Recognition and receptor-mediated endocytosis of the lysosomal acid lipase secreted by cultured human fibroblasts

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Abstract We have studied the recognition and uptake of acid lipase by human fibroblasts in order to determine requirements for localization and function of the enzyme in lysosomes. Our approach was based on evidence that a number of acid hydrolases involved in mucopolysaccharide metabolism are secreted from cultured fibroblasts and endocytosed by a phosphomannosyl-dependent, receptor-mediated process. Acid fatty acid ester hydrolase activity secreted from human fibroblasts was separable into two major forms by hydrophobic chromatography. The dominant form from normal cells was deficient in fibroblasts from patients with Wolman's disease, an inherited disorder of lysosomal cholesteryl ester and triglyceride metabolism. The time- and temperature-dependent, saturable uptake of this enzyme by fibroblasts was competitively inhibited by mannose 6-phosphate and was destroyed by pretreatment of the enzyme with phosphatase. Internalized lipase had a half-life of 1 day. Application of the enzyme to Wolman's disease fibroblasts reduced cholesteryl ester storage; this effect was blocked by ammonium chloride, a general inhibitor of lysosomal hydrolysis. Our results indicate that extracellular acid lipase is transported to fibroblast lysosomes by the same receptor-mediated process that functions in the packaging of several lysosomal glycosidases.—**Sando, G. N., and V. L. Henke.** Recognition and receptor-mediated endocytosis of the lysosomal acid lipase secreted by cultured human fibroblasts. *J. Lipid Res.* 1982. **23:** 114–123.

Supplementary key words cholesteryl ester hydrolysis • Wolman's disease • mannose 6-phosphate recognition • extracellular lipase

Lysosomal acid lipase activity is essential for the hydrolysis of cholesteryl esters that enter cells as part of the low density lipoprotein complex. The enzyme thus has a critical role in the regulation of cholesterol metabolism at control sites that depend on the level of free sterol (1). A genetic deficiency of the lipase, in patients afflicted with Wolman's disease, or with the clinically less severe disorder, cholesteryl ester storage disease, leads to excessive accumulation of lysosomal triglycerides and cholesteryl esters in several tissues (2).

In studies of lysosomal storage disorders that result from the deficiency of a specific glycosidase or sulfatase, faulty mucopolysaccharide degradation in cultured fibroblasts derived from affected patients was restored to normal when deficient fibroblasts were cocultured with nondeficient cells or when the missing enzyme activity was added to the culture medium (3). Correction was explained by the finding that cultured fibroblasts secrete and take up lysosomal hydrolases required for mucopolysaccharide turnover. The uptake process is mediated by the recognition of a phosphomannosyl component of the hydrolases by a fibroblast receptor. This conclusion is supported by evidence from kinetic studies of hydrolase uptake (4–7), the demonstration of phosphomannosyl-specific binding of glycosidases to cells and membrane preparations (8, 9), detection of mannose 6-phosphate in preparations of lysosomal enzymes (10–14), and the recent isolation and characterization of a phosphomannosyl-specific hydrolase receptor (15).

A similar approach was used to correct abnormal lipid metabolism in acid lipase-deficient fibroblasts (16, 17). Those results suggested that lysosomal lipase activity was acquired from the culture medium by the deficient cells. To determine if an acid lipase is transported by the same processes as are the mucopolysaccharide-degrading enzymes, and to investigate the role of those processes in the regulation of lipid metabolism, we have studied the secretion and uptake of acid lipase by human fibroblasts. We now report on the fatty acid ester hydrolase activity in fibroblast secretions, characteristics of its recognition and uptake by cultured human cells, and the relationship of lipase uptake to intracellular cholesteryl ester hydrolysis.

MATERIALS AND METHODS

Materials

Bovine serum albumin, alkaline phosphatase (EC 3.1.3.1, Type III-R, *E. coli,* 38 U/mg), cholesterol ox-

Abbreviations: MEM, Eagle's Minimal Essential Medium with Earle's salts; EDTA, ethylenediaminetetraacetic acid; 4-MUO, 4-methylumbelliferyl oleate.

idase (EC 1.1.3.6, from *Nocardia erythropolis,* 20 U/mg), phosphatidylcholine (Type V-E, egg yolk), sodium taurodeoxycholate, cholic acid, chloroquine, sugar phosphates, 4-methylumbelliferone, and decyl-agarose were obtained from Sigma Chemical Co., St. Louis, MO. Cholesteryl esterase (EC 3.1.1.13, from microorganisms, ca. 20 U/mg) was from Boehringer Mannheim Biochemicals, Indianapolis, IN. Horseradish peroxidase (EC 1.11.1.7, RZ = 3, 1070 U/mg) was purchased from Worthington Biochemical Corp., Freehold, NJ. Cholesterol and cholesteryl oleate were obtained from Applied Science, State College, PA, and 4-methylumbelliferyl oleate (4-MUO) was from Research Products International Corp., Elk Grove Village, IL. *p*-Hydroxyphenylacetic acid was obtained from Aldrich Chemical Co., Milwaukee, WI and purified as described by Heider and Boyett (18). Phenyl-α-L-iduronide was a gift from Dr. E. F. Neufeld, National Institutes of Health, Bethesda, MD. Other reagents and chemicals were of the highest grade commercially available. Tissue culture media and additives were purchased from the Grand Island Biological Company (Gibco), Grand Island, NY.

Cell culture

Normal human skin fibroblasts (GM 1603) and cells derived from patients with Wolman's disease (GM 1606, 2211) or with I-cell disease (GM 1586) were obtained from the Human Genetic Mutant Cell Repository, Institute for Medical Research, Camden, NJ. Another line of I-cell fibroblasts (ICH) and a culture of L-iduronidase-deficient fibroblasts derived from a patient with the Hurler syndrome were provided by Dr. E. F. Neufeld. A line of normal human foreskin fibroblasts (FSF) was derived from a primary culture by the State Hygienic Laboratory, University of Iowa. Cultures were maintained in 75 or 150-cm^2 plastic flasks at 37°C in Eagle's Minimal Essential Medium (MEM) with Earle's salts (Gibco No. 410–1100) supplemented with 10% heat-inactivated fetal bovine serum, 0.2 mM MEM nonessential amino acids (Gibco No. 320-1140), 2% MEM vitamins (Gibco No. 320-1120), 2 mM L-glutamine, 100 units/ml of penicillin, 100 μg/ml of streptomycin, and 8 units/ml of mycostatin. The medium was buffered at pH 7.4 with 0.015 M $NaHCO_3$ and 5% CO_2 in air. Cells were subcultured at a ratio of 1:4 with 0.5% trypsin (Gibco No. 610-5090) in 0.9% NaCl.

Measurement of acid lipase activity

A modification of the procedure of Cortner et al. (19) was used. Substrate was prepared by evaporating to dryness under nitrogen 8 μmol of phosphatidylcholine and 5 μmol of 4-methylumbelliferyl oleate in hexane; just prior to use, the 4-methylumbelliferyl oleate solution was extracted with 0.01 M sodium phosphate buffer, pH 8, to remove nonesterified methylumbelliferone. Following the addition of 12.5 ml of 2.4 mM sodium taurodeoxycholate in sterile water, the lipid was dispersed with the use of a vortex mixer, and the mixture was sonicated for 5 min at 0°C using a Bronwill Biosonik III sonic oscillator (4 mm probe, ~100 watts). The reaction was initiated by addition of sample to 0.2 M sodium acetate buffer, pH 4.2, which contained 0.05 ml of the substrate preparation in a final volume of 0.4 ml. The rate of change in fluorescence intensity at 31°C was monitored with a Perkin-Elmer Model 203 fluorescence spectrophotometer (excitation = 330 nm; emission = 445 nm). Readings were standardized against a solution of 4-methylumbelliferone in 0.2 M acetate buffer, pH 4.2. A unit is defined as that amount of enzyme which resulted in the formation of 1 nmol of 4-methylumbelliferone/min under the specified conditions.

Preparation of acid lipase from fibroblast secretions

Fibroblasts were grown to confluence in 490-cm^2 roller bottles as described above. To maintain cells free from serum proteins the original growth medium was replaced with Waymouth medium (752/1, Gibco No. 430-1400) supplemented with antibiotics and L-glutamine, as above, and with 8 μg/ml of crystalline bovine insulin (Gibco No. 890-8125). An initial overnight rinse with the serum-free medium was discarded and fresh medium supplemented with 15 mM ammonium chloride was added. Secretions were harvested every 3–4 days thereafter; collections were continued for as long as 4–6 weeks. Secretions were treated with a buffer solution to give as a final concentration of additives in the sample: 0.01 M sodium phosphate, pH 7.0; 1 mM EDTA; 10 mM 2-mercaptoethanol; and 0.02% sodium azide. (This is also the composition of buffer A). The buffered sample was filtered (Whatman #1) to remove cell debris. Routinely, samples were treated with hydrophobic chromatography as is described in Results. For some experiments, crude secretions were concentrated as follows: 15 mg of bovine serum albumin was added, sample volume was reduced to 5–10 ml by ultrafiltration (Amicon, YM-10 membrane), and proteins were precipitated with 80% ammonium sulfate. Following centrifugation of the sample at 27,000 *g* for 30 min, the precipitate was reconstituted to a volume of 1–2 ml with buffer A, and dialyzed in that buffer (two changes of a 1-liter bath). Preparations of lipase from secretions were stored at 6°C; lipase activity from crude or partially purified secretion preparations was inactivated by freezing at −20° or −60°C.

Measurement of acid lipase uptake by fibroblasts

Routinely, cells (passage 10–25) derived from a patient with Wolman's disease, thus deficient with respect to endogenous lipase activity, were seeded at a ratio of 1:4 in 35-mm diameter culture dishes and grown to confluence in the standard growth medium. After 4 days MEM

was replaced with serum-free Waymouth medium. Cells were maintained in this medium for about 1 week in order to further decrease endogenous 4-MUO hydrolase activity. The lipase sample, 1.5 mg of albumin, and other test substances in 0.25 ml of buffer, 0.01 M sodium phosphate (pH 6.0)–0.15 M NaCl, were combined with 1.25 ml of fresh Waymouth medium and applied to the well-drained cell monolayers. For incubations longer than 4 hr, the albumin concentration was increased to approximately 5 mg/dish to allow for sterilization by filtration (Millipore membrane, 0.45 μm pore size) without loss of enzyme. After incubation with the cells at 37°C in 5% CO_2–95% air, medium was removed, cells were rinsed twice with 0.9% NaCl, and then removed from dishes by treatment with 0.5% trypsin in 0.9% NaCl. Cell suspensions were centrifuged for 30 sec in a Beckman Microfuge B, and cell pellets were washed twice with 0.9% NaCl. Pellets could be stored for at least 1 week at −20°C with no apparent loss of acid lipase activity. To cell pellets on ice was added 0.2 ml of 0.9% NaCl. Cells were disrupted by sonication for 10 sec (4 mm probe of Biosonik III, ~60 watts). Aliquots containing 2–10 μg of protein (20) were assayed for acid lipase activity as described above.

Methods for the purification and assay of L-iduronidase, which was tested as an inhibitor in the lipase uptake system, have been described (5).

Analysis of cholesterol in fibroblasts

An aliquot of fibroblast sonicate was extracted with chloroform–methanol (21) immediately after disruption of cells in 0.9% NaCl. The extract was dried under nitrogen and redissolved in 95% ethanol. Aliquots were analyzed for free and total cholesterol by a coupled enzyme procedure which involved fluorimetric measurement of hydrogen peroxide generated from sample ± cholesteryl ester hydrolase by the action of cholesterol oxidase (22).

RESULTS

Fibroblast acid lipase activity

Cultured fibroblasts derived from patients with Wolman's disease had about 20% of the acid fatty acid ester hydrolase activity of normal fibroblasts, as determined by 4-methylumbelliferyl oleate hydrolysis (**Table 1**). The lipase deficiency was also expressed in extracellular enzyme levels. By contrast, although intracellular acid lipase activity was reduced in fibroblasts from patients with I-cell disease, the activity was elevated in I-cell secretions. The abnormal localization of acid lipase is therefore typical of that reported for most of the acid hydrolases that have been examined in I-cell fibroblasts (23, 24). The accumulation of extracellular lipase from normal fibroblasts increased 5–10 fold when ammonium chloride was added to the culture medium. This treatment was routinely employed to improve the yield of lipase for use in uptake studies.

TABLE 1. Acid lipase activity of cultured human skin fibroblasts

Fibroblasts	Acid Lipase Activity[a]		
		Secretions[c]	
	Intracellular[b]	−NH_4Cl	+NH_4Cl
Normal			
GM 1603	8.2 ± 1.1 (6)	5	30–50
FSF	12.6 ± 0.3 (6)	10–20	150–250
Wolman's disease			
GM 1606	1.8 ± 0.1 (5)		2–4
GM 2211	2.5 ± 0.1 (5)	2–10	2–5
I-Cell disease			
GM 1586	2.0 ± 0.2 (4)		
ICH	1.8 ± 0.1 (6)	100–150	

[a] The rate of 4-methylumbelliferyl oleate hydrolysis was measured as described in Methods.

[b] Cells were grown in 60-mm culture dishes for 3 days in MEM + 10% fetal bovine serum, then for 4 days in serum-free Waymouth medium. Lipase activity and protein content were measured in sonicates of cells harvested by trypsin treatment. Lipase activity per mg of cell protein is expressed as the mean ± standard error (number of dishes assayed).

[c] Extracellular lipase is expressed as the amount of activity found in the medium exposed to fibroblasts in a 490 cm^2 roller bottle for a 3-day period. The cells were originally grown to a confluent monolayer (approximately 6–7 mg of cell protein per bottle). The range of values obtained from several collections over the first 2 weeks of exposure of cells to serum-free medium is given for each cell line. Where indicated, 15 mM ammonium chloride was added to the collection medium.

When fibroblast secretions were applied to a column of decyl-agarose and subsequently eluted with a cholic acid gradient, two peaks of acid fatty acid ester hydrolase activity were detected, one eluting with 5 mM, and the other with 10 mM cholate (**Fig. 1**). The more retarded form predominated (80–90%) in secretions from normal fibroblasts whereas the opposite was true for Wolman cell secretions; of the small amount of lipase activity in secretions from the mutant cells (5–10% of that in normal secretions), approximately 20% was present as the more retarded form. The relative distribution of the two peaks in secretions from normal and deficient fibroblasts was not significantly altered by addition of ammonium chloride to the medium. Thus only the more retarded form from decyl-agarose chromatography was markedly reduced in secretions from Wolman fibroblasts, where it was present at less than 2% of the level in normal cell secretions. For studies of enzyme uptake, we used this form of the lipase from secretions of normal fibroblasts that were treated with ammonium chloride. This enzyme had a pH optimum of 4.0–4.2 with the 4-methylumbelliferyl oleate hydrolase assay; less than 1% of the op-

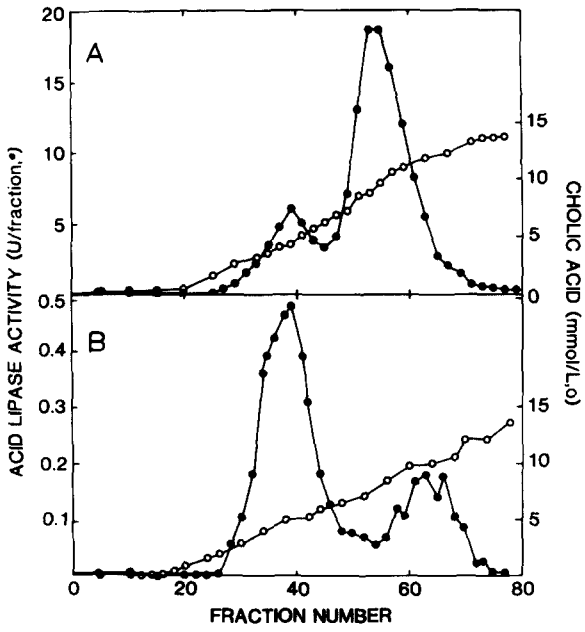

Fig. 1. Separation of two forms of acid lipase by decyl-agarose chromatography of fibroblast secretions. Secretions were applied to a column (2.5 × 30 cm) of decyl-agarose equilibrated at 4°C with buffer, pH 7.0, which contained 0.01 M sodium phosphate, 0.15 M NaCl, 1 mM EDTA, 0.02% NaN_3, and 10 mM 2-mercaptoethanol. The column was washed and eluted with a gradient of cholic acid in the same buffer. Fractions of approximately 7 ml were collected. Lipase activity was assayed by monitoring the rate of 4-methylumbelliferyl oleate hydrolysis. The cholate content of fractions was determined by comparing the absorbance at 397 nm of a 5-μl aliquot in 1.5 ml of concentrated sulfuric acid to that of a cholate standard. Cell secretions were collected for 3 days in serum-free Waymouth medium supplemented with 15 mM NH_4Cl: A, secretions from normal fibroblasts (GM 1603), twelve 490-cm^2 roller bottles; B, secretions from Wolman's disease fibroblasts (GM 1606), four 490-cm^2 roller bottles.

timal activity was detectable at pH 7–8 (data not shown). The specific activity of lipase in this preparation was approximately 5 times that in crude secretions and 50 times that in sonicates of normal fibroblasts.

Time course of uptake

Uptake of lipase by Wolman's disease fibroblasts was time-dependent and, under the conditions described in **Fig. 2,** was complete in 6 hr. For kinetic studies of uptake which required initial velocity measurements, the shortest incubation period compatible with adequate sensitivity of assays (about 2 hr) was chosen. In contrast to results at 37°C, where uptake measurements were routinely made, acid lipase was not significantly internalized at 0–5°C (data not shown). Preliminary results from our laboratory indicate that the enzyme binds to fibroblasts in the lower temperature range.

Saturation of uptake

As the concentration of acid lipase applied to cells were increased, the rate of uptake approached a maximum

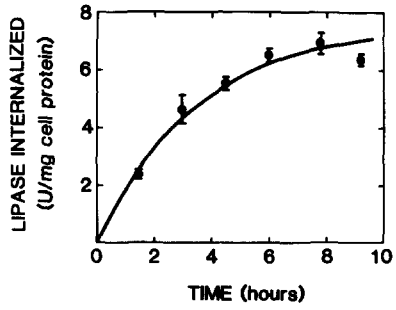

Fig. 2. Time course of lipase uptake. Acid lipase (the major form from decyl-agarose chromatography of normal fibroblast secretions, as in Fig. 1A) was dialyzed in buffer, pH 6, that contained 0.01 M sodium phosphate, 0.15 M NaCl, and 10 mM 2-mercaptoethanol. Enzyme (3 units/ml of Waymouth medium that contained 1 mg/ml of bovine serum albumin) was applied to Wolman's disease fibroblasts (GM 1606), grown as described in Methods. At the indicated times, cells were rinsed with 0.9% NaCl, harvested by trypsinization, and assayed for lipase activity and protein. The endogenous cellular lipase activity, normally about 1 unit/mg of cell protein, has been substracted. Results shown are means ± standard errors of triplicate uptake measurements.

(**Fig. 3**). This result, along with the high efficiency of uptake and the inhibition results discussed below, provides kinetic evidence that lipase is taken up by a receptor-mediated process (5). The uptake of lipase that had not been purified by decyl-agarose chromatography was saturable at a lower enzyme concentration and approached a lower maximum velocity than did that of purified enzyme (K_{uptake}, units per ml, of crude versus purified = 0.8 ± 0.9, 3.3 ± 0.5, respectively; V_{max}, units per mg cell protein per hr, of crude versus purified = 0.19 ± 0.05, 2.3 ± 0.1, respectively). An inhibitor of lipase uptake could be demonstrated in a macromolecular concentrate from the wash fraction of the decyl-agarose column.

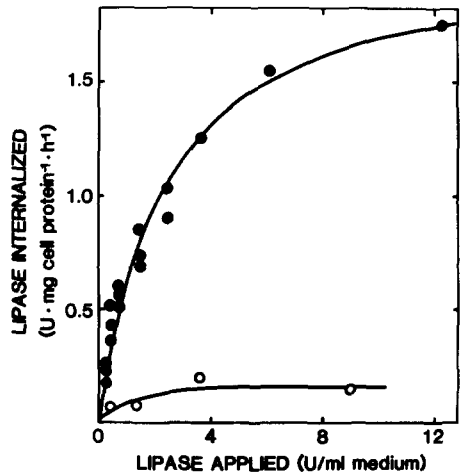

Fig. 3. Saturation of lipase uptake. Lipase uptake by Wolman's disease fibroblasts was measured as in Fig. 2. Enzyme in crude secretions from normal fibroblasts (open circles), or a preparation partially purified by decyl-agarose chromatography (closed circles) was applied to cells for 4.5 hr. Kinetic constants were obtained as described previously (5) from a direct least squares fit of the data to the equation: $v = VE/(K_{uptake} + E)$.

Inhibitors of lipase uptake

In addition to the endogenous macromolecular inhibitor, specific sugar phosphates also blocked lipase uptake (**Table 2**). Mannose 6-phosphate and fructose 1-phosphate were effective at approximately the same concentrations found to inhibit glycosidase uptake by fibroblasts (4–7). Other sugar phosphates were not inhibitory when tested at similar concentrations. Inhibition by mannose 6-phosphate was competitive, with a value for K_i of 5.0×10^{-5}M (**Fig. 4**). The use of a more highly purified lipase preparation in this study resulted in increased values for both K_{uptake} (4.1 ± 0.3 units per ml) and V_{max} (5.7 ± 0.2 units per mg cell protein per hr), which is consistent with the further removal of inhibitors of lipase uptake. Ammonium chloride and chloroquine blocked lipase uptake (Table 2) at approximately the same concentrations that inhibited the uptake of a lysosomal glycosidase, L-iduronidase, by fibroblasts (5). A "high uptake" form of the human urinary L-iduronidase, which shares many of the uptake properties described here for acid lipase, inhibited the lipase uptake rate approximately 50% when it was included in the assay at a concentration equivalent to its K_{uptake} (5) and when lipase was present at 1 unit per ml of medium. This result is consistent with the idea that the two hydrolases compete for the same cellular recognition site, although linear competitive inhibition kinetics were not rigorously demonstrated in this case.

TABLE 2. Inhibition of lipase uptake by sugar phosphates and amines[a]

Substance	Concentration	Rate of Lipase Uptake[b]
	mM	*per cent of control*
Sugar phosphate		
Mannose-6-P	0.05	32, 42
	0.5	2, 4
Fructose-1-P	0.05	68, 70
	0.5	29, 35
Mannose-1-P	0.05	96, 104
	0.5	103, 107
Fructose-6-P	0.05	90, 93
	0.5	85, 89
Glucose-6-P	0.05	66, 86
	0.5	92, 96
Glucose-1-P	0.05	101, 109
	0.5	99, 109
Galactose-6-P	0.05	70, 126
	0.5	83, 93
Amine		
Ammonium chloride	10	20, 30
	50	0, 4
Chloroquine	0.01	17, 25
	0.05	0, 4

[a] Acid lipase uptake was assayed as was described in Fig. 2. Decyl-agarose-purified enzyme (1 U/ml of medium) and potential inhibitors were applied to Wolman's disease fibroblasts (GM 1606) for 2.5 hr.
[b] Values from duplicate dishes are given. The rate of uptake by control cells was 0.63 units per mg of cell protein per hr.

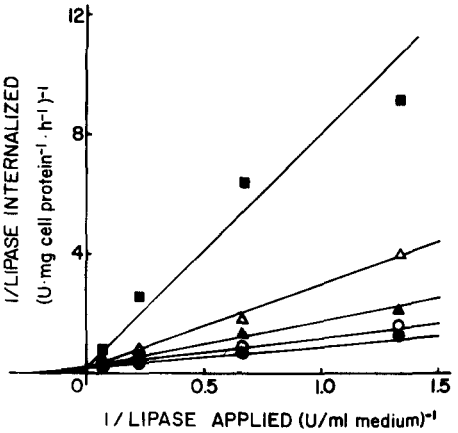

Fig. 4. Inhibition of lipase uptake by mannose 6-phosphate. Uptake measurements were made as described in Fig. 2. For this study, lipase from the decyl-agarose step was purified approximately 5-fold by gel filtration using a column (2.5 × 90 cm) of Sephacryl S-200 equilibrated with buffer A which was modified by the addition of 10% glycerol, 0.05% Triton X-100, and 5 mM cholic acid. Bovine serum albumin (2mg/ml) was added to the lipase preparation when it was dialyzed prior to uptake, as in Fig. 2. Lipase was applied for 2 hr to Wolman's disease fibroblasts at concentrations of 0.75, 1.5, 4.5, and 15 units/ml of medium, and mannose 6-phosphate, at concentrations of 0 (●), 20 (○), 60 (▲), 150 (△), and 500 μM (■). Positions of the lines and kinetic constants were determined, as described previously (5), from a direct fit of the data to the equation for competitive inhibition: $v = VE/[K_{uptake}(1 + i/K_i) + E]$.

Destruction of lipase uptake by phosphatase

Pretreatment of acid lipase with alkaline phosphatase under conditions in which greater than 90% of the lipase

TABLE 3. Destruction of acid lipase uptake by phosphatase[a]

Lipase Applied	Inorganic Phosphate in Preincubation	Lipase Internalized[b]
units/ml of medium		*units/ml cell protein/hr*
0.47	+	0.25, 0.27
1.2	+	0.46, 0.70
3.5	+	0.81, 0.99
0.42	−	0
1.1	−	0, 0.10
3.2	−	0.02, 0.10

[a] To a sample of acid lipase (100 units/2 ml of 0.05 M Tris-Cl buffer, pH 8.0) which had been partially purified from fibroblast secretions by decyl-agarose chromatography was added 3.6 units of *E. coli* alkaline phosphatase. Half of the sample was dialyzed for 18 hr at 6°C in 1 liter (with one bath change) of 0.05 M Tris-Cl buffer, pH 8.0, containing 1 mM $MgCl_2$, and the other half, in this buffer to which 0.01 M sodium phosphate had been added to inhibit phosphatase activity. Dialysis was continued at 25°C for 6 hr with two additional bath changes. Samples were then dialyzed for 18 hr at 6°C in 0.01 M sodium phosphate buffer, pH 6.0, which contained 0.15 M NaCl and 10 mM 2-mercaptoethanol. Aliquots from each preincubation mixture were applied to Wolman's disease fibroblasts for 2 hr for analysis of lipase uptake.
[b] Values obtained from duplicate dishes are given.

catalytic activity was retained, abolished uptake (**Table 3**). Uptake was preserved when a phosphatase inhibitor, inorganic phosphate, was included in the preincubation mixture, making it unlikely that a contaminant of the preparation, rather than phosphatase itself destroyed the lipase recognition marker. In addition, pretreatment of the "high uptake" L-iduronidase with phosphatase diminished its ability to inhibit lipase uptake, supporting the notion that a phosphorylated component of the glycosidase mediated its inhibitory effect.

Uptake of lipase by other fibroblast lines

The mannose 6-phosphate inhibitable uptake of acid lipase by normal fibroblasts could be demonstrated, although the sensitivity of the assay was limited by a high endogenous lipase level. The rate (approximately 1 unit internalized per mg cell protein per hr) was approximately the same as that with Wolman cells at a comparable concentration of lipase applied in the medium (4 units per ml). The same uptake rate was observed in I-cell fibroblasts (approximately 25 and 50% of the applied lipase concentration was internalized per hr per mg of cell protein at concentrations of lipase in the medium equivalent to, and less than half of K_{uptake}, respectively).

Lifetime of internalized lipase

Following uptake, the activity of the lipase in Wolman cells decayed to the cell blank with a half-life of 1.0 ± 0.1 d (**Fig. 5**). A similar value was obtained in several experiments with two lines of Wolman fibroblasts and in the presence of 10% fetal bovine serum or 1 mM mannose 6-phosphate in the culture medium. However, treatment of cells with ammonium chloride after initial uptake of the lipase resulted in an increased intracellular lifetime of internalized enzyme activity (**Table 4**).

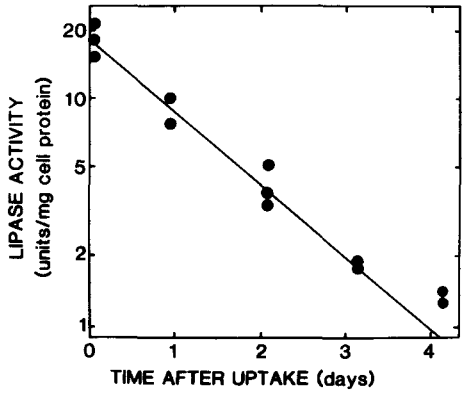

Fig. 5. Decay of internalized lipase activity. Partially purified acid lipase was applied (18 units/ml of medium) to Wolman's disease fibroblasts for 19 hr. At that time (day 0) cells were refed fresh serum-free Waymouth medium without enzyme, then sampled at the indicated times for lipase activity. The half-life value was obtained from a direct least squares fit of the data to the equation for first-order decay, $y = ae^{-kt}$ (5); computed values for the constants, k and a, were used to plot the line.

TABLE 4. Intracellular cholesteryl ester hydrolysis following uptake of acid lipase[a]

Treatment		Time After 5.5 hr		Cholesterol	
Lipase	NH_4Cl	Pulse	Internalized Lipase	Free	Esterified
		days	*units/mg cell protein*[b]	*nmol/mg cell protein*	
−	−	0	−	124 ± 7	59 ± 2
−	−	1.6	−	126 ± 16	56 ± 12
−	+	1.6	−	138[c]	54[c]
+	−	0	6.1 ± 0.9	127 ± 10	51 ± 6
+	−	1.6	1.2 ± 0.3	166 ± 4	3 ± 2
+	+	1.6	3.8 ± 0.5	145 ± 4	25 ± 3

[a] Skin fibroblasts derived from a patient with Wolman's disease (GM 1606) were grown in 35-mm dishes in MEM containing 10% fetal bovine serum for 3 days, and were then kept in serum-free Waymouth medium for 3 days. Cells were incubated for 5.5 hr with 1.25 ml of Waymouth medium ± acid lipase (7.5 units of decyl-agarose purified enzyme in 0.25 ml of buffer, pH 6.0, which contained 0.01 M sodium phosphate, 0.15 M sodium chloride, and 4.5 mg fatty acid-free bovine serum albumin). Samples from each group were harvested (0 time), and the pellets stored at −20°C. The remaining dishes were divided into two additional groups: cells previously incubated ± acid lipase were kept on Waymouth medium containing 3 mg/ml albumin, ±15 mM ammonium chloride for 1.6 days. Cells were analyzed for protein, acid lipase activity, and free and total cholesterol as described in Methods. Results are expressed as the mean ± standard error of values obtained from four dishes.

[b] The mean value (1.4 units/mg cell protein) for the endogenous lipase blank detected in cells that were not given enzyme was subtracted from the specific activity of those cells that received a 5.5 hr lipase pulse.

[c] Values obtained from one dish.

Effect of mannose 6-phosphate on intracellular lipase activity

The importance of an extracellular route for hydrolase packaging has been questioned in part by observations that intracellular levels of a number of glycosidases are not reduced in response to high concentrations of mannose 6-phosphate in the culture medium (25, 26). To examine this point with respect to acid lipase we applied mannose 6-phosphate in serum-free Waymouth medium to cultured skin fibroblasts (GM 1603) for 3 days, with renewal of the medium every 8–12 hr. The acid lipase activity (average of duplicate 35-mm dishes) of cells treated with 0, 0.3, 1.0, 3.0, and 10 mM mannose 6-phosphate was 11.6, 12.0, 10.2, 11.0, and 11.2 units per mg of cell protein, respectively. Thus, in agreement with the previous findings for glycosidases, prolonged treatment of fibroblasts with mannose 6-phosphate at concentrations greatly exceeding its K_i value with respect to lipase uptake, failed to substantially lower intracellular acid lipase activity.

Intracellular cholesteryl ester hydrolysis following lipase uptake

The abnormally high cholesteryl ester content of Wolman's disease fibroblasts was decreased following acid lipase uptake (Table 4). This was accompanied by a marked decrease in the abundant abnormal membrane-bound inclusions in these cells (2) observed by transmission electron microscopy (data not shown). Internalized enzyme was therefore functional in the cell. A small rise in cellular free cholesterol accompanied the decrease in esterified sterol, suggesting that lysosomal hydrolysis no longer limited the rate of cholesterol efflux. Addition of ammonium chloride to the medium once lipase was internalized interfered with cholesteryl ester clearance. This result was predicted from previous work which showed that exposure of cultured cells to weak bases inhibited lysosomal degradation of a variety of substances including proteins (27, 28), mucopolysaccharides (29, 30), and cholesteryl esters (31).

DISCUSSION

The importance of lysosomal acid lipase activity in cholesterol metabolism has been demonstrated in studies of cultured human fibroblasts genetically deficient with respect to the enzymes (16, 17, 32). The mutant cells accumulate intralysosomal cholesteryl esters, and a supply of free sterol from the lysosome is not available for the regulation of de novo cholesterol biosynthesis, receptor-mediated endocytosis of low density lipoprotein, and microsomal cholesterol esterification. Correction of these processes when lipase-deficient fibroblasts were co-cultured with nondeficient cells or were fed conditioned medium from them suggested that enzyme secreted from the normal cells was taken up and packaged into lysosomes by the deficient cells (16, 17). Our results strengthen that hypothesis, originally developed to explain metabolic correction in fibroblasts cultured from patients with mucopolysaccharide storage disorders (3). We have shown that normal fibroblasts secrete a form of acid lipase that is taken up by cultured cells with kinetics predicted for a receptor-mediated process (5). The internalized lipase catalyzes hydrolysis of lysosomal cholesteryl esters. Lipase uptake is destroyed by phosphatase treatment of the enzyme and is inhibited by mannose 6-phosphate in the medium, indicating that the fibroblast receptor interacts with a phosphomannosyl component of the enzyme (4–7). Taken with the findings that lipase uptake is inhibited by a lysosomal glycosidase, L-iduronidase, and that mannose 6-phosphate inhibits uptake of the lipase and a number of other hydrolases to the same extent, these results substantiate the view that the same receptor-mediated lysosomal packaging mechanism applies to a variety of acid hydrolases with diverse metabolic roles.

Our finding of decreased intracellular and increased extracellular acid lipase activity in cultures of I-cell fibroblasts is a further indication that the lipase shares with other hydrolases the requirement for a common recognition marker for lysosomal compartmentalization. Reduced intracellular acid acylhydrolase activity in tissues from I-cell patients has also been reported by Pittman et al. (33). The abnormal localization of several acid hydrolases in this inherited lysosomal storage disorder is thought to result from defective synthesis or attachment of the hydrolase phosphomannosyl recognition marker (13, 24). The ability of I-cells to take up normal hydrolases (23, 24) including the lipase, as noted above, is not impaired.

Besides providing information more directly applicable to the study of human disease, focusing on the human enzyme was advantageous in that genetically deficient human fibroblasts were available as a control for the various cellular forms of fatty acylhydrolase activity and for use as acceptors in the uptake assay. In addition, properties of the lipase uptake system can be more reliably compared with those of several other receptor-mediated uptake systems which have been characterized in human fibroblasts (34). Although human urine and platelets, in contrast to several other tissues, were good sources for a number of "high uptake" acid hydrolases (35), we found them to be poor sources of "high uptake" lipase. Secretions from cultured skin fibroblasts provided a substantial yield of "high uptake" lipase when the growth medium was supplemented with ammonium chloride. The rationale for this approach was that several amines inhibit the receptor-mediated uptake of acid hydrolases by fibroblasts (36, 37), and might therefore be expected to promote enzyme accumulation in the medium. Addition of ammonium chloride or chloroquine to the culture medium has recently been reported to stimulate the extracellular accumulation of newly synthesized fibroblast hydrolases (38–40). The mechanism of the amine effect on fibroblast lysosomal enzyme secretion and uptake is not known, although ammonium chloride and chloroquine have been suggested to interfere with receptor recycling, possibly through a pH-mediated inhibition of intracellular receptor-hydrolase dissociation (39).

Maximal rates of lipase uptake were observed once a nondialyzable inhibitory component (probably composed of other acid hydrolases that compete with lipase for binding to the phosphomannosyl receptor (5, 41)) was removed from crude cell secretions with decyl-agarose chromatography. The resulting five-fold purified lipase preparation was taken up by fibroblasts at approximately the same rate (based on the fraction of applied enzyme concentration internalized per hr per mg

cell protein) as that reported for purified "high uptake" human urinary L-iduronidase (5). Elution of the decyl-agarose column with a gradient of sodium cholate resolved fibroblast acid acylhydrolase activity, as measured with 4-methylumbelliferyl oleate substrate, into two forms. The predominant form from normal cells was deficient in Wolman's disease fibroblasts. Electrophoretically dissimilar forms of 4-methylumbelliferyl oleate hydrolase activity had previously been demonstrated in fibroblast (19) and leukocyte (42) extracts, and one of the forms was deficient in cells from patients with Wolman's disease or cholesteryl ester storage disease. We have obtained preliminary evidence that two forms are also separable by anion exchange gel chromatography. Differences in heat stability and substrate specificity of the acid acyl hydrolases have been indicated (19, 42, 43, and unpublished results from our laboratory). The lipase activity of Wolman's disease fibroblasts is relatively lower than that of normal cells when assayed with a cholesteryl oleate substrate (~2–10%) than when tested with 4-methylumbelliferyl oleate (~10–30%). The above results suggest that the 4-methylumbelliferyl oleate substrate is not specific for a single acid acylhydrolase and that the different forms may not be closely related structurally. However, a comparison of the biochemical, physical, and immunological properties of the purified, resolved enzymes is necessary to rule out the possibility that both forms arise from the same precursor or share a common structural component.

Following uptake, the acid lipase was capable of hydrolyzing cholesteryl esters that had accumulated in Wolman's disease fibroblasts, as was indicated from quantitative biochemical analysis (Table 4) and by lipid-staining properties of lipase-treated cells (data not shown). This uptake system thus holds promise as a model for studying the regulation of intracellular lipid hydrolysis and cholesterol efflux since the loading of cells with substrate, potential regulatory factors, and the acid lipase can be controlled. The reason for the lag in cholesteryl ester efflux during the period of enzyme application to the cells (Table 4) is not clear. A substantial amount of lipase activity (approximately 50% of that in normal fibroblasts) had accumulated intracellularly during that 5.5-hr period, yet little cholesteryl ester had apparently been hydrolyzed. This time period may have been required to distribute enzyme to compartmentalized substrate. Alternatively, the stored lipid may have been in a physical form that was not readily hydrolyzed by the enzyme. It is also possible that the cholesteryl ester content after 5.5 hr incubation with lipase represents, in part, cholesterol that had been liberated from the lysosome following hydrolysis and was then reesterified by the microsomal acyl-CoA:cholesterol acyltransferase. In that case, the cholesteryl ester that was cleared in the presence of ammonium chloride might represent extralysosomal material formed prior to application of the amine, since steps in the pathway of cholesterol efflux subsequent to lysosomal hydrolysis are not inhibited by a lysosomotropic amine (32). Studies are in progress to discriminate among these possibilities.

Although interaction of a number of acid hydrolases with a fibroblast phosphomannosyl receptor has been demonstrated and is generally accepted as a requirement for their efficient lysosomal compartmentalization, the route by which hydrolases reach the lysosome has not been firmly established. One argument against a model in which hydrolases must be secreted from the cell prior to packaging via endocytosis of enzyme bound to a cell surface receptor (36) is that treatment of fibroblasts with substances which interfere with hydrolase binding and uptake did not substantially reduce intracellular levels of several lysosomal glycosidases (25, 26). Addition of high concentrations of mannose 6-phosphate to the culture medium also failed to lower cellular lipase activity, suggesting that under the culture conditions described, the extracellular route does not have a major role in lysosomal packaging of newly synthesized acid lipase. The validity of this argument could be questioned if intracellular hydrolase lifetime were long compared to the time of exposure of cells to the sugar phosphate. This is not likely in the case of the lipase since enzyme internalized by Wolman's disease fibroblasts had a relatively short half-life, which was not altered by mannose 6-phosphate in the medium.

Findings of increased lysosomal lipid storage and altered arterial acid hydrolase activity in conjunction with atherosclerosis and related risk factors have focused attention on the involvement of lysosomes in vascular disease (44, 45). The key role of acid lipase in cholesterol metabolism led to the suggestion that modulation of its activity may be an important factor in atherogenesis (46–48). The ability to relate lysosomal function to disease processes requires an understanding of how intra- and extracellular acid hydrolase activity is regulated. Our studies of acid lipase transport and function in the cultured human fibroblast system provide a model that can be extended to the investigation of lipase regulation in vascular tissue cells, which are relevant to the pathogenesis of atherosclerosis.

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