer (17 mM NaHepes, 84 mM KCl, 40 mM Mes (free acid), pH 5.5). The samples were immediately applied to a Sephadex G50 fine (Amersham Pharmacia Biotech) column (Pasteur pipettes, 0.5 × 5cm) at 4 °C. Columns were equilibrated in cold incubation buffer, and vesicles were eluted with 1 ml of cold incubation buffer. Vesicle-associated radioactivity was determined by liquid scintillation counting in 10 ml of Insta-gel (Packard).

cis-Inhibition experiments were performed by incubating the proteoliposomes for 1 min at 37 °C with 2 μCi of [^{3}H]GlcA (final concentration, 10 μM) in 40 mM Mes (free acid), resulting in an inwardly directed proton gradient (pH_{in} = 7.4 > pH_{out} = 5.5), and 7 mM of the tested compound.

For *trans*-stimulation studies, a 60% proteoliposome solution (25 μl) was pre-incubated for 60 min at 37 °C with 17 mM NaHepes, 84 mM KCl, 40 mM Mes acid, pH 5.5, plus 10 μM monensin, 10 μM valinomycin (Boehringer Mannheim) in the presence or absence of 1 mM unlabeled substrate. The assay was started by adding 75 μl of an equivalent buffer containing 2 μCi of [^{3}H]GlcA at 37 °C with a final concentration of 0.25 mM. When the samples were pre-incubated without unlabeled substrate, the external final concentration was corrected as in the case of preloading (0.25 mM unlabeled compound). After 1 min, the reaction was stopped as described (6).

The experiments with [^{14}C]L-lactate (Amersham Pharmacia Biotech; specific activity, 152 mCi/mmol) were largely performed as described for [^{3}H]GlcA. However, incubation mixtures contained 0.066 μCi of [^{14}C]L-lactate (final concentration, 15 μM) and were performed at 20 °C instead of 37 °C. Blank values were determined by incubation of proteoliposomes with 40 mM Mes (free acid), 7 mM unlabeled sodium L-lactate and subtracted from all determinations. For protein side chain modification, proteoliposomes (100 μl) were incubated and treated as described (6).

Purification of the Rat Liver Lysosomal Membrane Sialic Acid Transport Protein

For a single purification, lysosomal membrane vesicles prepared from 150 g of rat livers (15 rats) were used.

Step 1: Solubilization—Solubilization of lysosomal membrane proteins was performed by mixing the lysosomal membrane vesicles 1:1 (v/v) with 1% Triton X-100 (especially purified for membrane research, Boehringer Mannheim), 20 mM Tris-HCl, pH 7.4. After 25 min of incubation at 0 °C, unextracted material was pelleted by ultracentrifugation at 150,000 × *g* in a Beckman SW 40 rotor for 20 min at 4 °C.

Step 2: Hydroxyapatite—The Triton X-100 extract was applied to hydroxyapatite columns (Pasteur pipettes containing 0.5 g of dry material, Biogel HTP, Bio-Rad, packed by 15 s of tapping) at 4 °C, with a maximum of 500 μl solubilized material/column. Each column was washed with 3 ml of 20 mM Tris-HCl, pH 7.4, 0.1% Triton X-100 (buffer A). Elution was with 3 ml of buffer A, 25 mM Na_2HPO_4, NaH_2PO_4, pH 7.4. After pooling all the 3-ml eluates, a 2-ml sample was concentrated in a Centricon 10 device (Amicon, Inc., Beverly, MA) until 100–150 μl and desalted. A 50-μl aliquot was used for the reconstitution assay and a 20-μl aliquot was used for the protein assay. Desalting was performed on a 2-ml Sephadex G50 medium (Amersham Pharmacia Biotech) column equilibrated in buffer A (10).

Step 3: Lentil Lectin—The eluates of the different hydroxyapatite columns were pooled and applied to a 2-ml lentil lectin affinity chromatography column (lentil lectin-Sepharose 4B, Amersham Pharmacia Biotech) pre-equilibrated in buffer A. After washing the lentil lectin column with 2 ml of buffer A, the flow-through fraction (unretained material) was applied to a 2-ml DEAE-Sephacel (Amersham Pharmacia Biotech) anion exchanger pre-equilibrated in buffer A containing 10% glycerol (buffer B). A 2-ml sample of the lentil lectin flow-through fraction was concentrated in a Centricon 10 device to 100–150 μl and desalted (10). A 50-μl aliquot was used for the reconstitution assay, and a 20-μl aliquot was used for the protein assay.

Step 4: DEAE-Sephacel—After extensive washing the DEAE-Sephacel column with 20 ml of buffer B and 20 ml of buffer B with 40 mM NaCl, bound material was eluted with 6 ml of buffer B with 100 mM NaCl. This fraction was stored at −70 °C.

Step 5: Hydroxyapatite—After the DEAE-eluate was thawed, a 0.5 ml sample was concentrated in a Centricon 10 device until 100 μl and (5.5 ml) was adjusted to pH 6.0 with 0.5 M Mes (free acid) and applied to a prepacked hydroxyapatite column (1-ml EconoPac HTP cartridge, Bio-Rad) pre-equilibrated in 300 mM NaCl, 20 mM NaMes pH 6.0, 0.1% Triton X-100. Transport activity was eluted with 6 ml of 1 mM Na_2HPO_4, NaH_2PO_4, pH 6.0, 300 mM NaCl, 0.1% Triton X-100. This eluate was concentrated in Millipore ultrafree-15 centrifugal filters 10K (Millipore Corporation, Bedford) to approximately 300 μl and desalted (10). A 50-μl aliquot was used for the reconstitution assay, and a 100-μl aliquot was used for the protein assay.

Step 6: Mono Q—The concentrated hydroxyapatite eluate (150 μl) was applied to a 0.10-ml Mono Q anion exchange column attached to a Amersham Pharmacia Biotech SMART system. This column was equilibrated in buffer B, and bound material was eluted with a linear gradient of 0–210 mM NaCl in buffer B. Fractions of 0.1 ml were collected and pooled pairwise, buffer was exchanged for 20 mM Na-Hepes, 100 mM KCl, 0.1% Triton X-100 by the desalting procedure as described above, and a 50-μl aliquot was used for the reconstitution assay. All column procedures were performed at 4 °C.

Protein Characterization and Determination

The purity of the various active fractions was determined by SDS-polyacrylamide gel electrophoresis according to Laemmli (11) of methanol/chloroform precipitated samples (12), followed by Coomassie Brilliant Blue R-250 or the silver nitrate staining according to Amersham Pharmacia Biotech. Protein concentration was determined by the procedure of Lowry *et al.* as modified by Peterson (13) for the presence of Triton. Protein concentrations in eluates of the second hydroxyapatite column were determined after methanol/chloroform precipitation (12). Protein concentrations in Mono Q eluates were too low to be determined by the above assay and were therefore estimated from silver-stained SDS-PAGE gels.

For the endoglycosidase F/*N*-Glycosidase F (Boehringer Mannheim) treatment of the purified protein, the Mono Q fractions 19–23 were pooled, concentrated, and incubated with 25 milliunits endoglycosidase F, 100 μl in the presence of 20 mM potassium phosphate buffer, pH 7.4, 50 mM EDTA, 2% Triton X-100, 0.2% SDS, 2% β-mercaptoethanol for 2 h at 37 °C. Proteins were precipitated with methanol/chloroform (12). The pellet was resuspended in sample buffer and analyzed by SDS-PAGE (10% gel).

RESULTS

Purification of the Lysosomal Sialic Acid Transporter—Various membrane (transport) proteins have been successfully purified using hydroxyapatite as well as ion exchange chromatography in the presence of detergents (14–18). In addition, affinity chromatography with oligosaccharide-specific lectins has been used to identify the major heavily glycosylated lysosomal membrane proteins: LAMPs (lysosomal-associated membrane proteins) and LIMPs (lysosomal integral membrane proteins) (19–22). Based on the success of these purification methods for membrane proteins we developed a purification protocol for the lysosomal sialic acid transporter. Previously, we have reported a successful reconstitution procedure for the rat liver lysosomal sialic acid transporter that now provided the functional assay to follow fractionation and purification of the solubilized transporter (6). At all steps of the purification procedure, samples were collected and reconstituted into proteoliposomes, and their transport activities were measured using radiolabeled GlcA as a substrate (Table I).

The Triton X-100 solubilized lysosomal membrane proteins were applied to small columns of dry hydroxyapatite material. The columns were washed with equilibration buffer at pH 7.4, and about 20% of the transport activity was eluted with 25 mM sodium phosphate buffer at pH 7.4. This resulted in a 4-fold purification. The next step consisted of lentil lectin affinity chromatography. Almost all activity of the sialic acid transporter was recovered from the column flow-through. Lentil lectin recognizes α-D-glucose and α-D-mannose residues and therefore binds glycoproteins. Consequently, a number of major lysosomal membrane glycoproteins bound to the column

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TABLE I

Purification of the sialic acid transporter from rat liver lysosomal membrane vesicles

Lysosomal membrane vesicles (approximately 25 mg of protein) derived from 150 g of rat livers were used as starting material. The purification procedure, reconstitution, and transport assay were performed as described under "Experimental Procedures." Activity is expressed as uptake of [^{3}H]GlcA in 1 min at 37 °C. Data represent the means of three separate isolations.

Fraction	Protein	Total protein	Total Activity	Yield	Specific activity	Fold enhancement
	μg/ml	*μg*	*pmol/min*	*%*	*pmol GlcA/mg/min*	
Solubilized lysosomal membrane extract	200	12000	2102.4	100	175.2	1
First hydroxyapatite eluate	16.4	591	441.5	21	747.3	4
Lentil lectin eluate	13.6	489	378.4	18	774.4	4.4
DEAE eluate	16.6	99.5	252.3	12	2534.9	14.5
Second hydroxyapatite eluate	0.5	3.0	42.1	2	14171.4	80
Mono Q eluate	0.14	0.028	2.1	0.1	75757.6	432

despite the fact that it did not lead to an increase in specific activity.

The lentil lectin flow-through fraction was applied to a DEAE-Sephacel anion exchange column. With 100 mM NaCl, 12% of the total transport activity was eluted. As depicted in Table I, this resulted in a ≈14.5-fold increase in specific activity over the starting material. Analysis of the protein composition of fractions obtained from these initial purification steps is shown in Fig. 1. Many different protein bands were still present.

The next purification step consisted of chromatography on hydroxyapatite. This time the column was pre-equilibrated at pH 6.0 in the presence of 300 mM NaCl. Under these conditions, acidic proteins are retained and are eluted with low phosphate buffers. This step provided an important purification of the sialic acid transport protein with an 80-fold enrichment in specific activity (Table I). SDS-PAGE protein analysis using silver staining showed at least four distinct protein bands (Fig. 2*A*). One of these proteins has a molecular mass of 85 kDa and based on its N-terminal amino acid sequence represented one of the major lysosomal membrane glycoproteins, the Lgp85 or LIMP II (23). Another major 67-kDa protein represented the lysosomal membrane-bound subunit of acid phosphatase (24). The other proteins were considered as candidates for the lysosomal sialic acid transporter.

The next purification step consisted of a strong anion exchange Mono Q column attached to the SMART system of Amersham Pharmacia Biotech. Retained proteins were eluted with a gradient of 0–210 mM NaCl. SDS-PAGE analysis by silver nitrate staining of the eluted proteins showed a predominant protein band with a molecular mass of ≈57 kDa in the fractions 20/21 in which also the highest GlcA transport activity was observed (Fig. 2*B*). In addition, quantitative image analysis of the SDS-PAGE protein elution pattern from the Mono Q column demonstrated a correlation between the 57-kDa protein and the transport activity (data not shown). All other visualized proteins could not represent the sialic acid transporter, because they became more prevalent in following fractions, where lower or no transport activity was detected (Fig. 2*B*).

In the final protein preparations (fractions 20–21 from the Mono Q column) transport activity was 432-fold enriched over the activity in the initial lysosomal membrane extract (Table I). Considering that the lysosomal membrane marker β-glucosidase is about 100-fold enriched in the lysosomal membrane vesicles (used as a starting material), the sialic acid transport protein is about 40,000-fold purified in the final eluate of the Mono Q column.

Properties of the Purified Lysosomal Sialic Acid Transporter—

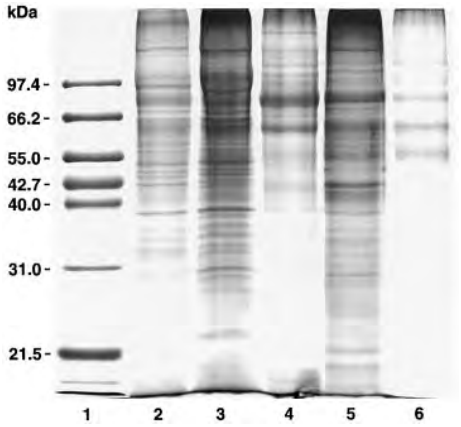

FIG. 1. **SDS-PAGE of protein fractions during the initial steps of the purification of the functional lysosomal sialic acid transporter from rat liver.** Protein fractions were analyzed by SDS-PAGE (10% gel) and Coomassie Brilliant Blue R-250 stained. Per lane, an aliquot of approximately 30 μg of total protein was loaded. *Lane 1*, mid-range protein molecular weight markers (Promega). *Lane 2*, rat liver lysosomal membrane vesicles. *Lane 3*, Triton X-100-solubilized lysosomal membrane vesicles. *Lane 4*, 25 mM sodium phosphate eluate of first hydroxyapatite column. *Lane 5*, lentil lectin unretained fraction. *Lane 6*, 100 mM NaCl eluate of DEAE-Sephacel column.

ture endoglycosidase F/*N*-glycosidase F. After treatment, the apparent molecular mass of the 57-kDa protein was not decreased. The apparent molecular mass of a control glycoprotein was decreased as a result of cleavage of glycosydic chains (data not shown). This, together with the observation that this protein did not interact with lentil lectin, indicates that the carrier is apparently not glycosylated. Analysis by SDS-PAGE in the presence or absence of the thiol-reducing agent 2-mercaptoethanol did not show any alteration of the electrophoretic behavior of the purified transport protein (data not shown). This indicates that the transporter is not functional as a (homo)dimer or polymer linked by disulfide bridges.

Substrate Specificity of the Lysosomal Sialic Acid Transporter—Because the final yield of the highly purified sialic acid transporter was very low, detailed kinetic studies were difficult to perform. Therefore, most kinetic characterization of the lysosomal sialic acid transporter was performed using partially purified preparations (DEAE-Sephacel eluates). Subsequently, some key experiments were repeated in a concise manner with the highly purified transport preparation.

Interaction of the Lysosomal Sialic Acid Transporter with Iduronic Acid—In earlier substrate specificity studies with the crude lysosomal sialic acid transporter, we have shown that this transporter recognizes structurally different types of acidic

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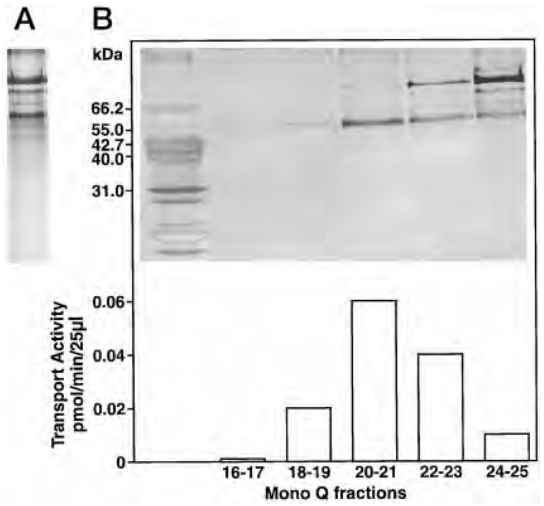

FIG. 2. **A 57-kDa protein correlates with the transport activity.** *Panel A* shows the SDS-PAGE protein pattern of the preparation, which was applied to the Mono Q column. *Panel B*, *bottom*, elution profile of Mono Q column. Transport activity was measured in reconstituted proteoliposomes of the respective Mono Q fractions. Proteoliposomes (25 μl) were incubated 1 min at 37 °C with 10 μM [^{3}H]GlcA in the presence of an inwardly directed proton gradient. Transport activity is expressed as pmol [^{3}H]GlcA/min/25 μl. *Top*, SDS-PAGE and silver staining of corresponding Mono Q fractions.

like GlcA, a major component of glycosaminoglycans. These are degraded in lysosomes, and thus free IdoA is like GlcA expected to be transported across the lysosomal membrane. The recent commercial availability of free IdoA made it now possible to investigate by *cis*-inhibition and *trans*-stimulation studies whether this uronic acid is also a substrate for the lysosomal sialic acid transporter (Table II). IdoA inhibited [^{3}H]GlcA uptake, although less efficiently than Neu5Ac and GlcA. Furthermore, IdoA was able to induce, like its isomer GlcA, almost a 2-fold *trans*-stimulation (Table II). These experiments indicate that IdoA is indeed a substrate for the sialic acid transporter.

Interaction of the Lysosomal Sialic Acid Transporter with Small Monocarboxylates—We investigated the interaction of the transport protein with other known substrates for organic anion carriers. Initially, mono-, di-, and tricarboxylic acids were tested for their *cis*-inhibition effect on the initial linear rate of proton-driven [^{3}H]GlcA uptake in a partially purified preparation (Table II). Most of these organic anions are known substrates for the proton-driven monocarboxylate transporters MCT1, MCT2, and MCT3 of the plasma membrane and for the pyruvate and the dicarboxylate transporters of the outer mitochondrial membrane. The monocarboxylic and dicarboxylic acids were all strong inhibitors, except for the amino acid glutamate and the Krebs cycle intermediate α-ketoglutarate. L-Lactate and the anti-epileptic drug valproic acid (dipropyl acetate), among the monocarboxylates, and succinate, among the dicarboxylates, were the strongest inhibitors (Table II). The tricarboxylate citrate showed no significant inhibition. To test whether inhibition represents interaction at the substrate binding site and consequently transport, we investigated the *trans*-stimulation effect of some representative mono- and dicarboxylate inhibitors on the uptake of [^{3}H]GlcA. Partially purified protein preparations were reconstituted in proteoliposomes and preloaded with an unlabeled compound at concentrations of 1 mM, just above the K_m of GlcA (0.4 mM) (6), and the uptake of [^{3}H]GlcA was followed for 1 min. As shown in Table II, L-lactate as well as GlcA itself *trans*-stimulated the uptake of [^{3}H]GlcA. Mevalonate and succinate did not cause *trans*-

TABLE II
cis-Inhibition and trans-stimulation of [^{3}H]GlcA uptake by mono-, di-, or tricarboxylic acids

The partially purified (DEAE-Sephacel eluate) sialic acid transporter was reconstituted, and proteoliposomes were incubated 1 min at 37 °C with 10 μM [^{3}H]GlcA in the presence of an inwardly directed proton gradient and 7 mM of the indicated compounds. Data represent the means of four independent determinations ± S.D. In *trans*-stimulation experiments partially purified proteoliposomes were preincubated for 60 min at 37 °C in the presence or absence of 1 mM unlabeled GlcA, IdoA, L-lactate, mevalonate, or succinate in 20 mM NaHepes, 100 mM KCl, 40 mM Mes, pH 5.5, 10 μM valinomycin and monensin. The transport assay was started by a 4-fold dilution in pH 5.5 incubation buffer with 2 μCi of [^{3}H]GlcA and allowed to proceed for 1 min. In the samples that were preincubated without unlabeled compound, 0.25 mM unlabeled compound was added together with radiolabeled substrate to give the same extravesicular substrate concentration in both experiments.

	Transport activity		trans-Stimulation
	pmol/mg/min	*% of control*	*% of not trans-stimulated*
Control	2280.5 ± 132.6		
Acidic monosaccharides			
GlcA	0	0	200
Neu5Ac	0	0	
IdoA	916.1 ± 272.4	40	180
Monocarboxylates			
Oxamate	553.0 ± 45.5	24	
Pyruvate	534.6 ± 46.0	24	
L-Lactate	195.6 ± 67.8	8	150
4-OH-butyrate	369.4 ± 107.2	16	
Mevalonate	510.0 ± 24.0	22	109
Valproate	0	0	
Dicarboxylates			
Succinate	0	0	79
Malate	366.0 ± 21.8	16	
Malonate	685.3 ± 37.5	30	
Maleate	592.5 ± 79.5	26	
Fumarate	234.3 ± 81.0	10	
α-Ketoglutarate	1090.2 ± 199.6	48	
Glutamate	2437.8 ± 195.4	107	
Tricarboxylate			
Citrate	1665.9 ± 30.6	73	

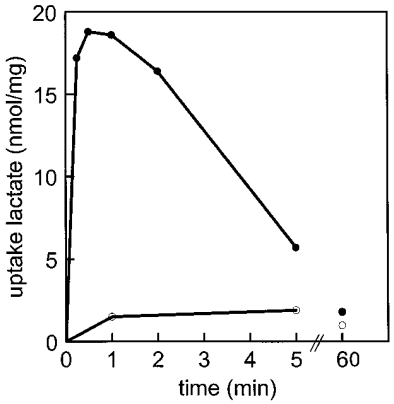

FIG. 3. **Proton gradient-dependent uptake of [^{14}C]L-lactate.** Proteoliposomes of DEAE-Sephacel eluate prepared in 20 mM NaHepes, 100 mM KCl, pH 7.4, were incubated with 15 μM [^{14}C]L-lactate at 20 °C in 40 mM Mes (free acid), 10 μM valinomycin, pH 5.5 (with proton gradient, pH_{in} = 7.4 > pH_{out} = 5.5, ●) or in 20 mM NaHepes, 10 μM valinomycin, pH 7.4 (no proton gradient, pH_{in} = pH_{out} = 7.4, ○).

diolabeled [^{14}C]L-lactate. The presence of an inwardly directed proton gradient (pH_{in} = 7.4 > pH_{out} = 5.5) stimulated initial uptake rates of lactate above equilibrium level (Fig. 3). At the top of overshoot, approximately 2% of external lactate was taken up inside the vesicles. This overshoot phenomenon was abolished in the absence of a proton gradient (pH_{in} = pH_{out} = 7.4), indicating that the transport of lactate is proton gradient-

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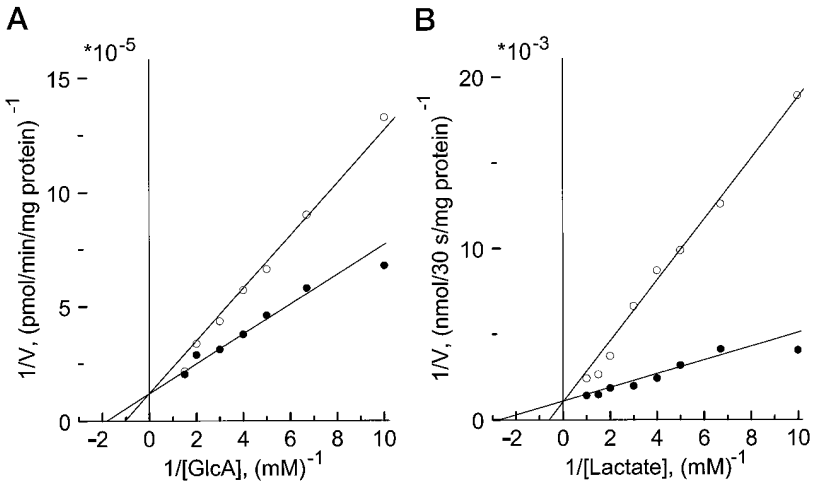

FIG. 4. **Competitive inhibition of [^{3}H]GlcA transport by L-lactate and of [^{14}C]L-lactate transport by Neu5Ac.** Initial proton-dependent transport rates of [^{3}H]GlcA (1 min, 37 °C) and [^{14}C]L-lactate (30 s, 20 °C) were measured in proteoliposomes of DEAE-Sephacel eluates. The uptake medium contained increasing concentrations of the respective substrates in the presence or absence of the inhibitors L-lactate or Neu5Ac. Data were plotted double reciprocally. *A*, [^{3}H]GlcA uptake with (○) or without (●) cold 2 mM L-lactate. *B*, [^{14}C]L-lactate uptake with (○) or without (●) cold 7 mM Neu5Ac.

trations of lactate (15 μM), lower temperature (20 °C), and the use of a pH far above the pK for the tested compounds limit the contribution of aspecific diffusion on the net uptake. Proton-driven [^{14}C]L-lactate transport under apparent zero-*trans* conditions was saturable with a K_m of approximately 0.4 mM and a V_{max} of 500 nmol/30 s/mg protein. An Eadie-Hofstee plot of the kinetic data indicated a linear process, suggesting that only one type of transport system operates (data not shown).

Although these studies provide evidence that the partially purified protein preparation is able to transport, in addition to acidic monosaccharides, many other small monocarboxylic acids, it cannot be excluded that other proteins in this preparation are present. However, the exchange of GlcA with L-lactate in the *trans*-stimulation experiments is strong evidence for transport of both compounds by the same protein. To provide further evidence that lactate and GlcA can be transported by the same carrier in the lysosomal membrane, competitive inhibition experiments were performed. Proton-dependent transport of [^{3}H]GlcA or [^{14}C]L-lactate was measured in voltage clamped membranes with K^+/valinomycin in the absence or presence of cold L-lactate or cold Neu5Ac as inhibitors, respectively. The results were fitted to a double reciprocal plot, showing a clear mode of competitive inhibition of lactate (calculated K_i of 2.5 mM) on GlcA transport and of Neu5Ac (calculated K_i of 2 mM) on L-lactate transport (Fig. 4). Definite evidence that transport of lactate is performed by the lysosomal sialic acid transporter was obtained from *cis*-inhibition and concentration-dependent inhibition studies with the highly purified sialic acid transporter preparation. Proton-driven [^{3}H]GlcA transport under apparent zero-*trans* conditions was completely inhibited in the presence of 7 mM unlabeled GlcA or Neu5Ac or L-lactate (Table III). Proton-driven [^{14}C]L-lactate transport under apparent zero-*trans* conditions was inhibited totally by L-lactate and significantly by Neu5Ac. It is interesting to note that under these conditions GlcA did not inhibit. Because the GlcA transport assays are performed at 37 °C and the lactate transport assays at 20 °C, these apparent inconsistencies can be explained by differences in affinities at different temperatures. Inhibition of [^{3}H]GlcA transport by L-lactate was a clear concentration-dependent process (Table III). Clearly, the highly purified transporter preparation contains a transporter that carries all three substrates, GlcA, Neu5Ac, and L-lactate (see also the above competitive inhibition experiments).

Sensitivity to Covalent Protein Modifiers—In previous experiments studying the effect of protein modifiers on GlcA transport in native lysosomal membrane vesicles and reconstituted

TABLE III

cis-Inhibition of [^{3}H]GlcA and [^{14}C]L-lactate uptake by GlcA, Neu5Ac, and L-lactate in proteoliposomes of the highly purified lysosomal sialic acid transporter

In the upper part of the table, the Mono Q eluate was reconstituted, and proteoliposomes were either incubated 1 min at 37 °C in the case of [^{3}H]GlcA assay or 30 s at 20 °C in the case of [rosup;14C]L-lactate assay, both in the presence of an inwardly directed proton gradient and with 7 mM of the indicated compounds. In the lower part of the table, proteoliposomes of the highly purified sialic acid transporter were incubated with 10 μM [^{3}H]GlcA for 1 min at 37 °C in the presence of an inward directed proton gradient (pH_{in} = 7.4, pH_{out} = 5.5) and with various concentrations of L-lactate. Data represent the means of two independent determinations.

Compound	Transport activity			
	[^{3}H]GlcA		[^{14}C]L-Lactate	
	pmol/min/assay	*% of control*	*pmol/30 s/assay*	*% of control*
Control	0.082		15.4	
GlcA	0	0	16.2	105
Neu5Ac	0	0	8.8	57
L-Lactate	0	0	0	0
L-Lactate				
10 μM	0.082	100		
20 μM	0.075	92		
50 μM	0.066	81		
150 μM	0.059	72		
500 μM	0.010	12		

6). To investigate whether L-lactate transport is similarly affected by some of these protein modifiers, inhibition and substrate protection experiments were performed. 1 mM *N*-ethylmaleimide, a thiol-modifier, irreversibly inactivated transport of both GlcA (75% inhibition) and L-lactate (86% inhibition) in proteoliposomes from partially purified preparations. Phenylglyoxal, under these conditions an arginine modifier, significantly inhibited GlcA transport (44% of uninhibited rate). The inactivation of GlcA transport could be partially rescued when phenylglyoxal treatment was performed in the presence of the substrates GlcA, Neu5Ac, and L-lactate (96, 96 and 56% of uninhibited rate, respectively). Similarly, L-lactate transport was significantly inhibited by phenylglyoxal (59% of uninhibited rate) and protected in the presence of L-lactate, GlcA, and Neu5Ac (91, 88, and 71%, respectively, of uninhibited rate). Apparently, GlcA, Neu5Ac, and L-lactate all use the same substrate-binding site of the transport protein.

DISCUSSION

In this paper we describe the purification of the lysosomal

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