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Detection and Characterization of Carrier-mediated Cationic Amino Acid Transport in Lysosomes of Normal and Cystinotic Human Fibroblasts

ROLE IN THERAPEUTIC CYSTINE REMOVAL?*

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The discovery of a *trans*-stimulation property associated with lysine exodus from lysosomes of human fibroblasts has enabled us to characterize a system mediating the transport of cationic amino acids across the lysosomal membrane of human fibroblasts. The cationic amino acids arginine, lysine, ornithine, diaminobutyrate, histidine, 2-aminoethylcysteine, and the mixed disulfide of cysteine and cysteamine all caused *trans*-stimulation of the exodus of radiolabeled lysine from the lysosomal fraction of human fibroblasts at pH 6.5. In contrast, neutral and acidic amino acids did not affect the rate of lysine exodus. *trans*-Stimulation of lysine exodus was observed over the pH range from 5.5 to 7.6, was specific for the L-isomer of the cationic amino acid, and was intolerant to methylation of the α-amino group of the amino acid. The lysosomotropic amine, chloroquine, greatly retarded lysine exodus, whereas the presence of sodium ion was without effect. The specificity and lack of Na^+ dependence of this lysosomal transport system is similar to that of System y^+ present on the plasma membrane of human fibroblasts. In addition, we find cystine exodus from the lysosomal fraction of cystinotic human fibroblasts to be greatly retarded as compared to that of normal human fibroblasts with half-times of exodus similar to those reported for the lysosomes of cystinotic and normal human leukocytes (Gahl, W. A., Tietze, F., Bashan, N., Steinherz, R., and Schulman, J. D. (1982) *J. Biol. Chem.* 257, 9570–9575). In contrast, normal and cystinotic human fibroblasts did not show any differences with regard to lysine efflux or its *trans*-stimulation by cationic amino acids. An important mechanism by which cysteamine treatment of cystinosis allows cystine escape from lysosomes may be the ability of the mixed disulfide of cysteine and cysteamine formed by sulfhydryl-disulfide exchange to migrate by this newly discovered system mediating cationic amino acid transport.

A question of biological importance is the manner in which amino acids formed by proteolysis within the lysosomal compartment of eukaryotic cells escape into the cytoplasm of the cell. The importance of this process is accentuated in the genetic disease, nephropathic cystinosis, in which the amino acid cystine accumulates in the lysosomes of affected persons. This accumulation results in crystal deposition in various body tissues and organs and gives rise to the appearance of renal tubular characteristics of the Fanconi syndrome with progressive renal glomerular damage leading to end-stage kidney failure (1). The demonstration by Gahl *et al.* (2–4) with human leukocytes and Jonas *et al.* (5) with human lymphoblasts that a lysosomal carrier-mediated system for transporting the amino acid cystine was defective in cystinosis suggests that specific systems for catalyzing the transport of other amino acids across the lysosomal membrane of eukaryotic cells may be found. In this report, we present evidence for a transport system in the lysosomes of human fibroblasts which has a specificity for cationic amino acids at least superficially similar to that of System y^+ present in many eukaryotic plasma membranes. This system is not defective in the lysosomes of cystinotic fibroblasts and appears to provide a route by which the therapeutic agent, cysteamine, may lower lysosomal cystine accumulations in cystinosis by allowing its escape in the form of the mixed disulfide of cysteine and cysteamine, which we find transported by the new system. Several of the amino acids transported by this system have molecular masses considerably smaller than 230 Da, indicating that aside from their largely mediated passage, lysosomal membranes are rather impermeable to small molecular weight amino acids.

EXPERIMENTAL PROCEDURES

Cell Culture and Preparation of Lysosomal-enriched Fraction—Normal (GM 0010) and cystinotic (GM 0090A) human fetal fibroblast cell lines were obtained from the Human Genetic Mutant Cell Repository. Fibroblasts were grown and maintained on 100-mm tissue culture dishes in Coon's modification of Ham's F-12 medium (GIBCO) supplemented with 10% fetal bovine serum as previously described (6).

Granular fractions enriched in lysosomes were prepared from human skin fibroblasts by the method of Harms *et al.* (7), proceeding only far enough to yield their fraction C. Two exceptions were made: first the buffer for cell lysis, 50 mM MOPS,[1] 0.25 M sucrose, was adjusted to pH 7.6 rather than 7.4, with Tris-free base (this buffer solution is designated MST); second, fraction B was not passed through filter paper. The MST also contained 1 mM disodium EDTA.

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[1] The abbreviations used are: MOPS, 3-(*N*-morpholino)propanesulfonic acid; MST, 50 mM MOPS in 0.25 M sucrose solution adjusted to the indicated pH with the free base, Tris; PBS, 10 mM sodium phosphate pH 7.4 buffer containing 0.154 M NaCl; EDTA, (ethylenedinitrilo)tetraacetate.

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The granular pellet was then usually resuspended in several volumes of MST, again at pH 7.6, for loading of radioactive amino acid methyl esters. Membrane integrity was conserved to the extent of 85% in most granular preparations as judged by the latency of hexosaminidase activity, which was determined by the difference in hexosaminidase activity in the presence or absence of 0.1% Triton.

Amino Acid Methyl Ester Uptake—Loading of human fibroblast granular fractions with L-[^{14}C]lysine methyl ester was performed by first taking an aliquot of a 250 μM stock solution of L-[^{14}C]lysine methyl ester (330 mCi/mmol) in methanol to complete dryness under a stream of N_2, redissolving the residue in 40 μl of MST, pH 7.6, and then immediately adding the solution to 140 μl of a fresh suspension of the fibroblast granular pellet in buffer, to give a final concentration of 50–80 μM L-[^{14}C]lysine methyl ester in the suspension. The suspension was incubated at 25 °C for 25 min, then diluted to 1.5 ml with ice-cold MST at pH 6.5 and centrifuged at 20,000 × *g* for 10 min at 4 °C. The supernatant was discarded and the pellet, resuspended in 1.5 ml of ice-cold buffer, was centrifuged once more and the resulting supernatant again discarded. The L-[^{14}C]lysine-loaded granular pellet was resuspended in ice-cold buffer and kept on ice until used in exodus experiments. In experiments in which the time course of loading was followed, 20 μl of the suspension were removed at timed intervals during the 25 °C incubation period and added to 10 ml of ice-cold PBS and filtered as described below for measurement of exodus. A 20-μl aliquot was also removed to which was added 20 μl of 1% Triton to serve as a blank in these experiments. [^{35}S]Cystine dimethyl ester was loaded into lysosomes in the manner described above with the exceptions that the loading and wash buffers were both at pH 7.0 and the incubation was extended to 45 min at 37 °C.

Amino Acid Exodus—Exodus of L-[^{14}C]lysine from lysosomes was measured by adding 30-μl aliquots of ice-cold L-[^{14}C]lysine-loaded lysosomal suspension to 970 μl of MST at pH 6.5 or to the same solution containing 2 mM amino acid, in both cases already at 25 °C. The suspension was incubated at 25 °C and 90-μl aliquots, removed at 3-min intervals, were added to tubes containing 10 ml of ice-cold PBS and filtered through a 24-mm Whatman GF/A filter. The filter was subsequently washed twice with 10-ml portions of ice-cold PBS, care being taken to minimize exposure of the filters to the air during filtration as suggested by Reeves and Reames (8). The filters were dried and soaked in 10 ml of scintillation fluid for 4 h prior to counting. From each incubation mixture, a 90-μl aliquot was removed, mixed with 20 μl of 1% Triton solution, and filtered as above to serve as a blank.

Exodus of L-[^{35}S]cystine from lysosomes was studied by adding 100 μl of ice-cold L-[^{35}S]cystine-loaded lysosomal suspension to 720 μl of MST pH 7.0 buffer prewarmed to 37 °C, and incubating the suspension to 37 °C. A series of duplicate 80-μl aliquots, removed at timed intervals, were added to microcentrifuge tubes containing 1.4 ml of ice-cold buffer and centrifuged at 15,600 × *g* with a Model 5414 Eppendorf centrifuge for 10 min at 4 °C. The supernatant was discarded, and pellets, resuspended in 1.5 ml of ice-cold buffer, were resedimented; the resulting supernatant again was discarded, and the pellet was resuspended in 80 μl of 10 mM sodium phosphate buffer, pH 7.0, containing 10 mM *N*-ethylmaleimide. The suspension was frozen and thawed in sequence three times, a 20-μl aliquot was removed for assay of hexosaminidase activity, and then one-tenth of a volume of 40% sulfosalicylic acid added to the remaining contents of each tube. The tubes were placed at 4 °C for 30 min and centrifuged at 15,600 × *g* for 7 min at 4 °C. A 40-μl aliquot from each sulfosalicylic supernatant was spotted on paper along with 30 nmol each of L-cystine and L-cysteine-NEM (*N*-ethylmaleimide derivative of L-cysteine prepared by reacting cysteine with a 2-fold molar excess of *N*-ethylmaleimide in 10 mM phosphate buffer, pH 7.0) to serve as internal standards. The paper was subjected to high voltage electrophoresis in 6% formic acid at 3500 V for 25 min and the radioactive cystine spots were cut out and counted. The experiments involving cystine exodus were performed at a higher temperature than those for lysine exodus because the system mediating exodus of cystine from the fibroblast lysosomes was observed to be much slower than that for exodus of lysine.

Calculation of Half-times of Exodus—Blank values were subtracted from the radioactivity for each time point (typically about 5% of the total) and the logarithm of the radioactive cpm/unit of hexosaminidase activity was plotted on the *y* axis *versus* time of exodus. The slope and *y* intercept of the line were determined by a BASIC computer program for linear regression analysis on a Radio Shack TRS-80 Model I microcomputer and the half-time of exodus was obtained by dividing 0.301 by the slope of the line.

Miscellaneous Assays—Radiolabeled amino acid methyl esters were synthesized according to Steinherz *et al.* (9) with a purity of at least 98% in most preparations as determined by high voltage electrophoresis.

β-Hexosaminidase activity was assayed by a modifiction of the method of Hall (10) in which 15 μl of a given sample were added to 100 μl of 4 mM *p*-nitrophenyl-β-D-*N*-acetylglucosaminide in 0.043 M citric acid containing 0.11 M Na_2HPO_4 pH 4.90 buffer. Incubations were performed at 37 °C for periods of up to 15 min and terminated by adding 350 μl of 0.8 M glycine/NaOH pH 10.4 buffer. The absorbance at 400 nm was read, and a unit of hexosaminidase activity was defined as 1 nmol of *p*-nitrophenol formed per min at 37 °C.

Protein was measured by a modified Lowry procedure described by Zak and Cohen (11), with sodium dodecyl sulfate having been added as suggested by Wang and Smith (12).

The mixed disulfide of cysteine and cysteamine was prepared by first forming cystine disulfoxide as described by Emiliozzi and Pichat (13); 0.45 g of the dried cystine disulfoxide was mixed with 0.10 g of cysteamine in 10 ml of 0.1 M HCl containing 0.01 M formic acid according to the procedure of Eriksson and Eriksson (14). The suspension was stirred for 2.5 h at room temperature, filtered through Whatman No. 1 filter paper, and then washed four times with distilled H_2O. The filtrate was then applied to a Dowex 50W-X8 (H^+ form) 100–200 mesh column equilibrated in 0.01 M formic acid with an effluent pH of 2.85 and a column volume of 13 ml. Following sample application, the column was washed with 50 ml of 0.01 M formic acid followed by 350 ml of 10% pyridine, and the mixed disulfide was then eluted with 1 liter of 0.1 M aqueous ammonia. The ammonia was removed by rotoevaporation under vacuum. The cysteinyl/cysteamine mixed disulfide so obtained migrated as a single spot on high voltage electrophoresis in 6% formic acid with an R_F of 1.73 relative to that of cystine, in good agreement with the value reported by Jonas and Schneider (15).

Materials—Unlabeled amino acids and amino acid methyl esters were obtained from Sigma. The labeled amino acids, L-[^{14}C]lysine HCl (330 mCi/mmol) and L-[^{35}S]cystine (278 mCi/mmol) were purchased from Amersham.

RESULTS

Initial studies by Goldman and Kaplan (16), and later by Reeves (17), indicated that amino acids can be specifically trapped within the lysosomes of crude granular fraction by prior incubation with the corresponding amino acid methyl esters. Lysosomes, as compared to other cellular organelles, contain high levels of esterase activity, enabling the conversion of the various amino acid methyl esters to amino acids, which then escape from the lysosomes much more slowly than their methyl ester derivatives. L-[^{14}C]Lysine was loaded into lysosomes of the granular fraction from human fibroblasts by incubating the granular fraction with L-[^{14}C]lysine methyl ester. Nearly complete conversion of L-[^{14}C]lysine methyl ester to L-[^{14}C]lysine occurred when loading the human fibroblast lysosome (Fig. 1, *A* and *B*). When loaded with 80 μM L-[^{14}C]lysine methyl ester, the amount of L-[^{14}C]lysine accumulated increased with time until a steady state was reached in approximately 25 min at 25 °C (Fig. 2). The degree of radioactive loading obtained was directly related to the concentration of L-[^{14}C]lysine methyl ester up to a concentration of 2 mM L-lysine methyl ester (Fig. 3). The system showed evidence of saturation when loading concentrations exceeded 10 mM L-lysine methyl ester. A nearly identical concentration curve was obtained when human fibroblast granular fractions were loaded instead with L-[^{3}H]methionine methyl ester (data not shown). The degree of loading increased as the pH of the loading buffer was raised over the pH range from 6 to 9 (data not shown) in agreement with Goldman and Kaplan (16), who have suggested that it is the uncharged form of the amino

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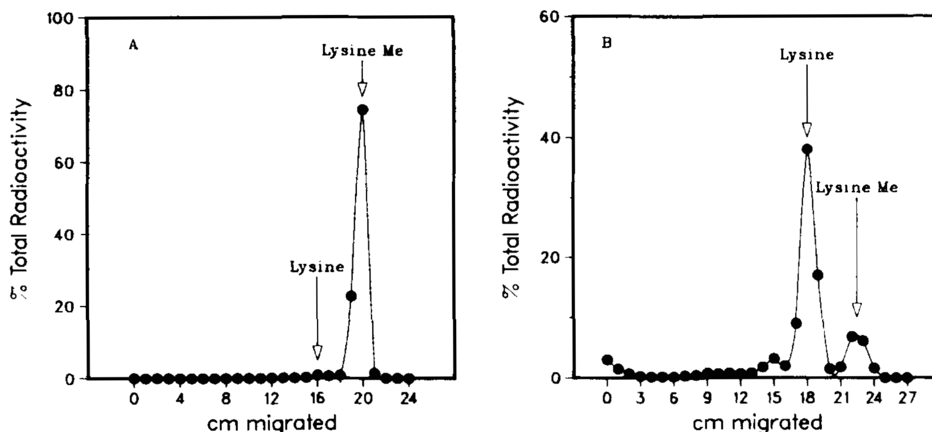

FIG. 1. **High voltage electrophoresis of L-[^{14}C]lysine methyl ester preparation (*A*) and radioactivity accumulated by fibroblast lysosomal fraction (*B*).** *A*, an aliquot of a L-[^{14}C]lysine methyl ester preparation was spotted on paper along with 30 nmol each of lysine and lysine methyl ester standard and subjected to high voltage electrophoresis. Strips of 1 cm each were counted for ^{14}C and the positions of standards (*arrows*) were visualized with a ninhydrin spray. *B*, lysosomal fraction was incubated at 25 °C in MST pH 7.6 buffer containing 0.048 mM L-[^{14}C]lysine methyl ester. After 30 min, the contents of the tube were diluted 50-fold with ice-cold buffer and centrifuged in an Eppendorf Microfuge for 12 min at 4 °C. The resulting pellet was suspended in 1.5 ml of ice-cold buffer and sedimented by centrifugation in the Eppendorf Microfuge for 12 min at 4 °C. The supernatant was discarded and the pellet resuspended to 20 μl in buffer containing 0.5% Triton. Sulfosalicylic acid was added to give a final concentration of 4%, followed by centrifugation in the Eppendorf Microfuge for 3 min. An aliquot of the supernatant was spotted for high voltage electrophoresis along with standards as indicated above. A control of L-[^{14}C]lysine methyl ester incubated with buffer but not lysosomes did not show any appreciable hydrolysis of the methyl ester. *Me*, methyl.

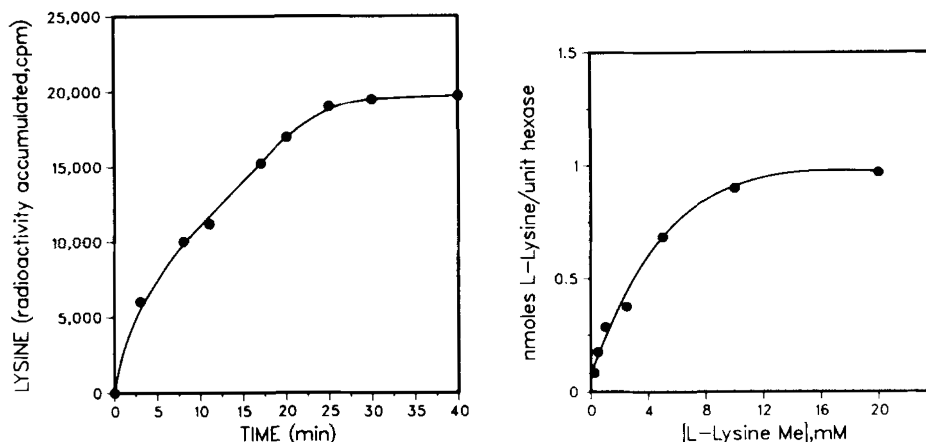

FIG. 2 (*left*). **Time course of the accumulation of L-[^{14}C]lysine from loading radiolabeled lysine methyl ester into lysosomal fraction isolated from human fibroblasts.** The lysosomal fraction was isolated from human fibroblasts and incubated at 25 °C with L-[^{14}C]lysine methyl ester to give a final concentration of 0.048 mM in a final volume of 0.2 ml. Aliquots were removed at the indicated time points and filtered through a GF/A filter to determine the amount of accumulated radioactivity as described under "Experimental Procedures."

FIG. 3 (*right*). **Lysine accumulation by fibroblast lysosomal preparations as a function of the L-[^{14}C] lysine methyl ester concentration during loading.** The fibroblast lysosomal preparation was incubated for 25 min at 15 °C in 0.025 ml of MST pH 7.6 buffer containing L-[^{14}C]lysine methyl ester at the concentrations indicated in the range from 0.25 to 20 mM. Incubation was terminated by the addition of 75 μl of ice-cold MST buffer from which aliquots were removed for determination of accumulated radioactivity by filtration through a GF/A filter and assay of latent hexosaminidase activity as described under "Experimental Procedures." *Me*, methyl.

acid methyl ester which is able to diffuse passively across the lysosomal membrane.

Exodus of L-[^{14}C]lysine from loaded human fibroblast lysosomes was measured as described under "Experimental Procedures" and the half-time of exodus determined from a linear regression analysis of a semi-logarithmic plot of the cpm of L-[^{14}C]lysine/unit of hexosaminidase remaining in the lysosomes as a function of time. A representative linear semi-logarithmic plot for the exodus of L-[^{14}C]lysine from loaded human fibroblast lysosomes at 25 °C and pH 6.50 is shown in Fig. 4 with a half-time for L-[^{14}C]lysine efflux of 26 min. No significant differences in the half-times for efflux were seen when lysosomes were loaded with various concentrations of L-[^{14}C]lysine methyl ester over the range from 20 to 350 μM, or when exodus was performed in the presence or absence of sodium ion (data not shown). Efflux of L-[^{14}C]lysine from loaded lysosomes, however, was observed to be highly temperature-dependent, giving a linear Arrhenius plot with an E_a = 17 kcal/mol and a Q^{10} of 2.2 (Fig. 5). Half-time values of exodus for duplicate conditions within an experiment from

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the same lysosomal preparation typically showed a standard deviation of less than 5%. Approximately twice this degree of variability, however, was observed when comparing half-time values obtained in one experiment with those obtained in a different experiment, presumably due to differences between lysosomal preparations.

Efflux of L-[^{14}C]lysine from loaded lysosomes was measured in the presence and absence of external 2 mM L-lysine to determine if exodus was subject to *trans* effects, a property shown by most carrier-mediated transport systems, including System y^+ of plasma membrane. A large *trans*-acceleration of L-[^{14}C]lysine from loaded human fibroblast lysosomes was observed in the presence of external lysine (Fig. 4), an effect reducing the half-time of efflux from 26 min in the absence of external L-lysine to 13 min in the presence of external 2 mM L-lysine. This *trans*-acceleration effect depended on the

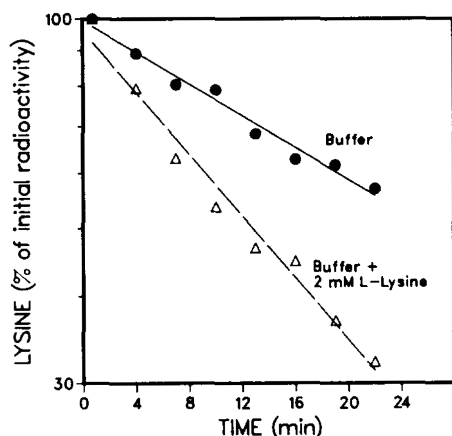

FIG. 4. **Exodus of L-[^{14}C]lysine from human fibroblast lysosomes in the absence or presence of external L-lysine.** Lysosomes from normal human fibroblasts were isolated and loaded with L-[^{14}C]lysine methyl ester as described under "Experimental Procedures;" 30-μl aliquots of the lysine-loaded lysosomal preparation were added to 970 μl of either MST pH 6.5 buffer or MST pH 6.5 buffer containing 2 mM L-lysine and L-[^{14}C]lysine exodus was then measured at 25 °C at 3-min intervals.

concentration of external L-lysine (Fig. 6) and appeared maximal at approximately 2 mM L-lysine. Efflux of L-[^{14}C]lysine from loaded human fibroblast lysosomes was pH-dependent, increasing as the pH became more alkaline over the pH range from 5.5 to 7.6 (Fig. 7). *trans*-Acceleration of L-[^{14}C]lysine exodus from human fibroblast lysosomes by external 2 mM L-lysine, however, was observed over this entire pH range. Various amino acids and amino acid analogs at a concentration of 2 mM (Table I) were tested for their ability to cause *trans*-acceleration of L-[^{14}C]lysine from human fibroblast lysosomes at pH 6.50 and 25 °C. The results indicate that all cationic amino acids tested (L-lysine, L-ornithine, L-arginine, 2-aminoethyl-L-cysteine, and diaminobutyrate) produced a significant *trans*-stimulation of L-[^{14}C]lysine exodus from human fibroblast lysosomes. L-Histidine, which has a pK′ of 6.04 for its imidazole side chain, caused a moderate *trans*-stimulation. At pH 6.50, 26% of L-histidine should be present as a cationic amino acid. In further support of shared transport, when lysosomes were loaded with L-[^{14}C]histidine, a nearly 2-fold *trans*-stimulation of histidine exodus was observed in the presence of external cationic amino acids (data not shown). None of the neutral or anionic amino acids tested had any effect on the efflux of L-[^{14}C]lysine from human fibroblast lysosomes. The D-isomer of lysine produced only a marginal *trans* effect in comparison with the L-isomer of lysine. In addition, *N*-methylation of the α-amino group of L-lysine resulted in a large reduction in its ability to produce *trans*-stimulation of L-[^{14}C]lysine efflux from human fibroblast lysosomes. Minor amounts of L-lysine as an impurity in the D-lysine and α-*N*-methyl-L-lysine preparations could be responsible for the marginal *trans*-stimulation effects observed with these latter two compounds (Table I) (18). Cystine at a concentration of 0.5 mM had no effect on L-[^{14}C]lysine efflux in a test in which 0.5 mM L-lysine produced a significant *trans*-stimulation of lysine efflux.

Some substances are selectively accumulated by lysosomes due to the unique properties and functions of this organelle, such as the pH gradient it maintains between the lysosomal interior and either the normal extralysosomal environment, the cytoplasm, or an experimental medium. One such lyso-

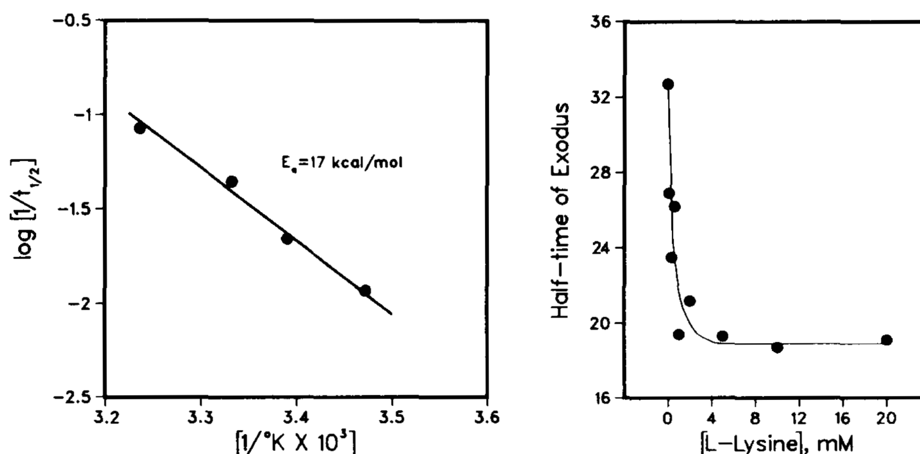

FIG. 5 (*left*). **Arrhenius plot of lysine exodus from fibroblast lysosomal preparations.** Exodus of L-[^{14}C]lysine from a lysine-loaded lysosomal preparation was measured in MST pH 6.5 buffer at 15, 22, 28.5, and 36 °C. Values for the half-time of exodus at each temperature were used in the Arrhenius plot to derive the relationship between the respective rate constants of efflux at each temperature.

FIG. 6 (*right*). **Dependence of *trans*-stimulation of L-[^{14}C]lysine exodus from human fibroblast lysosomal preparations on the concentration of external L-lysine.** Exodus of L-[^{14}C]lysine from lysine-loaded lysosomes was measured at intervals of 3 min at 25 °C in MST pH 6.5 buffer containing unlabeled L-lysine at the concentrations indicated in the range from 0 to 20 mM. Values for the half-time of exodus were determined by a linear regression analysis of a semi-logarithmic plot as described under "Experimental Procedures."

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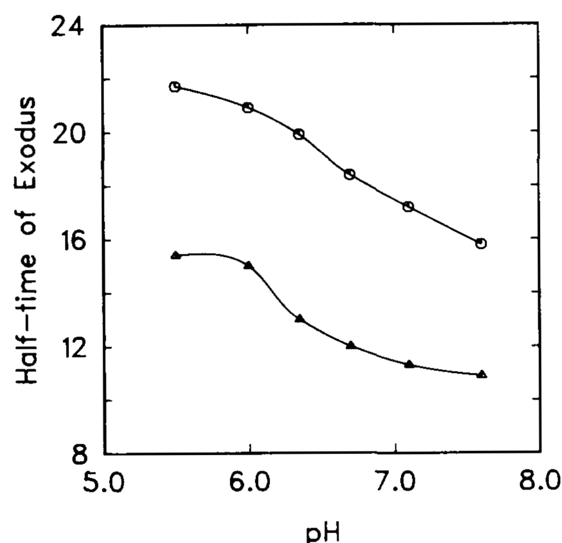

FIG. 7. **pH profile of the lysine exodus from human fibroblast lysosomal preparations in the presence or absence of external 2 mM L-lysine.** Thirty microliter aliquots of L-[^{14}C]lysine-loaded lysosomal preparation were added to 970 μl of either MST buffer (○) or the same buffer containing 2 mM L-lysine (△) at the indicated pH and L-[^{14}C]lysine exodus was then measured at 25 °C at 3-min time intervals. Half-time values of exodus were determined by a linear regression analysis of semi-logarithmic plots.

TABLE I

trans-Stimulation of L-[^{14}C]lysine exodus from normal human fibroblast lysosomal preparations by external amino acids at 25 °C and pH 6.5

Isolated lysosomal fraction from normal human fibroblasts was loaded with 0.065 mM L-[^{14}C]lysine methyl ester as described under "Experimental Procedures." Exodus of L-[^{14}C]lysine from lysosomes was measured at intervals of 3 min at 25 °C in MST pH 6.5 buffer containing the indicated amino acid at the concentration shown in parentheses. Half-time values were determined by a linear regression analysis of the semi-logarithmic plots as described under "Experimental Procedures." Experiments A and B, shown below, were performed with different lysosomal preparations.

Compound	$t_{1/2}$
Experiment A	
Buffer (50 mM MOPS, 0.25 M sucrose, pH 6.5)	21.5
L-Phenylalanine (2 mM)	24.9
D-Lysine (2 mM)	19.6
α-*N*-Methyl-L-lysine (2 mM)	18.7
L-Cystine (0.5 mM)	22.2
L-Lysine (0.5 mM)	15.4
L-Lysine (2 mM)	11.5
L-Arginine (2 mM)	12.2
L-Ornithine (2 mM)	12.7
2-Aminoethyl-L-cysteine (2 mM)	12.1
L-Diaminobutyrate (2 mM)	14.2
L-Histidine (2 mM)	16.8
Experiment B	
Buffer (50 mM MOPS, 0.25 M sucrose, pH 6.5)	25.6
L-Aspartate (2 mM)	30.2
L-Glutamate (2 mM)	25.0
L-α-Aminoadipate (2 mM)	31.2
L-Methionine (2 mM)	27.3
L-Threonine (2 mM)	24.6
L-Lysine (2 mM)	13.1
2-Aminoethyl-L-cysteine (2 mM)	11.7

somotropic substance is the weak base, chloroquine, which has been shown to inhibit various lysosomal activities causing vacuolization and alkalinization of the lysosomal interior (19). In a test of the effect of chloroquine on lysine exodus from human fibroblast lysosomes, shown in Table II, chloroquine

TABLE II

The effect of chloroquine on lysine exodus from normal human fibroblast lysosomes

Isolated lysosomal fraction from normal human skin fibroblasts was loaded with 0.058 mM L-[^{14}C]lysine methyl ester and washed by centrifugation in an Eppendorf Microfuge as described under "Experimental Procedures." The lysine-loaded pellet was resuspended in ice-cold MST pH 8.5 buffer and to the other half was added 6 μl of 10 mM chloroquine in MST pH 8.5 buffer. Both suspensions were incubated at 10 °C for 45 min, then diluted to 0.5 ml with ice-cold MST pH 6.5 buffer and centrifuged in an Eppendorf Microfuge for 10 min at 4 °C. The resulting pellet was washed once more by centrifugation in 0.5 ml of MST pH 6.5 buffer. Exodus of L-[^{14}C]lysine from lysosomes was measured at intervals of 3 min at 25 °C in MST pH 6.5 buffer with or without the addition of 2 mM L-lysine and the half-time values for exodus were determined.

Reagent present during prior incubation	Exodus conditions	Half-time of exodus
		min
Chloroquine	MST buffer, pH 6.5	66
Chloroquine	MST buffer with 2 mM L-lysine, pH 6.5	31
No chloroquine	MST buffer, pH 6.5	27
No chloroquine	MST buffer with 2 mM L-lysine, pH 6.5	15

greatly retarded exodus, resulting in an increase in the half-time from 27 to 66 min. The *trans*-stimulation property of the lysosomal lysine transporting system was retained in the presence of chloroquine, although exodus in the presence of external lysine was retarded by half by chloroquine treatment.

Human cystinotic polymorphonuclear leukocytes have been shown by Gahl *et al.* (2) to be defective in the carrier-mediated system for transporting cystine across the lysosomal membrane, displaying half-times of exodus of 26 and 81 min for normal and cystinotic leukocyte lysosomes, respectively. Jonas *et al.* (20) have previously shown that intact normal fibroblasts, when loaded with cystine by treatment with high concentrations of the mixed disulfide (CSSG) of cysteine and glutathione, lose their cystine with a half-time of 20 min, whereas no change in the cystine content of human cystinotic fibroblasts was observed within 90 min. In this experiment, the presumption is made that the lysosomal membrane barrier limits the rate of cystine escape from the intact fibroblasts. Cystine loss could not be demonstrated, however, from lysosomes isolated from the cystinotic fibroblasts or from normal fibroblasts treated with CSSG. When isolated fibroblast granular fractions were loaded by incubation with 30 μM L-[^{35}S] cystine dimethyl ester, we obtained half-times of cystine exodus (Table III) for normal and cystinotic fibroblast lysosomes similar to the corresponding values reported by Gahl *et al.* (2) for leukocyte lysosomes (Fig. 8). Depletion of endogenous cystine within the granular fraction of normal and cystinotic cells by cysteamine treatment prior to [^{35}S]cystine loading did not alter the observed difference between these cells with regard to lysosomal cystine transport, provided that the fractions were incubated with *N*-ethylmaleimide prior to the cysteamine treatment. Thus, the defective cystine transport system involved in cystinosis has now been demonstrated in lysosomes isolated from fibroblasts, where the lysosomes have been loaded directly using low concentrations of radiolabeled cystine dimethyl ester. The hyperbolic form of the semi-logarithmic plot shown by Fig. 8 for the lysosomes of normal fibroblasts when cystine is the test amino acid might arise from heterogeneity in the compartmentation of cystine in the lysosomal phase. In contrast to the differences in cystine efflux observed for normal and cystinotic fibroblast lysosomes, it was found (Table III) that the exodus of L-[^{14}C] lysine from cystinotic human fibroblast lysosomes took place

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TABLE III

trans-Stimulation of L-[^{14}C]lysine exodus from cystinotic human fibroblast lysosomal preparations by external amino acids at 25 °C and pH 6.5

Isolated lysosomal fraction from cystinotic human fibroblasts was loaded with 0.065 mM L-[^{14}C]lysine methyl ester as described under "Experimental Procedures." Exodus of L-[^{14}C]lysine from lysosomes was measured at intervals of 3 min at 25 °C in MST pH 6.5 buffer containing the indicated amino acid at the concentration given in parentheses. Half-time values were determined by a linear regression analysis of semi-logarithmic plots as described under "Experimental Procedures."

Compound	$t_{1/2}$
Buffer (50 mM MOPS, 0.25 M sucrose, pH 6.5)	19.94
L-Phenylalanine (2 mM)	21.33
L-Phenylalanine (20 mM)	21.07
D-Glutamate (2 mM)	19.41
D-Glutamate (20 mM)	21.90
L-Lysine (2 mM)	13.82
L-Lysine (20 mM)	13.95
2 mM ATP in 2 mM $MgCl_2$	10.94

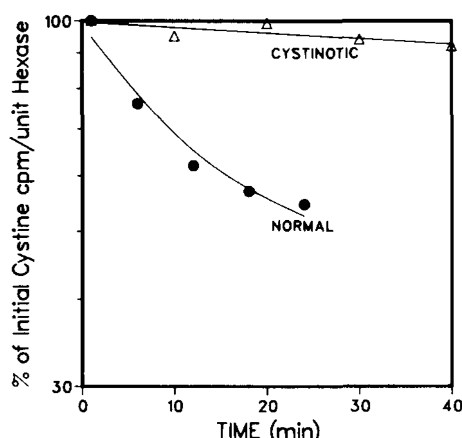

FIG. 8. **Exodus of L-[^{35}S]cystine from isolated lysosomal fraction of normal and cystinotic human fibroblasts.** The lysosomal fraction from either normal or cystinotic human fibroblasts was isolated and loaded with 0.03 mM [^{35}S]cystine dimethyl ester as described under "Experimental Procedures." Exodus of L-[^{35}S]cystine from lysosomes was performed at 37 °C in 0.82 ml of MST pH 7.0 buffer, removing duplicate 80-μl aliquots at each time point and centrifuging in an Eppendorf model 5414 Microfuge. Pellets were resuspended in *N*-ethylmaleimide, frozen and thawed, assayed for hexosaminidase, precipitated with sulfosalicylic acid, and aliquots of the sulfosalicylic acid supernatants were subjected to high voltage electrophoresis for quantitation of L-[^{35}S]cystine as described under "Experimental Procedures."

with the same half-time as in normal human fibroblast lysosomes. Furthermore, L-lysine and other basic amino acids, but not neutral or acidic amino acids, produce *trans*-stimulation effects very similar to those found in normal human fibroblast lysosomes. Therefore, by these criteria the system for transporting basic amino acids appears not to be defective in human cystinotic fibroblast lysosomes.

Our attention was drawn toward the mode of action of the therapeutic agent, cysteamine, used to reduce lysosomal cystine accumulation in individuals with cystinosis. Cysteamine was first postulated by Thoene *et al.* (6), and recently confirmed by Gahl *et al.* (21), to react with cystine in the organelle to form cysteine and the mixed disulfide of cysteine and cysteamine by sulfhydryl-disulfide exchanges. Based on the early work of Ehrenreich and Cohn (30) it was speculated that this mixed disulfide, having a molecular mass smaller than 230 Da, escapes from the lysosome by diffusion (6). The structural similarity of this mixed disulfide of cysteine and cysteamine (Fig. 9) to that of L-lysine and 2-aminoethyl-L-cysteine suggested to us that it may be transported by the system serving dibasic amino acids in human fibroblast lysosomes. In a test of its ability to cause *trans*-stimulation of L-[^{14}C]lysine efflux, the mixed disulfide of cysteine and cysteamine produced a large *trans* effect (Table IV) for both cystinotic and normal fibroblast lysosomes. Therefore, an important mode by which cysteamine treatment of cystinosis allows cystine escape from lysosomes appears to be the ability of the mixed disulfide of cysteine and cysteamine, formed by sulfhydryl-disulfide exchanges, to migrate by the dibasic amino acid transporting system.

Our attention was directed to the possible effects of ATP on lysine exodus from the work of Jonas *et al.* (5, 22), who have demonstrated that external ATP accelerates cystine exodus from Epstein-Barr virus transformed human lymphoblast lysosomes. Human fibroblast lysosomes were loaded with L-[^{14}C]lysine methyl ester either in the presence or absence of 2 mM MgATP with subsequent exodus measured in the presence or absence of 2 mM MgATP for each of the above loading conditions. The results shown in Table V indicate that MgATP, when added only at the initiation of exodus, can almost double the rate of lysine exodus. ATP does

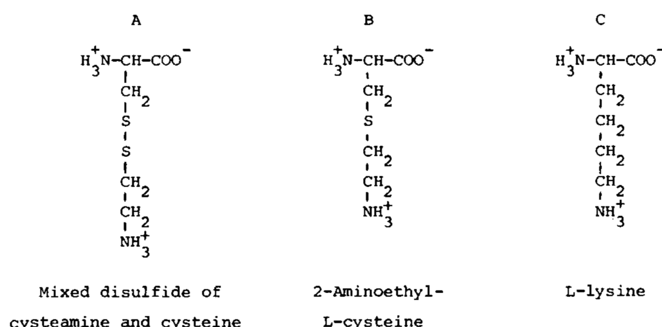

FIG. 9. **Structural comparison of lysine and the analogs, 2-aminoethylcysteine and the mixed disulfide of cysteine and cysteamine.**

TABLE IV

trans-Stimulation of L-[^{14}C]lysine exodus from normal and cystinotic human fibroblast lysosomal preparations by the mixed disulfide of L-cysteine and cysteamine at 25 °C and pH 6.5

Isolated lysosomal fraction from either normal (Experiment A) or cystinotic (Experiment B) human fibroblasts was loaded with 0.061 mM L-[^{14}C]lysine methyl ester as described under "Experimental Procedures." Exodus of L-[^{14}C]lysine from lysosomes was measured at intervals of 3 min at 25°C in MST pH 6.5 buffer containing the indicated amino acid at the concentration shown in parentheses. Half-time values were determined by a linear regression analysis of the semi-logarithmic plots as described under "Experimental Procedures."

Compound	$t_{1/2}$
Experiment A: normal	
Buffer (50 mM MOPS, 0.25 M sucrose, pH 6.5)	24.6
L-Cystine (0.5 mM)	22.3
2-Aminoethyl-L-cysteine (2 mM)	9.7
Mixed disulfide of L-cysteine and cysteamine (2 mM)	13.6
L-Lysine (2 mM)	8.7
Experiment B: cystinotic	
Buffer (50 mM MOPS, 0.25 M sucrose, pH 6.5)	25.6
L-Cystine (0.5 mM)	24.7
2-Aminoethyl-L-cysteine (2 mM)	14.7
Mixed disulfide of L-cysteine and cysteamine (2 mM)	14.7
L-Lysine (2 mM)	14.2

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TABLE V

The effect of the presence of ATP during either the incubation to load lysosomes with lysine or the subsequent incubation to measure the rate of L-[^{14}C]lysine exodus from lysosomes of normal human fibroblasts

The isolated lysosomal fraction from normal human fibroblasts was divided in half, and one-half was incubated at 25 °C for 25 min with 0.066 mM L-[^{14}C]lysine methyl ester in MST pH 7.6 buffer and the other half loaded under the same conditions but with the addition also of 2 mM ATP and 2 mM $MgCl_2$. At the completion of the loading incubation, each mixture was washed twice by centrifugation in the Eppendorf Microfuge as described under "Experimental Procedures." Exodus was then measured to correspond to each of the above loading conditions by incubating the lysine-loaded lysosomes at 25 °C in MST pH 6.5 buffer with or without the addition of 2 mM ATP and 2 mM $MgCl_2$. Aliquots were removed at 3-min intervals and the half-times of exodus were determined by a linear regression analysis of semi-logarithmic plots.

Condition of loading	Exodus conditions	Half-time of exodus
		min
Without ATP	MST buffer, pH 6.5	40
Without ATP	MST buffer with 2 mM MgATP, pH 6.5	26
With 2 mM ATP and 2 mM $MgCl_2$	MST buffer, pH 6.5	18.5
With 2 mM ATP and 2 mM $MgCl_2$	MST buffer with 2 mM MgATP, pH 6.5	18.3

not appear, however, to be directly involved in this stimulation of lysine efflux because the stimulatory effect of ATP upon lysine efflux can also be obtained by loading fibroblast lysosomes with L-[^{14}C]lysine methyl ester in the presence of 2 mM MgATP and measuring subsequent exodus in the absence of ATP. This effect indicates that the changes caused by ATP during loading which lead to a stimulation of lysine efflux, whatever they are, can be preserved through the two wash steps preceding exodus. This effect of ATP may somehow coincide with the promotion by ATP of the function of the lysosomal proton pump; detailed experiments with highly purified fibroblast lysosomes will be necessary, however, in order to gain an understanding of this effect of ATP on lysine efflux. A similar stimulatory effect of ATP on lysine exodus was observed with lysosomes from human cystinotic fibroblasts.

DISCUSSION

The discovery of a *trans*-stimulation property associated with exodus of L-[^{14}C]lysine from human fibroblast lysosomes has allowed the characterization of a carrier-mediated system for the transport of dibasic amino acids across the human fibroblast lysosomal membrane. Demonstration of a *trans* effect in a transport process provides strong evidence that the process is carrier-mediated, although not all carrier-mediated transport systems show *trans* effects. Further evidence that this lysosomal amino acid transport system is carrier-mediated is indicated by its selectivity seen so far only for cationic amino acids, its intolerance to methylation of the α-nitrogen group of the amino acid, and its stereoselectivity for the L-isomer of the amino acid. The characteristics of this human fibroblast lysosomal amino acid transport system show considerable similarity to those cited for System y^+ associated with the plasma membrane of human fibroblasts, Ehrlich cells, rabbit reticulocytes, and rat hepatoma cells (23–29). In these cells, System y^+ is a high-affinity, Na^+-independent transport system serving for cationic amino acids, which shows a strong *trans*-stimulation property (23). An inference from the presence of these similar cationic amino acid transport systems in both the plasma membrane and lysosomal membrane of the human fibroblast is that a mechanism may exist in fibroblasts whereby amino acid transport proteins can be targeted either to the plasma membrane or to the lysosomal membrane following synthesis. Differences in the observed kinetic parameters for these systems may be expected because of differences in the membrane environments, pH gradient, and membrane potential of the plasma membrane and lysosomal membrane. Whereas System y^+ of the plasma membrane has been found to be relatively insensitive to changes in pH between pH 5.5 and 7.5, we now observe efflux of L-[^{14}C]lysine from the lysosome to increase in a steady manner as the pH is increased over this same pH region. Since there is little change in the ionization of the titratable groups on lysine within this pH range, this effect is not likely to arise from the titration of the substrate, lysine. Titration of a group on the carrier molecule could, however, alter its affinity for lysine in a manner analogous to what happens when System ASC is protonated. Alternatively, the response to change in pH could be an effect of the increasing pH gradient on lysine exodus.

A major difficulty in characterizing amino acid transport systems in crude granular fractions is that evidence for the presence of an amino acid transport system appears, for the present, to be limited to the detection of a *trans* effect for a given system. Reeves (17) did not observe a *trans* effect of external leucine on leucine exodus in rat liver lysosomes. Likewise, with human fibroblast lysosomes, we have not detected a *trans* effect by several neutral amino acids on phenylalanine exodus.[2] These results, however, do not exclude the presence of amino acid transport systems for leucine and phenylalanine in lysosomes, but delay their possible detection until more suitable methods of investigation can be applied.

Based on vacuolization of mouse macrophage lysosomes as a signal for their retention of test osmolites, Ehrenreich and Cohn (30) have previously proposed that the lysosomal membrane is permeable to amino acids or dipeptides smaller than approximately 230 Da. This interpretation depends on special assumptions, and on a rather limited range of test solutes. How widely applicable the factor of pore size is for the permeation of biological membranes remains somewhat controversial. Questions arose early regarding the validity of the proposal of Ehrenreich and Cohn from the experiments of Goldman (31), demonstrating that dipeptides as large as 286 Da were capable of crossing the lysosomal membrane, and the observations of Lloyd (32) that dipeptides cross the lysosomal membrane more rapidly than amino acids and in a pH-dependent manner. Particularly the latter results should perhaps transfer attention to mediated peptide transport through somewhat specific molecular recognition. The evidence for the mediated exodus of cystine (2–5), and the discovery of the present system for transport of cationic amino acids across the lysosomal membrane, provide direct evidence that lysosomal membranes are not porous to amino acids, but that their passage depends on the presence of suitable transport facilities. A carrier-mediated system for sugar transport in rat liver lysosomes has recently been suggested from the work of Docherty *et al.* (33, 34) and Maguire *et al.* (35). Examination of published evidence for passage of dipeptides across lysosomal membranes indicates that dipeptides composed solely of neutral amino acids cross the lysosomal membrane, whereas dipeptides containing an amino acid residue bearing a charged side chain experience difficulty in such passage (30–32). Recognition for mediated transport by a peptide transport

[2] R. Pisoni and R. Wolf, unpublished observations.

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system seems likely to be involved. This situation appears also to exist for monosaccharides, in that Lloyd (36) has shown uncharged monosaccharides to be much more capable of passage across the lysosomal membrane than anionic monosaccharides such as D-glucuronate and D-gluconate. Decisive effects of electrical charge for membrane transport are theoretically to be expected and regularly observed. The vacuolization caused by the poor passage of $(\text{D-glutamate})_2$ across the mouse macrophage lysosomal membrane in the experiments of Ehrenreich and Cohn (30) may have arisen because this dipeptide carries a net molecular charge rather than because of its larger molecular size.

The presence of a system for transporting cationic amino acids across the human fibroblast lysosome membrane provides encouragement in the search for the means of transport of other amino acids across such membranes. In addition, questions arise regarding the importance of the lysosomal pH gradient and membrane potential in the function of individual transport systems, the manner in which protein receptor sites for transport may be targeted to the plasma membrane and to lysosomal membranes of eukaryotic cells, and how amino acid transport systems of the plasma membrane and of the lysosomal membrane differ so as to serve their different biological functions. Finally, other congenital defects in amino acid transport from the lysosome may remain to be discovered.

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