The Mannose 6-Phosphate Receptor of Chinese Hamster Ovary Cells

COMPARTMENTALIZATION OF ACID HYDROLASES IN MUTANTS WITH ALTERED RECEPTORS*

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The localization of acid hydrolases was examined in Chinese hamster ovary cells with defective mannose 6-phosphate receptors; these mutants had been shown to exhibit reduced uptake and altered binding of exogenously added acid hydrolase (Robbins, A. R., Myerowitz, R., Youle, R. J., Murray, G. J., and Neville, D. M., Jr. (1981) J. Biol. Chem. 256, 10618-10622). Cells were grown in the presence of [3H]mannose, [3H]-a-l-iduronidase and [3H]-beta-hexosaminidase, which were immunoprecipitated sequentially, electrophoresed on polyacrylamide gels containing sodium dodecyl sulfate, and detected by fluorography. About 55% of the [3H]-a-l-iduronidase and [3H]-beta-hexosaminidase synthesized by the mutants in 12 h was found in the growth medium; parental cells secreted only ~15%. The mutants also secreted 2 to 6 times more alpha-mannosidase, beta-glucuronidase, and a-l-fucosidase than the parent as determined by measurements of enzyme activity. Intracellular levels of these enzymes were reduced in the mutants. The mutants secreted acid hydrolases in the precursor forms, within the cells these enzymes resided in lysosomes and were processed normally; thus, the mutants appeared aberrant only with respect to distribution of hydrolases between intracellular and extracellular compartments. [3H]S-Gluthionyl-labeled beta-hexosaminidase and alpha-l-iduronidase secreted by the mutants were taken up normally by both human fibroblasts and wild type CHO cells, and this uptake was inhibited by mannose 6-phosphate. Thus, the elevated secretion of acid hydrolases was not due to alteration of the mannose 6-phosphate recognition marker on the enzymes, but appears to result from alterations in the mannose 6-phosphate receptor.

Recognition of mannose 6-phosphate residues by a receptor on fibroblasts has been shown to mediate the binding and uptake of exogenously added acid hydrolases (1-6). It has been postulated that this same receptor functions in the translocation of newly synthesized endogenous acid hydrolases to the lysosomes (7-10), based primarily on the observation that mutant human fibroblasts in which lysosomal enzymes lack the mannose 6-phosphate recognition marker secrete abnormal amounts of newly synthesized hydrolases (11-13). These mutant fibroblasts are isolated from patients with mucolipidosis II (I-cell disease), a disorder in which the receptor, has not yet been observed in patients with lysosomal storage disorders.

In the preceding paper (15), we showed that mutant Chinese hamster ovary cells selected for resistance to a conjugate of ricin and alpha-(6-phospho)-pentamannose have altered mannose 6-phosphate receptors. In this report we examine the biosynthesis and localization of lysosomal enzymes in these receptor-defective mutants.

EXPERIMENTAL PROCEDURES

Materials—4-Methylumbelliferyl alpha-L-iduronide was synthesized (16) and supplied by Dr. Bernard Weissmann, University of Illinois; other 4-methylumbelliferyl substrates were purchased from Research Products International. [2-3H]Mannose (16 Ci/mmol), [35S]Methionine (1050 Ci/mmol), and [14C]Methylated proteins (3 to 30 Ci/mg) were obtained from Amersham. Antisera raised in goats against human kidney alpha-L-iduronidase (17) and human placental beta-hexosaminidase A, B, and the alpha subunit (18) were provided by Drs. Leonard H. Rome, Andre Hasilik and Elizabeth F. Neufeld (National Institutes of Health). Colloidal silica (Ludox HS 40) was a gift from DuPont. Bovine serum albumin (A grade) was obtained from Calbiochem; sodium dodecyl sulfate was purchased from Sigma. Other reagents were purchased from standard commercial suppliers.

Cells and Cell Culture—The CHO cells, media, reagents, and conditions of culture used in this study are described in the preceding paper (15). Human diploid fibroblasts were provided by Dr. Elizabeth F. Neufeld. For maintenance of cells in serum-free medium, we used Waymouth medium MAB 87-3 (19), formulated as described in the GIBCO catalogue, to which was added bovine serum albumin (1 mg/ml).

Labeling of Cells—Cells were grown on 100-mm dishes to 2-5 x 10^6 cells/dish. Before labeling with [2-3H]mannose, the cells were rinsed and incubated 1 h in 4 ml of Eagle's minimal essential medium with glucose reduced to 139 mM, supplemented with antibiotics, nonessential amino acids, 15 mM Tricine, and 10% dialyzed fetal bovine serum. Labeling was started by addition of 4 ml of this medium containing 0.4 mCl of [2-3H]mannose, 16 Ci/mmol; prior to use, the [3H]mannose was dried under nitrogen and redissolved in medium. In pulse-chase experiments, labeling was terminated by the addition of glucose to 5.6 mM. Before labeling with [35S]Methionine, the cells were rinsed three times with Eagle's minimal essential medium, lacking methionine and supplemented with antibiotics, nonessential amino acids and dialyzed fetal bovine serum (10%). Cells were labeled in 4 ml of this same medium containing 0.1 mCi of [35S]Methionine diluted with carrier to a final specific activity of 7.7 Ci/mmol.

At the indicated times, the growth medium was collected, and protein was precipitated with 70% (NH4)2SO4. After dialysis, the volume was adjusted to 0.5 ml with 10 mM NaPO4, pH 6.8, containing 0.15 M NaCl. Following addition of 2.5 ml of 20% Triton X-100, the samples were centrifuged for 3 h at 18,000 rpm in a Sorvall rotor SM24 and the clarified supernatants were used for immunoprecipitation.

Cells were harvested using 1 ml of 0.1% trypsin, and collected into growth medium containing 10% fetal bovine serum to stop the action of trypsin; the cells were then centrifuged and washed once in solution A (0.14 M NaCl, 7 mM KCl, 1 mM Na2P i B, pH 7.4). The cell pellet was resuspended in 0.5 ml of 80 mM Tris-HCl, pH 7.0, frozen and thawed.

After addition of 2.5 ml of 2% Triton X-100, the suspension was twice
subjected to sonication for 30 s in a cold bath (Ultrasonic cleaner, Laboratory Supplies Co, Hicksville, NY). To each tube was added 5 µl of 3% protamine sulfate. After 20 min on ice, the samples were centrifuged to a final concentration of 5.6 mM (Ch, chase). Each lane represents the immunoprecipitate from cells of two dishes. Fluorographic exposure time was 7 days.

Immunoprecipitation of Radioactive Enzymes, Polyacrylamide Gel Electrophoresis, and Fluorography—The method used for immunoprecipitation is a modification of that described by Hasilik and Neufeld (18). To prepare the clarified supernatant fractions obtained from growth medium or cells, 0.5 µg of partially purified human urinary α-1-iduronidase (2), 0.5 µg of purified human placental a-L-iduronidase (2), 0.5 µg of partially purified bovine serum albumin (69,000), ovalbumin (46,000), carbonic anhydrase (30,000), and lysozyme (14,000) were dialyzed against this same buffer, and subsequently used for assay of enzyme activities. Cells were harvested by trypsinization, washed once with solution A, then lysed by repeated cycles of freezing and thawing.

Enzyme Assays—α-L-Iduronidase (EC 3.2.1.76) was assayed using 4-methylumbelliferyl α-L-iduronide (4); one unit of enzyme activity represents 1 nmol of substrate hydrolyzed/h at ambient temperature. α-Mannosidase (EC 3.2.1.24), β-glucuronidase (EC 3.2.1.31), β-hexosaminidase (EC 3.2.1.30), and α-1-fucosidase (EC 3.2.1.51) were assayed as previously described (22). For these enzymes one unit of enzyme activity represents 1 nmol of substrate hydrolyzed/h at 37 °C. Protein was measured by the method of Lowry et al. (23).

Uptake of Radioactive Enzymes—Cells were labeled with [35S]methionine as described above with the addition of NH4Cl to 10 mM in the medium to maximize enzyme secretion (18, 24). After 12 h, the growth medium was collected and protein was precipitated with 70% (NH4)2SO4. The samples were centrifuged at 18,000 rpm for 1 h; aliquots of the clarified supernatants were counted. Recipient cells, grown on 100-mm dishes, were washed once with fresh growth medium. Growth medium (4 ml) containing 2.5 × 106 cpm of radioactive protein was filtered onto the cells through a Millex 0.22 µm filter (Millipore). After 15 h, recipient cells and growth medium were harvested as described above, with the single exception that human fibroblasts were removed from the dishes using 0.25% trypsin. Immunoprecipitation of radioactive enzymes,

| β-Hexosaminidase | α-L-Iduronidase |
|------------------|-----------------|
| P    | Ch  | P    | Ch  |
| 1h   | 14  | 3h   | 14  |
| 24h  | 14  | 24h  | 14  |
| Mr x 10^-3 | 200 | 200 |

FIG. 1. Synthesis of β-hexosaminidase (A) and α-L-iduronidase (B) in wild type CHO cells. Cells grown to 2 × 10⁶/100-mm dishes were incubated with [2-H]mannose for the times indicated (P, pulse) in glucose-deficient medium, then unlabeled glucose was added to a final concentration of 5.6 mM (Ch, chase). Each lane represents the immunoprecipitate from cells of two dishes. Fluorographic exposure time was 7 days.

1 The abbreviations used are: SDS, sodium dodecyl sulfate; CHO cells, Chinese hamster ovary cells.
RESULTS

Like human fibroblasts, wild type CHO cells first synthesized the acid hydrolases β-hexosaminidase and α-L-iduronidase as larger precursors which were subsequently processed (18, 25). The molecular weights of the two precursor polypeptides of β-hexosaminidase were 69,000 and 63,000; the Mₐ’s of the mature polypeptides were 59,000 and 52,000 (Fig. 1A). Using specific anti-α antiserum, the polypeptides of Mₐ = 69,000 and 55,000 were shown to correspond to the α chain of the human enzyme; with antiserum directed against β-hexosaminidase B, the polypeptides of 63,000 and 52,000 were shown to correspond to the human β-chain. The precursor of α-L-iduronidase had an Mₐ = 78,000, which was then processed through an intermediate, Mₐ = 74,000 to a final Mₐ = 72,000 (in Fig. 1B, the precursor and intermediate forms are seen as a doublet after a 3 h pulse).

Fig. 2 compares the distribution of β-hexosaminidase newly synthesized by mannose 6-phosphate receptor-defective mutants and parental cells. The mutants A10-2-4 and B4-2-1 secreted 57 and 51%, respectively, of the β-hexosaminidase labeled in a 9-h pulse, while the parent secreted only 13%. Enzyme secreted by the three cell types was in the precursor form, as has been reported for human fibroblasts (18). Most of the β-hexosaminidase found in extracts of parent and mutant cells was in the mature form (Fig. 2). Both precursor and processed forms of β-hexosaminidase from B4-2-1 migrated somewhat more rapidly on SDS-polyacrylamide gel electrophoresis than did enzyme from other CHO cells.

The mutants also secreted increased amounts of newly synthesized α-L-iduronidase (Fig. 3); media collected from A10-2-4, B4-2-1, and the parent N211-1-8 contained 64, 58, and 16%, respectively, of the enzyme labeled in a 12-h pulse. Secreted enzyme was in the precursor form, while enzyme in the cell extracts was found as the Mₐ = 74,000 intermediate. As with β-hexosaminidase, α-L-iduronidase synthesized by B4-2-1 moved somewhat more rapidly than enzyme from other CHO cells.

Consistent with the results of metabolic labeling, medium conditioned by the mutants contained elevated levels of acid hydrolase activities (Table I). Secretions collected from A10-2-4 had 3 to 4 times more activities of the 5 hydrolases tested than did secretions from the parent. With the exception of α-L-iduronidase, hydrolase activities in medium conditioned by B4-2-1 were also elevated (2- to 6-fold). Hydrolase activities

![Image](image_url)

**FIG. 3.** Synthesis of α-L-iduronidase by parent and mutant cells. Each of two 100-mm dishes of N211-1-8 (N), B4-2-1 (B) and A10-2-4 (A), at 4 x 10⁶ cells/dish, was incubated with [³²°H]mannose for 12 h. Contents of the two dishes were pooled for immunoprecipitation; total cell protein for the three cell types was 1.4 ± 0.1 mg. Fluorographic exposure time was 14 days.

**TABLE I**

| Source | Enzyme           | N211-1-8 | A10-2-4 | B4-2-1 |
|--------|------------------|----------|---------|--------|
|        |                  | units/mg cell protein |
| Media  | β-Hexosaminidase | 54       | 160     | 230    |
|        | α-L-Iduronidase  | 1        | 3       | 0.3    |
|        | α-L-Fucosidase   | 31       | 92      | 74     |
|        | β-Glucuronidase  | 17       | 65      | 110    |
|        | α-Mannosidase    | 47       | 140     | 100    |
| Cells  | β-Hexosaminidase | 460      | 350     | 290    |
|        | α-L-Iduronidase  | 18       | 12      | 4      |
|        | α-L-Fucosidase   | 630      | 550     | 400    |
|        | β-Glucuronidase  | 440      | 190     | 320    |
|        | α-Mannosidase    | 210      | 46      | 49     |

**FIG. 4.** Uptake of [³⁵S] β-hexosaminidase from mutant and parent cells into human fibroblasts. Secretions prepared from A10-2-4 (A), B4-2-1 (B) and N211-1-8 (N) after growth of those cells with [³⁵S]methionine, were added to two dishes each of human fibroblasts. One dish of each pair also received mannose 6-phosphate (M₆P), to a final concentration of 2 mM. Recipient cells and growth media were harvested after 15 h and enzyme was immunoprecipitated. Total cell protein was 1.2 ± 0.1 mg. Fluorographic exposure time was 4 days.
in cell extracts of A10-2-4 and B4-2-1 ranged from 21 to 87% and 22 to 77%, respectively, of the activities measured in extracts of the parent N211-1-8 (Table I). While hydrolase activity found in the medium represents enzyme synthesized over the 12-h period of the experiment, activity measured in cell extracts reflects both new and previously synthesized enzyme; due to the long lifetimes of acid hydrolases, newly synthesized enzyme contributes only a small fraction of total intracellular activity.

Cell-associated acid hydrolases were compartmentalized in lysosomes in the receptor-defective mutants. After centrifugation of extracts from mutant, parent, and wild type cells on gradients of colloidal silica (22,26), a-L-fucosidase, a-mannosidase, and a-hexosaminidase activities were found in the two peaks which are characteristic for lysosomes on this gradient (data not shown).

Previous studies have shown that the secreted forms of acid hydrolases are internalized by recipient cells via recognition of the mannose 6-phosphate marker on those enzymes (22, 27, 28). Secretions prepared from parent and mutant cells metabolically labeled with [35S]methionine were added to human fibroblasts, more than 60% of the a-hexosaminidase present in secretions from the three types was internalized and uptake was inhibited by mannose 6-phosphate (Fig. 4). Similar results were obtained in studies of a-L-iduronidase uptake, and using wild type CHO cells rather than human fibroblasts as recipients. Thus, unlike the elevated secretion of hydrolases observed with fibroblasts from patients with 1-cell disease (11-13), increased secretion by A10-2-4 and B4-2-1 is not associated with lack of the mannose 6-phosphate recognition marker on those hydrolases.

Note in Fig. 4 that the labeled polypeptides immunoprecipitated from the recipient human cells were located in the same positions on the SDS-polyacrylamide gel as the mature a and b chains of the hexosaminidase found in the CHO cell donors (Fig. 2). Results of the reciprocal experiment, i.e. uptake into CHO cells of the hexosaminidase from human fibroblasts, are shown in Fig. 5. As would be expected, the receptor-defective mutants internalized less enzyme than the parent but both parent and mutant cells processed the human enzyme to polypeptides of molecular weights 54,000 and 29,000, the sizes of the mature a and b chains found in human fibroblasts (12). Thus, the differences observed between human fibroblasts and CHO cells with respect to the molecular weights of mature hexosaminidase appear to reflect differences in the polypeptides being processed rather than in the processing systems.

**DISCUSSION**

Exogenously added acid hydrolases are taken up by cultured fibroblasts (1-3) and delivered to lysosomes (26). Compelling evidence exists for the participation of the mannose 6-phosphate receptor in enzyme uptake (1-7). Current theories on the compartmentalization of endogenous acid hydrolases into lysosomes invoke the participation of the same receptor (7-10).

We show here that CHO mutants with defective receptors secrete 2 to 6 times more newly synthesized lysosomal enzyme than does the parental cell line. Acid hydrolases secreted by the mutants are taken up into recipient cells via the mannose 6-phosphate receptor. This rules out the possibility that the mutants studied here have a defect analogous to that of human I-cells, in which incorrect compartmentalization of lysosomal enzymes is associated with the absence of the mannose 6-phosphate recognition marker from those enzymes (12, 13). That portion of the mutants' acid hydrolases that is retained in the cells is located in lysosomes, and cell-associated hydrolases are processed normally by the mutants. Both mutants exhibit residual mannose 6-phosphate receptor activity (15); thus, we cannot distinguish whether the presence of mature hydrolases in the mutants' lysosomes reflects residual receptor activity or a secondary mechanism in CHO cells for delivery of enzymes to the lysosomes.

Our results suggest that the same receptor functions in both internalization of extracellular enzymes and compartmentalization of endogenous enzymes, or that the receptors involved in the two functions share a common subunit. At present we do not know whether the receptor-defective mutants have undergone a change in the primary structure of the receptor or in some post-translational modification (e.g. glycosylation) essential for receptor function. In addition, we cannot yet rule out that the receptors involved in uptake and compartmentalization are distinct entities pleiotropically affected by a defect in some modifying enzyme. Discrimination between these possibilities awaits structural analyses of the normal and altered receptors.

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**Fig. 5.** Uptake of [35S] a-hexosaminidase from human fibroblasts into mutant and parent cells. Secretions prepared from human fibroblasts grown with [35S]methionine were added to two dishes each of A10-2-4 (A), B4-2-1 (B), N211-1-8 (N); two dishes of N211-1-8 also received mannose 6-phosphate, to a final concentration of 2 mM (N'). Recipient cells were harvested after 15 h, pairs of dishes were pooled, and enzyme was immunoprecipitated. An aliquot of the human secretions equal to that applied to a pair of dishes was also immunoprecipitated (HS). Total cell protein for the recipient cells was 0.9 ± 0.1 mg. Fluorographic exposure time was 20 days.
Acid Hydrolase Distribution in Receptor-defective Mutants

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