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Synthesis of β-hexosaminidase in cell-free translation and in intact fibroblasts: An insoluble precursor α chain in a rare form of Tay–Sachs disease

(lysosomal enzymes/mannose 6-phosphate/intracellular transport/Sandhoff disease)

RICHARD L. PROIA AND ELIZABETH F. NEUFELD

Genetics and Biochemistry Branch, National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases, Bethesda, Maryland 20205

Contributed by Elizabeth F. Neufeld, July 22, 1982

ABSTRACT **RNA was isolated from human term placenta or cultured fibroblasts and translated in a rabbit reticulocyte system in the presence of [^{35}S]methionine; the translation products were immunoprecipitated with antisera made against β-hexosaminidase or its isolated α and β chains and analyzed by polyacrylamide gel electrophoresis. The largest translated α and β chain polypeptides had M_rs of 65,000 and 59,000, respectively. These are ≈2,000 greater than the M_rs of precursor chains synthesized by intact fibroblasts and deglycosylated with *endo*-β-*N*-acetylglucosaminidase H suggesting the presence of a signal sequence. RNA of fibroblast cultures from two patients with Sandhoff disease did not direct the translation of immunoprecipitable β chain; RNA of fibroblast cultures from four patients with Tay–Sachs disease (three of Ashkenazi Jewish descent and one of non-Jewish descent) did not direct the translation of immunoprecipitable α chain. In contrast, a normal amount of α chain was made in the presence of RNA from the fibroblast culture of another non-Jewish Tay–Sachs patient (GM 1110). Intact fibroblasts from this patient also synthesized the α chain as shown by labeling with [^{3}H]leucine; however, strong detergent was required for extraction. The α chain could be labeled with [^{3}H]mannose but not with [^{32}P]-phosphate; it was neither secreted nor accumulated in the proteolytically processed form, and it disappeared within a day of synthesis. A plausible though not unique explanation is that the insoluble α chain is not transported from the endoplasmic reticulum (the site of glycosylation) to the Golgi apparatus (the site of phosphorylation) nor to further points of destination—lysosomes and the exterior of the cell.**

β-Hexosaminidase is a lysosomal enzyme relevant to several heritable diseases. The A isozyme of β-hexosaminidase is composed of α and β subunits, which are structurally dissimilar and encoded on different chromosomes; the B isozyme is composed of β subunits only. Tay–Sachs disease affects the α subunit and, therefore, the A isozyme; Sandhoff disease affects the β (or common) subunit and, therefore, both isozymes A and B. A third disease entity (AB variant) allows the production of normal β-hexosaminidase but not of its activator. These diseases are characterized by marked accumulation of G_{M2} ganglioside, the major natural substrate of β-hexosaminidase A, resulting in progressive loss of nervous system function; they are usually fatal in early childhood. Variant forms of later onset and reduced severity are also known. The clinical, genetic, and biochemical aspects of these disorders have been reviewed (1).

We previously have studied the synthesis and processing of the two chains of β-hexosaminidase in cultured human skin fibroblasts (2–4). The chains are synthesized as precursors of M_r 67,000 (α) and M_r 63,000 (β), which are processed proteolytically to the size that is characteristic of subunits of the enzyme isolated from tissues (α, M_r 54,000; β, M_r 29,000 plus smaller fragments). Similar studies with fibroblasts from two patients with Tay–Sachs disease and one patient with Sandhoff disease had shown the absence of α and β chains, respectively.

We have extended these studies to the level of cell-free translation by using RNA extracted from placenta, normal fibroblasts, and fibroblasts of several patients with G_{M2} gangliosidoses. In the course of this work, we found that cells of one of the Tay–Sachs patients previously reported to make no α chain (2) in fact did synthesize an altered α polypeptide.

MATERIALS AND METHODS

Reagents. Nuclease-treated rabbit reticulocyte lysate, L-[^{35}S]methionine (1,000–1,500 Ci/mmol; 1 Ci = 3.7 × 10^{10} becquerels), L-[4,5-^{3}H]leucine (40 Ci/mmol), [2-^{3}H]mannose (13.2 Ci/mmol), and [^{14}C]methylated M_r standards were purchased from Amersham. Carrier-free [^{32}P]phosphate was from New England Nuclear. Nonidet P-40 and fixed, protein A-bearing *Staphylococcus aureus* (Immuno-Precipitin, 10% wt/vol suspension) were from Bethesda Research Laboratories. $NaDodSO_4$ (No. 5750, containing other alkyl sulfates) and aprotinin were from Sigma. Goat antiserum to human fibronectin was from Cappel Laboratories (Cochranville, PA). Goat antisera to human placental β-hexosaminidase A and B and to isolated α and β chains of the enzyme had been prepared by A. Hasilik as described (2). *endo*-β-*N*-Acetylglucosaminidase H (*endo* H) was from Health Research (Albany, NY). Other reagents were purchased from standard commercial suppliers.

RNA Isolation and Cell-Free Translation. Human term placenta was obtained from a local hospital immediately after delivery, quickly frozen in liquid nitrogen, and stored at −70°C. Total RNA was isolated from this tissue by the guanidine·HCl procedure of Deeley *et al.* (5). Total RNA was isolated from cultured human fibroblasts by a similar method with the modifications of Tolstoshev *et al.* (6).

Cell-free translation was performed with a nuclease-treated rabbit reticulocyte lysate under conditions determined to be optimal for protein synthesis with respect to time, temperature, and RNA concentration. Total RNA and [^{35}S]methionine were added at 400 μg/ml and 2 mCi/ml, respectively. Translation was carried out at 30°C for 75 min. After this incubation, the translation mixtures (50–100 μl) were diluted to 1 ml with Tris/NaCl (0.01 M Tris·HCl/0.15 M NaCl/0.02% NaN_3, pH 7.4) containing 0.1% $NaDodSO_4$, 1% Nonidet P-40, and 0.01 M EDTA. Aprotinin (50 μg) and phenylmethylsulfonyl fluoride (1 μmol) were added to prevent proteolysis.

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Abbreviations: *endo* H, *endo*-β-*N*-acetylglucosaminidase H; Tris/NaCl, 0.01 M Tris·HCl/0.15 M NaCl/0.02% NaN_3, pH 7.4.

Cell Culture and Labeling. Fibroblasts from patients with Tay–Sachs disease were obtained from the Human Genetic Mutant Cell Repository (Institute for Medical Research, Camden, NJ). Fibroblasts from patients with Sandhoff disease were provided by G. Thomas (John F. Kennedy Institute, Baltimore, MD). Other fibroblasts were derived from samples submitted to this laboratory for diagnosis. The cells were maintained as described (2).

Conditions for labeling fibroblasts with [^{3}H]leucine, [2-^{3}H]-mannose, and [^{32}P]phosphate were as described by Hasilik and Neufeld (2, 3) except that the glucose concentration in the medium was decreased to 25 μg/ml for labeling with [^{3}H]mannose.

After labeling, cell extracts were prepared by one of three methods. The procedure in method 1 is the same as that previously described (2): briefly, the cells were harvested by trypsinization, washed, and suspended in 0.5 ml of 50 mM Tris·HCl (pH 7.0); the samples were frozen, thawed, and subjected to sonication. In method 2, the cell monolayers were gently rinsed with 0.9% NaCl and extracted with 1 ml of Tris/NaCl containing 1% Nonidet P-40 (7). In method 3, the cells were harvested by trypsinization, washed, and dissolved by heating at 100°C for 5 min in 0.3 ml of Tris/NaCl containing 1% $NaDodSO_4$; after being cooled, the mixture was diluted to 1 ml with Tris/NaCl containing 2% Nonidet P-40 and 10 mg of bovine serum albumin per ml.

The cell extracts prepared by each of these methods were treated with protamine sulfate (2).

Immunoprecipitation and Polyacrylamide Gel Electrophoresis. The β-hexosaminidase from concentrates of medium was immunoprecipitated directly as described (2). β-Hexosaminidase in cell extracts and in translation mixtures was immunoprecipitated with the aid of protein A-bearing *Staphylococcus aureus* after removal of major contaminants by a prior absorption step.

For this absorption, the translation mixtures were incubated with 5 μl of normal goat serum and 50 μl of the 10% *S. aureus* suspension for 15 min on ice. The cell extracts were first incubated with 10 μl of goat anti-human fibronectin (30 min on ice), followed by incubation with 100 μl of *S. aureus* suspension for 15 min on ice. In each case, after centrifugation, the supernatants were treated an additional two times with the *S. aureus* suspension only.

The absorption-purified translation mixtures and cell extracts were mixed with 2.5 μl of the appropriate antiserum and incubated for 16 hr at 4°C. After a 15-min incubation with 30 μl of *S. aureus*, the immune complexes were collected by centrifugation and washed four times with 0.01 M Tris·HCl, pH 8.6/0.6 M NaCl/0.1% $NaDodSO_4$/0.05% Nonidet P-40. The pellets were transferred to clean tubes with Tris/NaCl, centrifuged, suspended in 55 μl of gel sample buffer (125 mM Tris·HCl, pH 6.8/1% $NaDodSO_4$/10% glycerol) and heated for 5 min at 100°C. The bacteria were removed by centrifugation, and the supernatant solution was heated for an additional 5 min at 100°C after addition of dithiothreitol to a final concentration of 16.6 mM.

Polyacrylamide gel electrophoresis was performed by the method of Laemmli (8) with minor modifications (2). Radioactive bands were visualized by fluorography (9, 10).

***endo* H Digestion.** After the immunoprecipitated β-hexosaminidase was released from *S. aureus* by heating at 100°C for 5 min in 100 μl of 10 mM sodium acetate, pH 5.5/0.3% $NaDodSO_4$/10 μM pepstatin A, the bacteria were removed by centrifugation. The supernatant was diluted 1:3 with the same buffer without $NaDodSO_4$ and divided into two aliquots. One aliquot received 0.01 unit of *endo* H and the other received no enzyme. Both aliquots were incubated for 16 hr at 37°C, lyophilized, and dissolved in gel sample buffer for analysis by polyacrylamide gel electrophoresis.

RESULTS

Identification of the α and β Chains of β-Hexosaminidase Translated in the Presence of Placental RNA. Antiserum to β-hexosaminidase A, which reacts with both α and β chains, precipitated polypeptides of apparent M_rs of 65,000, 59,000, 35,000 (minor), 34,000, and 20,000. By use of antisera specific for the β chain (anti-β-hexosaminidase B and anti-denatured-β-chain) and for the α chain (anti-denatured-α-chain), the M_r 65,000 and M_r 35,000 polypeptides were identified as α chain, and those of M_rs 59,000, 34,000, and 20,000, as β chain. The two larger polypeptides (M_rs 65,000 and 59,000) were assumed to be the primary α and β translation products, respectively, and the smaller ones, proteolytic fragments, although we were unsuccessful in preventing their appearance by use of proteinase inhibitors. By comparing the intensity of the radioactive bands of β-hexosaminidase chains and of placental lactogen (11), we estimated that each chain represents 0.002–0.01% of the total translation products.

The translated α and β polypeptides (M_rs 65,000 and 59,000) were smaller than the corresponding precursor chains isolated from intact fibroblasts (α, M_r 67,000; β, M_rs 63,000 and 61,000) but larger than the same precursors after deglycosylation with *endo* H (α, M_r 63,000; β, M_r 57,000) (Fig. 1).

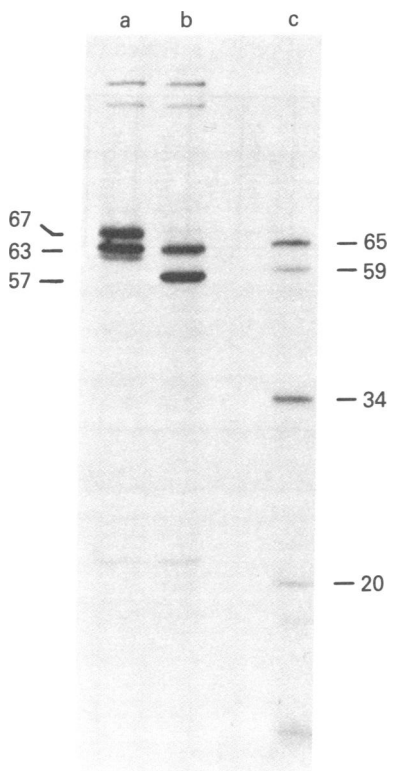

FIG. 1. Comparison of the α and β chains of β-hexosaminidase synthesized in cell-free translation and in intact fibroblasts. One 100-mm dish of normal fibroblasts was labeled for 1 hr with 0.5 mCi of [^{3}H]leucine. The cells were extracted by method 2 and treated with anti-β-hexosaminidase A. The immunoprecipitated β-hexosaminidase was solubilized and divided into two aliquots, one serving as a control (lane a) and the other receiving *endo* H (lane b). Human placental RNA was translated in a rabbit reticulocyte protein-synthesizing system, and the translation products were immunoprecipitated with anti-β-hexosaminidase A (lane c). Fluorography of the electrophoretic gels was for 7 days. M_rs are shown $\times 10^{-3}$.

Cell-Free Translation with RNA from Tay–Sachs and Sandhoff Fibroblasts. Translation products directed by the RNA of control fibroblasts (normal and Hurler) were essentially identical to those of placental RNA (Fig. 2). Those directed by RNA of fibroblasts from two unrelated patients with Sandhoff disease contained α chains (M_rs 65,000 and 35,000) but not β chains, whereas those directed by fibroblast culture GM 515 from a Tay–Sachs patient contained β chains (M_rs 59,000 and 34,000) but not α chain (Fig. 2). A similar absence of α chain was observed when RNAs from three other patients with Tay–Sachs disease (GM 221, GM 2968, and GM 502) were translated (not shown). By contrast, a normal amount of the M_r 65,000 α chain was directed by the RNA from Tay–Sachs fibroblasts GM 1110 (Fig. 2). This result was unexpected because it had been reported that intact GM 1110 fibroblasts do not synthesize the α chain (2), and prompted us to reinvestigate the synthesis of β-hexosaminidase in these cells.

Extraction of the α Precursor Chain Synthesized by GM 1110 Fibroblasts. Normal and GM 1110 fibroblasts were biosynthetically labeled with [^{3}H]leucine, and cell extracts were prepared in different ways. The procedure used previously (2), freeze-thawing and sonication, which efficiently extracted the α (M_r 67,000) and the β (M_r 63,000) precursor chains of β-hexosaminidase from normal fibroblasts, failed to extract the M_r 67,000 α chain from GM 1110 fibroblasts (Fig. 3, method 1). GM 1110 fibroblast extracts prepared with the nonionic detergent, Nonidet P-40, contained some α chain, although much less than extracts of normal fibroblasts contained (Fig. 3, method 2). Solubilizing the labeled cells by heating at 100°C in the presence of $NaDodSO_4$ revealed the presence of a normal amount of the α chain in the mutant fibroblasts (Fig. 3, method 3). Hot $NaDodSO_4$ extraction of biosynthetically labeled Tay–Sachs fibroblast lines GM 515, GM 221, GM 2968, GM 502, and GM 77 or of the two Sandhoff lines yielded no immunoprecipitable α or β chains, respectively (not shown).

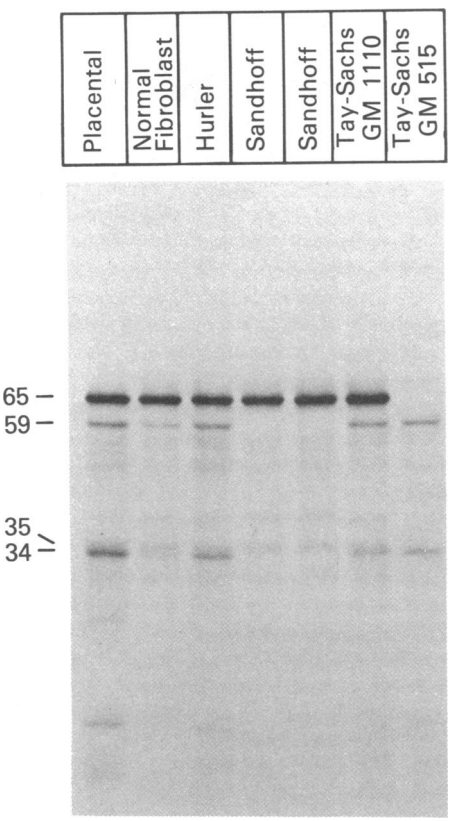

FIG. 2. Cell-free synthesis with RNAs from normal and mutant fibroblasts. Total RNA was isolated from the sources indicated and translated in a rabbit reticulocyte protein-synthesizing system; the translation products were immunoprecipitated with anti-β-hexosaminidase A. Fluorography of the electrophoretic gels was for 4 days. M_rs are shown $\times 10^{-3}$.

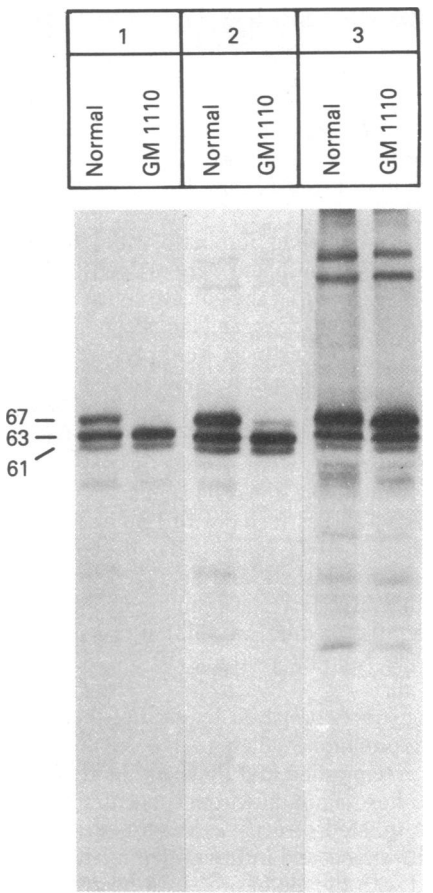

FIG. 3. Synthesis of β-hexosaminidase in intact normal and GM 1110 fibroblasts. Normal and GM 1110 fibroblasts were labeled with [^{3}H]leucine (0.5 mCi per 100-mm dish) for 2 hr. After this period, cell extracts were prepared by methods 1, 2, and 3 and immunoprecipitated with anti-β-hexosaminidase A. Fluorography of the electrophoretic gels was for 3 days. M_rs are shown $\times 10^{-3}$.

Fate of the α Precursor Chain in GM 1110 Fibroblasts. In normal human fibroblasts, the β-hexosaminidase chains mature through a series of proteolytic cleavages, with the α chain being converted from M_r 67,000 to M_r 54,000 and the β chain being processed from M_r 63,000 through an intermediate form of M_r 52,000 to M_r 29,000 and smaller fragments (2). In addition, a portion of the unprocessed chains are secreted into the medium. This is seen in Fig. 4, except that the small β chain fragments are not present after the extraction and immunoprecipitation conditions used here. The β chain of GM 1110 fibroblasts was processed and secreted normally. However, the M_r 67,000 α chain was not processed to the M_r 54,000 form in any significant amount and disappeared in a 24-hr chase; it was not found in the medium at any time, even if the medium was treated with $NaDodSO_4$ before immunoprecipitation or if the culture was labeled in the presence of 10 mM NH_4Cl.

The use of [^{3}H]mannose and [^{32}P]phosphate to label the enzyme biosynthetically showed that the GM 1110 fibroblasts could glycosylate but not phosphorylate the α chain (Fig. 5). Treatment of the GM 1110 α chain with *endo* H yielded a M_r 63,000 peptide electrophoretically indistinguishable from *endo* H-treated normal α chain (not shown).

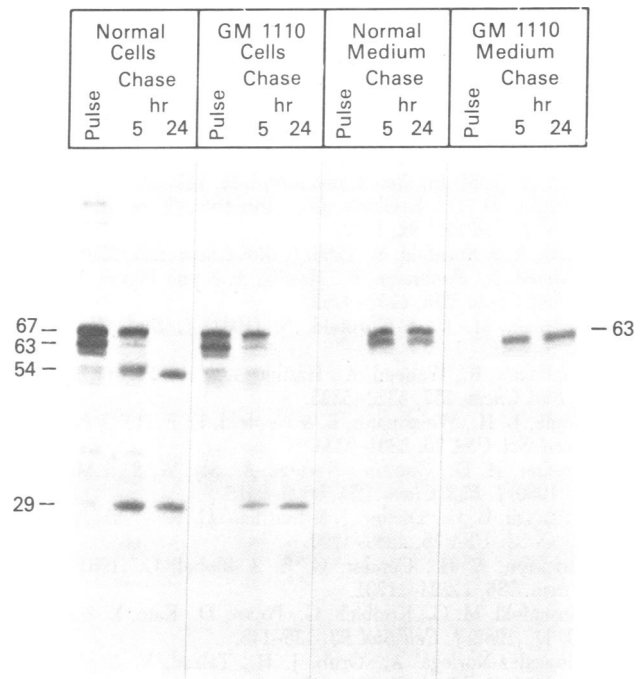

FIG. 4. Pulse–chase labeling of β-hexosaminidase in normal and GM 1110 fibroblasts. Three 100-mm culture dishes of normal and GM 1110 fibroblasts were labeled with 0.5 mCi of [^{3}H]leucine for 2 hr and, at the end of this period (pulse), one dish of each cell type was harvested. The remainder of the dishes received 50 μl of a sterile solution (5 mg/ml) of unlabeled leucine and were incubated for an additional 5 or 24 hr (chase). The cells were processed by method 3; cell and medium extracts were immunoprecipitated with anti-β-hexosaminidase A. Fluorography of the electrophoretic gels was for 2 days. M_rs are shown × 10^{-3}.

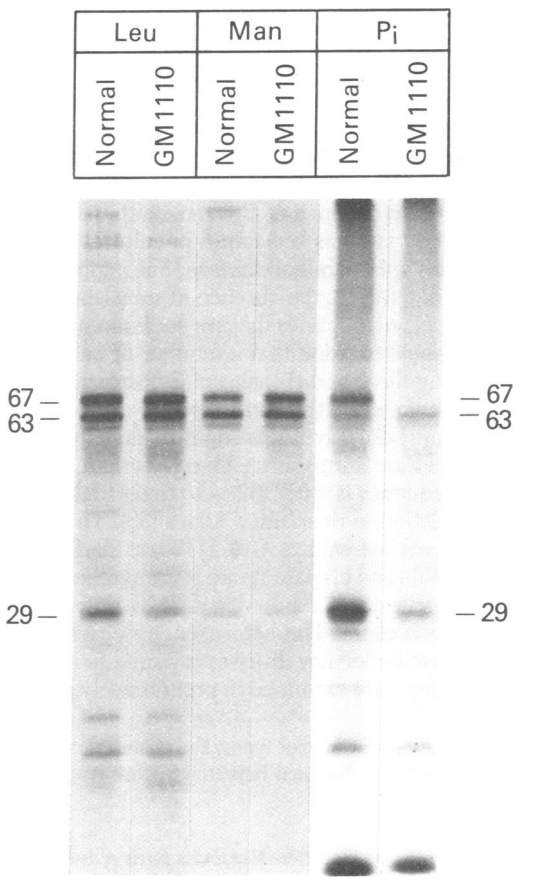

FIG. 5. Labeling of β-hexosaminidase with [^{3}H]leucine (left lanes), [^{3}H]mannose (center lanes), and [^{32}P]phosphate (right lanes) in normal and GM 1110 fibroblasts. Normal and GM 1110 fibroblasts in 100-mm dishes were labeled for 5 hr with 0.5 mCi of [^{3}H]leucine, 1.0 mCi of [2-^{3}H]mannose, or 0.85 mCi of [^{32}P]phosphate. The cells were processed by method 3, and the extracts were allowed to react with anti-β-hexosaminidase A. The immunoprecipitates were analyzed by polyacrylamide gel electrophoresis followed by fluorography for 2 days for [^{3}H]leucine and [2-^{3}H]mannose and for 20 days, without intensifying screen, for [^{32}P]phosphate. M_rs are shown × 10^{-3}.

DISCUSSION

The natural history of lysosomal enzymes starts much as that of secretory proteins—i.e., by synthesis on membrane-bound polysomes, followed by insertion of the nascent polypeptides into the endoplasmic reticulum, where the signal sequence is removed and high-mannose oligosaccharide chains are added (for reviews, see refs. 12 and 13). Enzymes destined for lysosomes are then equipped with mannose 6-phosphate recognition markers in the Golgi apparatus (14–17), bound to phosphomannosyl receptors (18–20), and transported to lysosomes. A portion of the newly formed phosphorylated lysosomal enzymes may be secreted, and a fraction thereof may be recaptured by the phosphomannosyl receptors on the cell surface. Both endogenous and endocytosed enzymes are subject to proteolytic processing after insertion into lysosomes (2, 4).

The synthesis of β-hexosaminidase in intact fibroblasts has been shown to include all of these steps except for the initial synthesis of a polypeptide with a signal sequence (2). The difference in size (ΔM_r = 2,000) between cell-free translation products and deglycosylated intracellular precursor chains suggests the existence of a transient signal sequence on both β-hexosaminidase polypeptides, as has been demonstrated for cathepsin D and for β-glucuronidase (21, 22).

The α chain is synthesized by GM 1110 fibroblasts in normal amounts but is not extracted in the absence of detergent. The altered α chain must be inserted into the endoplasmic reticulum because it becomes labeled by [^{3}H]mannose; the similar size of the glycosylated and *endo* H-deglycosylated α chain of GM 1110 and of normal fibroblasts suggests cleavage of the presumed signal sequence. Though glycosylated, the α chain of GM 1110 is not phosphorylated. It does not accumulate in the processed M_r 54,000 form, nor is it found in the medium. This is the case even in the presence of NH_4Cl, which enhances the secretion of most newly formed lysosomal enzymes (2, 23). It may be that the insolubility of the α chain prevents its transfer from the endoplasmic reticulum to the Golgi apparatus (the site of phosphorylation) and further transport to lysosomes or out of the cell. Alternatively, the altered α chain may be mistaken by the cell for a membrane protein or else bound to an inappropriate receptor. It also is possible that an insoluble α chain is a normal biosynthetic intermediate, which becomes soluble after a processing step to which the GM 1110 α chain is resistant.

Thus, GM 1110 belongs to a still small group of mutants in which the primary defect affects the transport of a protein through intracellular organelles. In I-cell disease and pseudo-Hurler polydystrophy, a deficiency of the first enzyme required for the formation of the mannose 6-phosphate recognition marker causes a major portion of lysosomal enzymes to be secreted (24–26). A less profound alteration in the distribution of enzymes between lysosomes and secretions occurs in Chinese hamster ovary cell mutants with defective phosphomannosyl receptors (27). In one type of α_1-antitrypsin deficiency, an insoluble variant form of antitrypsin aggregates and accumulates in the endoplasmic reticulum (28, 29). Some vesicular stomatitis

virus mutants blocked in G-protein maturation (30) and an altered HLA-A2 antigen of mutant lymphoblastoid cells (31) fail to reach the cell surface.

In contrast to GM 1110, four other Tay–Sachs disease cultures tested in intact cells and in cell-free translation, and a fifth culture tested in intact cells only, failed to synthesize the α chain. Three of these cultures were from patients of Jewish origin. An analogous absence of β chain synthesis in the whole cell and cell-free systems was found in cultures from two unrelated patients with Sandhoff disease. Such apparent absence of synthesis may reflect the absence of translatable mRNA or the formation of polypeptides that are not immunoprecipitable.

It is reasonable to think that a number of mutations in the genes encoding the α and β chains of β-hexosaminidase would have arisen independently, and to expect different defects in unrelated kindreds. Tay–Sachs disease is very rare except among Jews of East European (Ashkenazi) origin; in that group, the carrier frequency is 0.037 (32)—10 times higher than in the general population in the United States (33). The patient from whom the fibroblast culture GM 1110 was derived is of non-Jewish extraction and his parents are consanguineous. It is probable that his cells contain a double dose of a rare mutant gene for an altered α chain. The heterogeneity observed in the β-hexosaminidase deficiency diseases is bound to increase as the molecular defects are examined in progressively greater depth.

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