***N*-Acetyl-D-glucosamine countertransport in lysosomal membrane vesicles**

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Countertransport of GlcNAc was examined in membrane vesicles prepared from rat liver lysosomes which had been lysed by exposure to 5 mM-methionine methyl ester. These vesicles have a random orientation, have intact ATP-dependent acidification and are largely free of soluble hydrolases. Vesicular volume ($24.69 \pm 4.51 \mu\text{l}/\text{mg}$ of protein) was greater than that of lysosomes ($3.02 \pm 0.56 \mu\text{l}/\text{mg}$ of protein), corresponding to a doubling of diameter. Characteristics of GlcNAc transport in vesicles ($K_m = 1.3 \text{ mM}$) were similar to those observed in intact lysosomes ($K_m = 4.4 \text{ mM}$). Sulphation or phosphorylation of the substrate resulted in loss of recognition by the carrier. Hydroxyl group orientation at multiple positions did not appear to be critical, whereas orientation of the acetyl group appeared to have a fundamental role in recognition by the carrier. Based on these criteria, phenyl isothiocyanate–GlcNAc (PITC–GlcNAc) was identified as a possible substrate for transport. Under mild conditions, PITC–GlcNAc reversibly inhibited GlcNAc countertransport in lysosomes and vesicles. This and other modified substrates may be of value in identification of the GlcNAc/GalNAc lysosomal transporter. Lysosomal membrane vesicle preparation is a technique that should be useful for the study of other lysosomal transport systems.

INTRODUCTION

Free GlcNAc and GalNAc are generated during the lysosomal degradation of glycoproteins, glycosphingolipids and glycosaminoglycans. These and other products of degradative processes must then exit the lysosome to avoid osmotic imbalance with subsequent vesicular swelling and possible rupture. We have recently demonstrated a highly specific transport system in Percoll-gradient-purified rat liver lysosomes that recognizes only GlcNAc or GalNAc [1]. The GlcNAc/GalNAc carrier exhibits countertransport, is not dependent on pH gradients, divalent cations or MgATP and is inhibited by cytochalasin B.

As yet, lysosomal sugar and amino acid transport systems have not been successfully reconstituted into artificial vesicles [1–10]. The lysosomal membrane is markedly asymmetrical, with a highly glycosylated inner surface that provides protection against the large concentrations of soluble internal hydrolases [11]. Exposure of the outer membrane surface to these hydrolases may result in impaired membrane protein function. As a prelude to efforts to reconstitute the GlcNAc carrier in artificial vesicles, GlcNAc transport in resealed lysosomal vesicles was investigated. We have built upon a technique developed for the purification of lysosomal membranes that employs exposure to amino acid methyl ester derivatives to produce osmotic lysis of purified lysosomes. The spontaneously resealed vesicles are then collected by density-gradient centrifugation [12]. We now report the characteristics of GlcNAc countertransport in these vesicles.

MATERIALS AND METHODS

Materials

All chemicals, unless otherwise indicated, were obtained from Sigma Chemical Company. *N*-[acetyl- ^3H]Acetyl-D-glucosamine, [^3H]borohydride, [^3H]water and [^{14}C]sucrose were obtained from ICN. *N*-Acetylmuramic acid (MurNAc) and phenyl isothio-

cyanate (PITC) were obtained from Aldrich Chemical Co. Scintiverse II was obtained from Fisher. Female Sprague–Dawley rats (150–200 g) were obtained from Simonsen.

Lysosomal purification and loading

Lysosomes were purified from homogenized rat liver using differential centrifugation and Percoll-gradient centrifugation as described previously [13]. The lower-density fractions of the gradient containing membrane, Golgi and mitochondria were removed with a Pasteur pipette and discarded. The higher-density lysosomal fraction (approx. 1 ml) was removed and transferred to a clean centrifuge tube. Lysosomal purity was assessed by the specific activity of β -hexosaminidase and/or β -glucosidase [14]. Protein was determined spectrophotometrically on samples made Percoll-free as described previously [15].

Lysosomal GlcNAc loading

An appropriate volume of 250 mM-GlcNAc was added to the lysosomal Percoll-gradient fraction to yield a final concentration of 25 mM-GlcNAc. This was allowed to equilibrate for 60 min at 25 °C. Lysosomes were then diluted 10-fold with 0.25 M-sucrose/20 mM-Hepes, pH 7.0, at 4 °C and were collected by centrifugation at 13000 *g* for 10 min. The lysosomal pellet, now free of Percoll, was resuspended in 600 μl of cold buffer for countertransport studies.

Lysosomal countertransport

Lysosomal suspension (20 μl) was added to a 500 μl microcentrifuge tube and mixed rapidly with 2 μCi of [^3H]GlcNAc in buffer to yield a final volume of 50 μl , containing 1 mM-GlcNAc and any other additions as indicated. Whenever possible, additions were dissolved in Hepes/sucrose buffer and adjusted to pH 7.0. PITC derivatives were dissolved in methanol, dispensed in microcentrifuge tubes and dried under a stream of N_2 prior to

Abbreviations used: AllNAc, *N*-acetylallosamine; MurNAc, *N*-acetylmuramic acid; 3-GlcNAc, 3-acetamido-3-deoxyglucopyranose; 6-GlcNAc, 6-acetamido-6-deoxyglucopyranose; 3-AllNAc, 3-acetamido-3-deoxyallosopyranose; PITC, phenyl isothiocyanate; CCCP, *m*-carbonyl cyanide chlorophenylhydrazide.

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any other additions. Incubations were performed for 2 min at 25 °C. A portion of the mixture (45 μ l) was then removed, filtered through a GFC fibre filter and rinsed with 5 ml of ice-cold Dulbecco's phosphate-buffered saline without added Ca^{2+} . Filters were dried and the radioactivity was counted.

Lysosomal vesicle preparation

The Percoll-gradient fraction containing lysosomes was incubated for 15 min at 37 °C in 0.25 M-sucrose/20 mM-Hepes, pH 7.0, with 2 μ g of leupeptin/ml, 1 μ g of pepstatin A/ml, 2 mM- MgCl_2 , 2 mg of bovine serum albumin/ml, 5 mM-methionine methyl ester and 50 mM-GlcNAc. Resealed vesicles were collected on a second Percoll density gradient as described previously [12]. ATP-dependent acidification was determined by fluorescent assay using 17 μ M-Acridine Orange, with excitation of 480 nm and emission at 520 nm [16]. Vesicle orientation was determined by labelling with tritiated sodium borohydride before and after galactose oxidase treatment [17,18]. Vesicle volume was determined by incubation in tritiated water and [^{14}C]sucrose [18].

Vesicle transport

The gradient fraction containing vesicles was removed and diluted 1:20 (v/v) with 0.25 M-sucrose/20 mM-Hepes, pH 7.0. GlcNAc countertransport was measured in these vesicles as described for lysosomes, with the exception that 0.22 μ m Millipore filters were used for vesicle collection.

PITC-GlcNAc incubation

Unloaded Percoll-purified lysosomal fractions were incubated with or without 1 mM-PITC-GlcNAc for 15 min at 25 °C in 0.25 M-sucrose/20 mM-Hepes, pH 7.0. Samples were then diluted 10-fold with ice-cold buffer and collected by centrifugation at 13000 g for 10 min. Lysosomal pellets were then resuspended in 1 ml of buffer and loaded with GlcNAc for countertransport assay as described previously. Samples of GlcNAc-loaded lysosomes were assayed for protein content and β -hexosaminidase activity.

RESULTS

Lysosomes were purified by approx. 90-fold by Percoll-gradient centrifugation, similar to previously reported results, based on the specific activity of β -hexosaminidase [13]. After exposure to methionine methyl ester, the resealed vesicles had a density of 1.056 as compared with 1.111 for intact lysosomes [12]. The specific activity of soluble β -hexosaminidase in resealed vesicles (535 nmol per min/mg) was decreased by 86 % compared with that in lysosomes (3820 nmol per min/mg), whereas the specific activity of membrane-bound β -glucosidase was un-

Fig. 1. Acidification of lysosomal membrane vesicles

Vesicles were suspended in 0.2 M-sucrose/50 mM-KCl/20 mM-Hepes, pH 7.0, at 25 °C with 17 μ M-Acridine Orange. Addition of vesicles resulted in a rapid decrease of fluorescence until equilibrium was reached. The addition of 2 mM- MgCl_2 /ATP resulted in a small artifactual increase in fluorescence followed by a steady decrease reflecting acidification. The addition of the protonophore CCCP (10 μ M) caused vesicular alkalization.

changed at 10 nmol per min/mg. Exposure of vesicles to tritiated borohydride following galactose oxidase treatment resulted in labelling of 60 % of the glycoproteins (Table 1). Normally only 10 % of lysosomal membrane glycoproteins are on the external membrane surface and available for labelling [18]. The data are therefore consistent with a random vesicular membrane orientation. Vesicles incubated with 2 mM-ATP/ MgCl_2 were acidified, reflecting activity of the lysosomal proton-translocating ATPase (Fig. 1). The addition of CCCP in the presence of KCl resulted in alkalization similar to observations in intact lysosomes [19]. Internal volume, estimated by the relative distributions of [^3H]water and [^{14}C]sucrose, was $24.69 \pm 4.51 \mu\text{l/mg}$ of protein (mean $\pm$ S.D., $n = 4$) for vesicles as compared with $3.02 \pm 0.56 \mu\text{l/mg}$ of protein (mean $\pm$ S.D., $n = 4$) for intact lysosomes. This corresponded to a doubling of diameter in the vesicles.

Uptake of [^3H]GlcNAc by vesicles formed in the presence of 50 mM-GlcNAc (Fig. 2) was similar to countertransport results previously reported with intact lysosomes [1]. At 25 °C, uptake was completed by 3 min. Little accumulation of radiolabel was seen in vesicles formed without GlcNAc. In order to demonstrate that this represented true uptake, vesicles incubated under these conditions for 5 min at 25 °C were washed with buffer and collected by centrifugation. After three washes, supernatants were largely free of radiolabel and the vesicles were suspended in either iso-osmotic buffer or deionized water at 4 °C. Examination of the pellets after collection by centrifugation revealed 3.0 ± 10^5 d.p.m./mg of protein for buffer-treated vesicles and

Table 1. [^3H]Borohydride/galactose oxidase determination of membrane vesicle sidedness

Membrane vesicles were first treated with neuraminidase followed by galactose oxidase. Exposed glycoproteins were labelled with [^3H]borohydride. Controls were either without galactose oxidase to determine non-specific labelling or were sonicated during enzymic treatment to allow labelling of all glycoproteins. Pellets were collected by ultracentrifugation and washed before analysis. Values are expressed per mg of protein.

Condition	Membrane-bound radiolabel		
	Observed (c.p.m./mg)	Corrected (c.p.m./mg)	Glycoprotein labelling (%)
– Galactose oxidase	25 535	–	–
+ Galactose oxidase	41 822	18 287	60
Sonicated	54 257	30 722	100

Fig. 2. Uptake of [^3H]GlcNAc (1 mM) by membrane vesicles formed in the presence of 50 mM-GlcNAc

Uptake was performed at 25 °C in 0.25 M-sucrose/20 mM-Hepes, pH 7.0. Vesicles were collected by filtration. Points are means $\pm$ S.D. ($n = 4$).

0.2×10^5 d.p.m./mg of protein for water-treated vesicles. Over 90% of the label in the osmotically ruptured vesicles was in the supernatant, as opposed to 27% for the buffer-treated vesicles. These results are consistent with uptake of radiolabelled GlcNAc by countertransport. The K_m for GlcNAc countertransport was 1.3 mM (Fig. 3), similar although not identical to the previously reported value of 4.4 mM for GlcNAc uptake by intact lysosomes. Countertransport of GlcNAc by vesicles had similar specificity to results obtained previously with intact lysosomes (Table 2).

Additional characterization of transport in lysosomes and

Fig. 3. Lineweaver-Burk plot of [^3H]GlcNAc uptake by lysosomal membrane vesicles

Vesicles were preloaded with 50 mM-GlcNAc and were incubated for 1 min at 25 °C with [^3H]GlcNAc before collection by filtration. v is in pmol/min; $[S]$ is in μM . Points are means $\pm$ S.D. ($n = 3$).

Table 2. Competition for GlcNAc countertransport by other sugars

Vesicles formed in the presence of 50 mM-GlcNAc were incubated with 2 μCi of [^3H]GlcNAc (final concentration 1 mM-GlcNAc) in 45 μl of 0.25 M-sucrose/20 mM-Hepes, pH 7.0, for 2 min at 25 °C. Competitors were added as 5 μl of a 250 mM solution (final concn. 25 mM). Vesicles were collected by filtration through 0.22 μm filters. Uptake was calculated as pmol/min/ $(\mu\text{mol}$ of β -hexosaminidase/min) and then expressed as % of baseline. Values are means $\pm$ S.D. ($n = 3$). Lysosomal data were previously reported [1] and are presented for comparison.

Condition	Uptake (% of baseline)	
	Lysosomes	Vesicles
Control	100	100
GlcNAc	—	100
GalNAc	5 ± 5	0 ± 0
D-Glucose	111 ± 33	105 ± 9
D-Glucosamine	108 ± 6	101 ± 1
D-Mannose	117 ± 26	97 ± 15
L-Fucose	116 ± 4	92 ± 4
<i>N</i> -Acetylneuraminic acid	107 ± 16	52 ± 6
D-Glucuronic Acid	83 ± 6	71 ± 7

membrane vesicles was performed. The effects of substitutions to the GlcNAc moiety resulting in charged species are summarized in Table 3. Phosphorylation or sulphation of GlcNAc resulted in species that were not recognized by the carrier in either intact lysosomes or resealed vesicles. The site of the substitution did not appear to be nearly as important as the change in charge itself. MurNAc, which is similar to GlcNAc but has a carboxyl group in position 3, also failed to compete for countertransport of GlcNAc, attesting to the importance of charge in the recognition.

It had already been shown that GlcNAc and GalNAc, which differ only in orientation of the hydroxyl group at position 4, are competitive species for transport in lysosomes [1]. *N*-Acetyl-allosamine (AllNAc), which differs from GlcNAc in the orien-

Table 3. Charge, *N*-acetylation and competition for GlcNAc countertransport

Experimental conditions were as described for Table 2. Lysosomes were equilibrated with 25 mM-GlcNAc prior to experiments. All additions were neutralized to pH 7.0 with NaOH. All competitors were present at a final concentration of 25 mM. Values are means $\pm$ S.D. ($n = 3$).

Condition	Countertransport (% of baseline)	
	Lysosomes	Vesicles
Control	100	100
GlcNAc 1-phosphate	96 ± 12	68 ± 11
GlcNAc 6-phosphate	95 ± 15	59 ± 11
GlcNAc 3-sulphate	96 ± 23	88 ± 11
GlcNAc 6-sulphate	93 ± 8	93 ± 11
MurNAc	110 ± 6	85 ± 2
GlcNAc	7 ± 2	12 ± 1
3-GlcNAc	21 ± 12	10 ± 1
6-GlcNAc	54 ± 13	66 ± 7
2-AllNAc	9 ± 4	2 ± 13
3-AllNAc	63 ± 13	92 ± 11
ManNAc	60 ± 7	74 ± 5

Fig. 4. Inhibition of GlcNAc countertransport by cytochalasin B

Membrane vesicles were incubated for 2 min at 25 °C in 1 mM-GlcNAc and collected by filtration as described previously. Points are means $\pm$ S.D. ($n = 3$).

Table 4. Effect of PITC-GlcNAc on countertransport

Experimental conditions were as described for Table 2. PITC-GlcNAc was dispensed as a methanolic solution and dried under a stream of N_2 . Values are means $\pm$ S.D. ($n = 3$).

Addition	Countertransport (% of baseline)	
	Lysosomes	Vesicles
None	100	100
GlcNAc (25 mM)	5 $\pm$ 4	12 $\pm$ 1
PITC-GlcNAc (0.6 mM)	32 $\pm$ 3	30 $\pm$ 4
PITC (1 mM)	92 $\pm$ 3	—

tation of the hydroxyl groups at positions 1 and 3, also effectively competed for transport in both lysosomes and membrane vesicles (Table 3). Movement of the acetyl group from position 2 to position 6 of GlcNAc markedly affected recognition by the carrier. In contrast to the case for GlcNAc, acetylation of AllNAc in position 3 resulted in a poor substrate for transport. As reported previously for lysosomes, ManNAc, which differs from GlcNAc in acetyl group orientation, is also a weak competitor for GlcNAc transport in vesicles. Thus hydroxyl group orientation is flexible whereas charge and orientation of the acetyl groups are critical requirements for carrier recognition.

Cytochalasin B, an inhibitor of lysosomal GlcNAc transport, also inhibited vesicular transport, although much less effectively. A 4-fold increase in concentration of cytochalasin B was required to achieve inhibition comparable with that observed in lysosomes (Fig. 4). Since it conformed to the criteria for substrate recognition, and under proper conditions is capable of covalent protein binding, PITC-GlcNAc was examined for its effects on transport. Under mild conditions not favourable for covalent linkage (pH 7.0), PITC-GlcNAc inhibited GlcNAc countertransport by 70 %. In contrast, free PITC had little effect on transport (Table 4). Inhibition by PITC-GlcNAc was completely

reversible under these conditions (results not shown), consistent with a mechanism of competitive inhibition.

DISCUSSION

Using a technique established in our laboratory for the purification of rat liver lysosomal membranes, we have been able to prepare vesicles that exhibit ATP-dependent proton translocation and GlcNAc countertransport. The characteristics of GlcNAc countertransport in vesicles and intact lysosomes are virtually identical, with some minor exceptions. The K_m for countertransport in vesicles was somewhat lower, at 1.3 mM, than the previously obtained K_m of 4.4 mM for GlcNAc uptake by intact lysosomes. Phosphorylated derivatives of GlcNAc, although poor substrates for transport by both lysosomes and vesicles, had somewhat greater effects on countertransport by vesicles. This may be a consequence of the random membrane orientation of these vesicles, with exposure of membrane-bound acid phosphatase to the exterior. Some of the phosphorylated substrate may thus be subject to hydrolysis, resulting in release of free GlcNAc, a highly effective competitor for transport.

The membrane vesicles have roughly twice the volume of intact lysosomes, consistent with previously reported electron microscopic analysis of lysosomal membranes prepared in this manner [12]. Other methods yield vesicles with somewhat smaller volumes than intact lysosomes [16,18]. However, for reasons that are currently not understood, we have had difficulty demonstrating GlcNAc countertransport in these other preparations. These different techniques provide options that may simplify the study of lysosomal transport systems. Although many transport systems are bidirectional, this need not always be the case. It is also conceivable that under some circumstances the lysosomal proton gradient may be unfavourable for uptake of substrate. Preparation of inside-out vesicles might therefore be a very useful technique, eliminating the need for more cumbersome substrate-loading procedures.

Characterization of GlcNAc transport in lysosomes and membrane vesicles has revealed additional criteria for substrate recognition by the carrier. Although sulphated and phosphorylated substrates are not recognized, the carrier is not likely to be presented with these substrates under normal circumstances. Sulphated GlcNAc and GalNAc are enzymically cleaved by lysosomal sulphatases as part of proteoglycan degradation, and any phosphorylated sugar would similarly be degraded by lysosomal phosphatase. As might be predicted from its negative charge, sialic acid, although acetylated, is not carried by the GlcNAc system. A lysosomal transport system for sialic acid and other negatively charged sugars has previously been demonstrated. Unlike the GlcNAc system, the negatively charged sugar system appears to be dependent upon proton gradients [20,21].

Cytochalasin B, an inhibitor of lysosomal GlcNAc countertransport, has similar effects on membrane vesicle transport, although larger concentrations are required. This may be due to proteolytic modification of the carrier during vesicle formation. It is also possible that the random orientation of the vesicles contributes to this effect. Cytochalasin B can be cross-linked to transporters by photoactivation and has been used to identify a glucose transporter in plasma membranes [22,23]. We have found that similar experiments with lysosomal membranes are difficult to interpret due to background labelling (A. J. Jonas & H. Jobe, unpublished work). Binding of cytochalasin B to other proteins in the lysosomal membrane and the relative abundance of the GlcNAc/GalNAc transporter may represent complicating factors.

A specific, irreversible inhibitor of transport would be of great

value in identification of the GlcNAc/GalNAc transporter. Knowledge of the criteria for substrate recognition permit the selection or the design of probes which are capable of covalent modification of the transporter. PITC–GlcNAc, which has been used previously to glycosylate albumin [24], is one of a number of such possible probes. PITC, which under appropriate conditions can covalently modify cysteine or lysine residues, has been linked to GlcNAc at position 1 without affecting substrate recognition. Under mild conditions, we have shown that PITC–GlcNAc can reversibly inhibit GlcNAc transport in both lysosomes and membrane vesicles. This and other inhibitors of transport may be useful for labelling of the carrier.

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