Isolation of lysosomal α -mannosidase mutants of Chinese hamster ovary cells

(replica plating/fluorogenic substrates/enzyme uptake/cell density-dependent enzyme)

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ABSTRACT A method is presented for detection of lysosomal hydrolase activity *in situ* in colonies of Chinese hamster ovary cells. This method was used to screen for mutants deficient in lysosomal α -mannosidase. Mutagenized cells were replicated onto filter papers [Esko, J. D. & Raetz, C. R. H. (1978) *Proc. Natl. Acad. Sci. USA* 75, 1190–1193] and allowed to divide for 11–14 days; cells on the filter replicas were lysed, and the filters were incubated with 4-methylumbelliferyl- α -D-mannopyranoside at pH 4.4 and then photographed under UV light. Colonies for which the replicas exhibited little or no fluorescence were picked from the master plates and purified; mutants with decreased α -mannosidase activity were obtained at a frequency of 1 per 1500 mutagenized cells. Analysis of one of these mutants showed that lysosomal α -mannosidase activity was 18% of that from wild-type cells, whereas the activities of α -mannosidases not of lysosomal origin were similar in the wild type and mutant (these included both Golgi-associated and cytosolic enzymes as well as a novel acid α -mannosidase seen only in cells from confluent culture). The mutant contained normal levels of other lysosomal hydrolases. Both wild-type and mutant cells secreted α -mannosidase into the medium at levels proportional to those found inside the cells. Incubation of mutant cells with secretions from the wild type resulted in partial restoration of enzyme activity. Added enzyme was localized in the lysosomal fractions; uptake of added enzyme was inhibited by mannose 6-phosphate and fructose 1-phosphate, which are known to inhibit uptake of lysosomal enzymes into human diploid fibroblasts.

In cell culture, diploid fibroblasts isolated from patients with inherited lysosomal storage diseases manifest the enzymatic defect characteristic of the donor's disorder (1, 2). These mutant cells have played a crucial role in elucidation of the steps involved in lysosomal degradation of macromolecules. Moreover, some of the current models describing biosynthesis and modification of lysosomal enzymes have arisen from studies of mutant fibroblasts with a pleiotropic deficiency in lysosomal hydrolases, isolated from patients with I-cell disease (3–5).

That the source of lysosomal mutants is limited to patients with lysosomal storage diseases has certain disadvantages. First, one is limited to studying those defects that lead to defined clinical manifestations at the level of the whole organism. At the two extremes, deficiencies may be mild enough to escape detection or of such severity that the fetus does not come to term. Second, diploid fibroblasts are unsuited for genetic manipulations in cell culture; for example, the isolation of revertants or of second-step mutants from mutants defective in lysosomal enzymes is hampered by the finite life-span and low plating efficiencies of diploid fibroblasts.

In this report I present a simple method for isolation of lysosomal enzyme mutants using Chinese hamster ovary (CHO) cells.

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MATERIALS AND METHODS

Reagents. Fetal calf serum, tissue-culture-grade trypsin, and bovine pancreatic insulin were purchased from GIBCO. Tissue culture media were prepared in the Media Supply Unit of the National Institutes of Health. Ethyl methanesulfonate was purchased from Eastman. 4-Methylumbelliferyl substrates were purchased from Research Products International (Elk Grove Village, IL). Colloidal silica (Ludox HS 40) was a gift of Du Pont. Bovine serum albumin (A grade) was obtained from Calbiochem. All other reagents were purchased from standard commercial suppliers and were of the best grade available.

Cells and Cell Culture. WTB (6), a subclone of a CHO cell line that is a proline auxotroph (7), was the gift of R. M. Baker. Cells were grown in Eagle's minimal essential medium supplemented with antibiotics, nonessential amino acids, and 10% fetal calf serum (8) in 5% CO₂ at 35°C. Under these conditions, doubling of the cells occurred in 16 hr, and plating efficiency, determined by colony formation, was >90%. Cells were frozen at 2×10^6 cells per ml in growth medium plus 10% dimethyl sulfoxide and stored at -80°C . Stocks of cells were maintained, always at a cell density below confluence, by passing the cells at 1:100 dilution every 4 days. Cells were kept in culture for only 2 months; at this time, frozen cells were used to initiate new stock cultures. Waymouth medium MAB 87-3 (9), formulated as described by GIBCO, was used for maintenance of cells in serum-free medium.

Replica Plating. An inoculum of 2×10^5 WTB cells was grown in a 100-mm dish to a density of 1.5×10^6 cells and then treated with the mutagen ethyl methanesulfonate (200 $\mu\text{g}/\text{ml}$); after 18 hr the cells were harvested and replated at 400 cells per 100-mm dish in growth medium plus insulin (20 $\mu\text{g}/\text{ml}$). After 24 hr, a sterile Whatman no. 50 filter was added to each dish and weighted down with glass beads (10). In early experiments, sporadic episodes of cell death were encountered, apparently due to filters sterilized by autoclaving; filters sterilized by boiling in two changes of 95% ethanol and then dried under a stream of sterile air were not toxic (this modification was the helpful suggestion of C. Raetz).

Detection of Hydrolase Activity *In Situ*. Cells were grown in contact with the filter papers for 11–14 days. To minimize cross-feeding the growth medium was changed every 2 days for the first week, and every day thereafter; after the first week the medium did not contain added insulin. To remove the replicas, the growth medium was aspirated, the beads were poured off, and the filters were transferred to separate dishes. Growth medium was then added to the master dishes which were maintained at room temperature in 5% CO₂. The replicas were washed gently with solution A (0.14 M NaCl/7 mM KCl/1 mM NaKPO₄, pH 7.4) and cells were lysed by freezing and thawing in the following manner: 0.5–1.0 ml of 95% ethanol,

Abbreviation: CHO, Chinese hamster ovary.

previously chilled in a dry ice/ethanol bath, was added to the dishes containing the filter replicas and the dishes were floated immediately on the surface of the dry ice/ethanol; after 5 min the dishes were transferred to room temperature. As the dishes warmed, the ethanol was removed; after 15 min the dishes were again placed on dry ice/ethanol for 5 min and then warmed at room temperature. The filters were blotted dry and then transferred to dishes containing 0.15 ml of 1 mM 4-methylumbelliferyl substrate in 0.1 M Na acetate buffer, pH 4.4. When the filters appeared to be saturated with the assay mixture they were blotted dry, transferred to new dishes, and floated on the surface of a 37°C bath for 1.5–2 hr. The filters were then photographed under long-wave UV light with Polaroid 665 film and an exposure time of 0.5 sec. The filters were stained with Coomassie brilliant blue for 1 hr and then destained for 2 days with five changes of destaining mixture (11).

Purification of Potential Mutants. Colonies were detached from the master dishes onto 0.5-cm discs of sterile filter paper that had been soaked in 0.1% trypsin; the filters were then transferred to 60-mm dishes containing growth medium. Cultures of these cells were grown to densities of about 5×10^5 cells per dish, harvested, diluted in growth medium, and plated at 1 cell per 3 wells of a 96-well cluster dish (Costar). Wells were examined at 6 and 10 days to identify those which contained single colonies; these were trypsinized, and the cells were transferred to dishes and grown to densities suitable for quantitative assays of enzyme activity.

Cell Fractionation. Cells grown in a 150-cm² flask were washed with solution A; 2 ml of 0.1% trypsin in this same buffer was added for 10 min at 35°C. Growth medium (8 ml) was then added and the cell suspension was centrifuged 5 min at $600 \times g$. The cells were resuspended in 10 ml of fresh growth medium and allowed to recover for 1 hr at 35°C; they were then centrifuged at $600 \times g$ for 5 min and washed with 10 ml of solution A. Cells were disrupted in 0.25 M sucrose by the method of Rome *et al.* (12) with the one exception that the final volume of postnuclear supernatant was 6.0 ml. Of this, 4.5 ml was layered above 25 ml of colloidal silica mixture, which had in turn been layered above 4 ml of saturated sucrose solution; centrifugation and collection of fractions followed the procedure of Rome *et al.* (12).

Enzyme Assays. The acid hydrolases α -mannosidase (EC 3.2.1.24), β -galactosidase (EC 3.2.1.23), β -glucuronidase (EC 3.2.1.31), β -hexosaminidase (EC 3.2.1.30), and α -L-fucosidase (EC 3.2.1.51) were assayed in a final volume of 0.30 ml of 0.1 M Na acetate buffer, pH 4.4, containing the appropriate 4-methylumbelliferyl substrate at 1 mM. Unless otherwise specified, all assays contained 0.1% Triton X-100. After 30–60 min at 37°C, reactions were terminated with 1.0 ml of 0.5 M glycine/0.5 M Na₂CO₃, pH 10.0. Release of 4-methylumbelliferone was measured with a Farrand ratio fluorometer-2. One unit of enzyme activity represents 1 nmol of substrate hydrolyzed per hr at 37°C.

For determination of α -mannosidase activity at pH 6.0, 0.05 M Na cacodylate was used as buffer. The galactosyltransferase (EC 2.4.1.38) assay has been described elsewhere (12). Protein was measured by the Lowry method (13).

Preparation of Extracellular Secretions. Cells were grown to confluence in T150 flasks, growth medium was removed, and 25 ml of serum-free medium was added. After 4 hr, this medium was replaced with 25 ml of fresh serum-free medium; the cultures were then maintained for 40 hr. At this time the culture fluid was decanted into tubes and centrifuged at 500 rpm for 10 min to remove any floating cells. The medium (20 ml) was carefully removed and this material was used in assays of enzyme activity. For experiments involving cellular uptake of

secretions, bovine serum albumin (1 mg/ml) was added to the medium after centrifugation, followed by (NH₄)₂SO₄ to 70% saturation. The suspension was incubated for 8 hr at 4°C and then centrifuged in a Sorvall SS-34 rotor at 15,000 rpm for 25 min at 4°C. The precipitated material was resuspended in 0.15 M NaCl/10 mM Na phosphate, pH 6.0, at 1/40th the original volume and dialyzed at 4°C for 36 hr with three changes of buffer. For addition to cells the concentrated, dialyzed secretions were diluted in growth medium and sterilized by passage through cellulose nitrate filters (0.45 μ m, Millipore).

RESULTS

Isolation of α -Mannosidase Mutants. WTB cells mutagenized with ethyl methanesulfonate (200 μ g/ml) were plated on master dishes and then replicated onto filter papers (10). The toxicity of the mutagen was such that 270 of the 400 cells plated per master dish formed colonies. Replication from master dish to filter was $\geq 90\%$. Cells on the filter papers were lysed and the filters were incubated with 1 mM 4-methylumbelliferyl- α -mannopyranoside at pH 4.4, as described above. Of 6500 colonies identified on the filter papers by Coomassie blue staining, 12 exhibited little or no fluorescence after incubation with substrate. A stained filter replica containing one of these negative colonies is shown in Fig. 1, along with the corresponding UV photograph.

The 12 potential mutants were picked from the master dishes and cloned. Extracts of these cloned cells were assayed for α -mannosidase activity at pH 4.4. Seven of the original colonies picked yielded clones with decreased α -mannosidase activity (Table 1). The original colonies were contaminated with wild-type cells; for example, four of the five clones obtained from N12-5 exhibited α -mannosidase activity ranging from 25 to 31% of the level measured with WTB cells, and the fifth clone had enzyme activity equal to that of WTB. This may explain why deficient clones were obtained from only 7 of the 12 colonies identified in the initial screening.

The experiments presented henceforth were performed with N12-5-2, one of the four α -mannosidase-deficient clones obtained from N12-5.

Characterization of α -Mannosidase Mutant N12-5-2. α -Mannosidases have been purified from the lysosomes (14), Golgi apparatus (15), and cytosol (16) of rat liver. Both human and bovine mannosidases are thought to result from deficiencies in the lysosomal enzyme (e.g., refs. 17 and 18). To characterize the defect in N12-5-2, subcellular fractions of the wild type and mutant were prepared by centrifugation of cell extracts on gradients of colloidal silica (12). Two major peaks of lysosomal enzyme activity were found, represented in Fig. 2 by α -man-

FIG. 1. Detection of acid α -mannosidase activity in CHO cell colonies *in situ*. A filter paper replica was prepared and incubated at pH 4.4 with 1 mM 4-methylumbelliferyl α -mannopyranoside. (A) Photograph of the filter under UV light. (B) The same filter subsequently stained with Coomassie blue. Arrows indicate position of an acid α -mannosidase-deficient colony.

Table 1. Acid α -mannosidase activity in wild-type and mutant cells

Strain*	Enzyme activity	
	units/mg protein	%
WTB	37.6	100
N4-1 (4)	15.1-16.5	40-44
N7-1 (5)	9.5-12.0	26-32
N7-2 (2)	16.9-18.8	45-50
N12-5 (4)	9.4-11.6	26-31
N18-1 (5)	10.5-12.9	28-34
N19-1 (3)	11.9-13.8	32-37
N24-2 (4)	10.7-15.3	28-41

Cells were harvested by trypsinization and then washed once in growth medium and twice in solution A; after resuspension in 0.5 ml of solution A, the cells were lysed by repeated cycles of freezing and thawing. Total cell protein obtained from the various cultures ranged from 250 to 380 μ g; 12-19 μ g was used in assays of acid α -mannosidase activity. One unit of enzyme activity is defined as hydrolysis of 1 nmol of substrate per hr at 37°C.

* Five clones from each mutant were tested; the numbers in parentheses indicate the number of clones with enzyme activity in the range shown. The remaining clones, e.g. one clone from N4-1 and three clones from N7-2, exhibited acid α -mannosidase activity 92-117% of that measured with wild-type cells.

nosidase, α -L-fucosidase, and β -galactosidase, all assayed at pH 4.4. α -Mannosidase activity was decreased in both peaks obtained from the mutant N12-5-2; the heavier peak contained 18% of the activity measured in the corresponding peak from WTB, and the lighter peak contained 25% of the corresponding wild-type activity. Little difference was observed between WTB and N12-5-2 with respect to the activities of the acid hydrolases α -L-fucosidase and β -galactosidase (Fig. 2) or of

FIG. 2. Profiles of hydrolase activity from wild-type (Left) and mutant (Right) cells on density gradients of colloidal silica. One 150-cm² flask each of WTB (Left) and N12-5-2 (Right) was harvested, the cells were homogenized, and the homogenates were centrifuged as described in the text; 2.25 mg of protein from WTB was applied to one gradient (A-D); 2.11 mg of protein from N12-5-2 was applied to the other (E-H). α -MAN., α -L-FUC., and β -GAL. refer to α -mannosidase, α -L-fucosidase, and β -galactosidase activities, respectively; enzyme activities are reported in units/ml. Note the different scales used for enzyme activities. Fractions (1.1 ml) are numbered in the order of decreasing density. Bars in D and H indicate the position of galactosyl transferase activity.

β -glucuronidase and β -hexosaminidase (data not shown). (Unlike the other acid hydrolases examined, β -glucuronidase activity does not partition equally between the two lysosomal enzyme peaks; instead about 75% of this enzyme cosedimented with the lighter peak.)

When α -mannosidase activity was measured at pH 6.0, a single major peak was observed; this peak cosedimented with galactosyl transferase (see bars in Fig. 2D and H), a marker for the Golgi apparatus, and probably represents the Golgi α -mannosidase (15). Extracts of wild-type and mutant cells exhibited similar levels of this enzyme (Fig. 2D and H). Some of the acid α -mannosidase activity observed in the light acid hydrolase peak from N12-5-2 may reflect the residual activity of the Golgi α -mannosidase at pH 4.4; comparison of Fig. 2E and H shows a marked similarity between the two profiles at the position of the Golgi α -mannosidase.

Only small amounts of cytosolic α -mannosidase (15) are present in CHO cells. A minor peak of enzyme activity measured at pH 6.0 was coincident with the soluble material; the level of this activity was similar in gradients from WTB (Fig. 2D) and N12-5-2 (Fig. 2H). This soluble enzyme activity may result, in part, from release of enzyme from the Golgi.

The results presented in Fig. 2 were obtained with cells from subconfluent cultures. Extracts prepared from both wild-type and mutant cells that had been maintained in confluent culture for approximately half a generation time exhibited a significant increase in acid α -mannosidase activity cosedimenting with the light acid hydrolase peak. Unlike the α -mannosidase activity associated with the acid hydrolase peaks of subconfluent cells, the cell density-dependent α -mannosidase was inhibited by 0.1% Triton X-100. Fig. 3 shows the profiles of Triton-resistant and Triton-sensitive acid α -mannosidase activities in a gradient prepared with confluent WTB cells. Mutant and wild-type cells contained similar levels of the cell density-dependent enzyme. Results of preliminary experiments indicate that the cell density-dependent α -mannosidase can be distinguished from the cytosolic, lysosomal, and Golgi-associated species by pH profile and inhibitor sensitivity (data not shown).

The decreased levels of lysosomal α -mannosidase activity found in extracts of N12-5-2 could result from increased secretion of that enzyme into the extracellular fluid. Precedent for this comes from studies of fibroblasts isolated from patients with I-cell disease: these cells contain decreased levels of many acid hydrolases; the same enzymes are present in abnormal amounts in the culture medium (2). Table 2 shows the extracellular hydrolase activities obtained from parallel cultures of N12-5-2 and WTB. Medium from wild-type cells contained 2.5 times more acid α -mannosidase than was found in medium from N12-5-2. Similar levels of α -mannosidase activity assayed at pH 6.0 were observed in the two cultures, and no significant differences were observed between N12-5-2 and WTB with respect to secretion of the acid hydrolases β -galactosidase, α -L-fucosidase, β -glucuronidase, and β -hexosaminidase. Also shown in Table 2 are the activities of these hydrolases in extracts of the cells from which the secretions were collected. Confluent cultures were used in these experiments; although the cell-density dependent α -mannosidase was present in extracts of WTB and N12-5-2, the acid α -mannosidase activity measured in secretions from those cells was not inhibited by either Triton X-100 or *p*-chloromercuriphenylsulfonic acid. Thus, it appears that the cell density-dependent α -mannosidase is not secreted.

Uptake of Acid α -Mannosidase into Mutant N12-5-2. When added to fibroblasts defective in a lysosomal enzyme, secretions from normal human fibroblasts restore the activity of that en-

FIG. 3. Profiles of α -mannosidase activities from confluent wild-type cells. A 150-cm² flask was harvested 8 hr after the culture appeared to be confluent; 4.68 mg of protein from WTB was applied to the gradient. Acid α -mannosidase activity was assayed across the gradient in the presence and absence of 0.1% Triton X-100. (Upper) "Triton-resistant" represents α -mannosidase activity measured in the presence of detergent; (Lower) "Triton-sensitive" represents the difference obtained upon subtracting α -mannosidase activity measured in the presence of Triton from activity measured in the absence of detergent. Enzyme activities are reported in units/ml.

zyme in the mutant (2). A recent study has shown that addition of purified α -L-iduronidase to recipient cells missing that enzyme results in the appearance of α -L-iduronidase activity in the two acid hydrolase peaks defined by centrifugation in colloidal silica (12). Parallel cultures of N12-5-2 were incubated either with medium harvested from WTB cells and concentrated or with concentrated medium that had not been previ-

Table 2. Intracellular and extracellular hydrolase activities from WTB and N12-5-2

Enzyme	WTB		N12-5-2	
	Secre-tions*	Cells†	Secre-tions*	Cells†
α -Mannosidase, pH 4.4	428	601	188	261
β -Hexosaminidase, pH 4.4	375	967	363	1040
α -L-Fucosidase, pH 4.4	285	1370	334	1190
β -Glucuronidase, pH 4.4	263	1510	320	1481
β -Galactosidase, pH 4.4	103	1920	98	1640
α -Mannosidase, pH 6.0	234	795	220	795

Cells previously grown to confluence in 150-cm² flasks were maintained in serum-free medium for 40 hr. This medium was then removed and prepared as described for assays of extracellular enzyme activity. The cells were harvested by trypsinization, washed twice with solution A, and then lysed in 1 ml of solution A by repeated cycles of freezing and thawing.

* Results are presented in units per 25 ml, the volume of medium harvested from one flask. These results are corrected for blank values obtained with fresh serum-free medium.

† Results are presented in units per ml, the volume that contained cells from one flask; 4.3 mg of cell protein was obtained from WTB and 3.8 mg from N12-5-2.

ously incubated with cells. After 24 hr the cells were harvested and homogenized and the extracts were fractionated on gradients. Incubation with secretions from WTB resulted in a 2-fold increase in the acid α -mannosidase activity in the two lysosomal enzyme peaks, defined by the positions of acid β -galactosidase activity (Fig. 4A); quantitation of the change in α -mannosidase activity in the lighter acid hydrolase peak was obscured somewhat by the proximity of the Golgi α -mannosidase (Fig. 4B).

Uptake of lysosomal α -mannosidase activity into N12-5-2 cells incubated with secretions from WTB increased linearly with respect to the level of recipient cell protein. N12-5-2 cells grown to confluence (approximately 1.5 mg protein per dish) internalized 10% of the acid α -mannosidase activity added. When secretions from N12-5-2 were used as a source of enzyme, acid α -mannosidase activity in recipient cells was increased by 30%, compared to an 87% increase obtained with secretions from WTB.

Uptake of lysosomal enzyme into human fibroblasts is dependent on the presence of a phosphorylated carbohydrate on the enzyme; this is evidenced by loss of uptake after treatment of the enzyme with periodate or alkaline phosphatase and by the inhibition of uptake by certain phosphorylated sugars (19-21). To determine whether uptake of α -mannosidase activity into CHO cells occurs by a similar mechanism, N12-5-2 cells were incubated with WTB secretions in the presence of these sugars. Mannose 6-phosphate and fructose 1-phosphate completely inhibited uptake of α -mannosidase into N12-5-2, but mannose, mannose 1-phosphate, and fructose 6-phosphate did not inhibit (Table 3). This specificity of inhibition is the same as that reported for human fibroblasts.

FIG. 4. Profile of acid α -mannosidase activity from the mutant after uptake of secretions from wild-type cells. Serum-free medium from WTB was collected and concentrated; 0.45 ml of this concentrate, containing 153 units of acid α -mannosidase activity, was diluted with growth medium to a final volume of 10 ml and then added to N12-5-2 cells growing in a 150-cm² flask. After 24 hr the cells were harvested and homogenized, and 855 μ g of cell protein was applied to one gradient (solid bars; solid line). A parallel culture of N12-5-2 was incubated with a concentrate of fresh, serum-free medium, diluted in growth medium as above; 770 μ g of protein from these cells was applied to the second gradient (open bars; dotted line). Enzyme activities are reported in units/ml. Horizontal bars indicate the peaks of acid β -galactosidase activity.

Table 3. Inhibition of acid α -mannosidase uptake into N12-5-20

Secretions	Additions*	Enzyme activity, units/mg
—	—	10.9
+	—	20.2
+	Mannose	21.1
+	Mannose 1-phosphate	18.9
+	Mannose 6-phosphate	9.6
+	Fructose 1-phosphate	10.5
+	Fructose 6-phosphate	19.0

N12-5-2 cells were grown in 100-mm dishes; at 0 time the growth medium was removed and replaced with 5 ml of fresh growth medium. Included in this medium were, where indicated, 200 μ l of secretions from WTB cells, containing 204 units of acid α -mannosidase activity. After 24 hr the cells were harvested, washed, and lysed as described in Table 1. Total cell protein obtained from the various cultures ranged from 430 to 500 μ g; 21–25 μ g was used in assays of acid α -mannosidase activity.

* Each present at 1 mM.

DISCUSSION

The essential features of the screening procedure described in this report are: (i) replica plating of the cell colonies (10); (ii) lysis of the cells on the replicas in the presence of cold ethanol; (iii) determination of enzyme activity *in situ* with a fluorogenic substrate. This procedure is not limited to screening for acid α -mannosidase mutants; with the appropriate 4-methylumbelliferyl substrates I have demonstrated in colonies *in situ* the acid hydrolases β -galactosidase, β -hexosaminidase, α -L-fucosidase, and α -L-iduronidase. The method can also be extended to hydrolases not of lysosomal origin: Golgi α -mannosidase activity was detected by incubating the replicas with 4-methylumbelliferyl- α -mannopyranoside at pH 6.0. The intensity of 4-methylumbelliferone fluorescence is such that 5 pmol of product, concentrated in a spot the size of a colony (1–2 mm), is easily visible under UV light. If desired, a 4-fold increase in sensitivity can be achieved by spraying the replicas with pH 10 buffer after incubation with the substrate.

Diploid fibroblasts isolated from patients with lysosomal storage diseases have served as a model system for studies of function and biosynthesis of lysosomal enzymes. On comparison of the results of these studies with those presented here for CHO cells, a number of similarities emerge. In both diploid fibroblasts and CHO cells, lysosomal enzymes are distributed in two populations of particles, separable by density gradient centrifugation (12). α -Mannosidase activity is decreased to a similar extent in the two types of particles isolated from the CHO mutant N12-5-2. CHO cells secrete lysosomal enzymes into the growth medium, as do diploid fibroblasts (2). Exogenous addition of lysosomal enzyme to deficient fibroblasts results in internalization of the added enzyme (2, 19–21) and internalized enzyme accumulates in both types of lysosomal particles (12). Using secretions from wild-type CHO cells as a source of acid α -mannosidase, I obtained partial restoration of enzymatic

activity in N12-5-2; this activity sedimented in the two fractions corresponding to endogenous lysosomal enzymes. Finally, internalization of lysosomal enzyme by diploid fibroblasts is inhibited by mannose 6-phosphate and fructose 1-phosphate but not by other phosphorylated carbohydrates (19–21); similar results were obtained here in experiments measuring restoration of α -mannosidase activity in N12-5-2.

With refinement of the screening procedure presented here, it should be possible to identify mutants defective in posttranslational modification and uptake of lysosomal enzymes. The ability to isolate mutants of lysosomal function in cell culture will increase the repertoire of cells available for biochemical and genetic studies.

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