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IMPAIRED PROTEOLYTIC PROCESSING OF LYSOSOMAL N-ACETYL-β-HEXOSAMINIDASE IN CULTURED FIBROBLASTS FROM PATIENTS WITH INFANTILE GENERALIZED N-ACETYLNEURAMINIC ACID STORAGE DISEASE

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Cultured skin fibroblasts from patients suffering with infantile generalized N-acetylneuraminic acid (NeuAc) storage disease accumulate free NeuAc in a population of lysosomes less dense than those observed in normal fibroblasts (1.035 vs. >1.07 mean density), as assessed by the distribution of lysosomal enzyme activities and NeuAc on Percoll gradients after subcellular fractionation. In the present study, normal and affected fibroblasts were labeled with [^{35}S]methionine, and cell homogenates or subcellular fractions from Percoll gradients were immunoprecipitated with polyclonal antibodies to lysosomal N-acetyl-β-hexosaminidase (Hex); immunoprecipitated polypeptides were analyzed by SDS-polyacrylamide gel electrophoresis. The synthesis and initial processing of Hex polypeptides were comparable in normal and affected fibroblasts, but mature polypeptides were quantitatively localized in "buoyant" lysosomes of affected cells, along with Hex activity; moreover, mature α-chain of Hex was ~2 kDa larger than that observed in normal cells. The molecular weight difference was apparently due to impaired proteolytic processing of α-chain in affected fibroblasts, since treatment of immunoprecipitated α-chain from normal and affected cells with neuraminidase and endo-β-N-acetylglucosaminidase H failed to resolve the molecular weight difference. The impaired processing was observed to be persistent (after a chase of up to 200 h), but had no apparent effect on the turnover or activity of Hex in affected fibroblasts. The observed proteolytic processing defect may be primary or secondary in infantile NeuAc storage disease.

Infantile generalized N-acetylneuraminic acid (NeuAc) storage disease is an inherited (autosomal recessive) disorder marked by the lysosomal accumulation of NeuAc and hyperexcretion of NeuAc in the urine (1-6). A more benign variant, Salla Disease, is also marked by intracellular accumulation and hyperexcretion

Abbreviations

NeuAc, N-acetylneuraminic acid; Hex, N-acetyl-β-hexosaminidase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Endo H, endo-β-N-acetylglucosaminidase H (*S. plicatus*); Endo F, endo-β-N-acetylglucosaminidase F (*F. meningosepticum*); 4-MU-GlcNAc, 4-methylumbelliferyl-β-N-acetylglucosamine.

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of NeuAc, although in lower amounts than observed in patients suffering from the infantile variant (7-10). Considerable evidence suggests that impaired lysosomal transport of NeuAc may result in intralysosomal NeuAc accumulation in both disorders (2,9-12), although the molecular defect has not been demonstrated in either variant. In addition, the genetic relationship between the infantile and Salla variants has not yet been established, although on the basis of biochemical findings these two variants appear to be more closely related to each other than to a third variant, sialuria, which is marked by NeuAc hyperexcretion in the apparent absence of intracellular NeuAc accumulation (13).

While there has been to this point no evidence of impaired lysosomal function (aside from NeuAc transport) in the NeuAc storage diseases, the dramatic alteration in lysosomal buoyancy previously observed (3) suggested the possibility of interference with other lysosomal functions. In addition to catabolic processes, limited proteolytic processing of lysosomal enzymes is also carried out in the lysosome (or a prelysosomal compartment); in the case of lysosomal N-acetyl-β-hexosaminidase (Hex), processing involves the cleavage of a 67 kDa glycosylated propolypeptide of α-chain to a 54 kDa mature polypeptide, and (via internal protelytic cleavage) conversion of a 63 kDa glycosylated β-chain propolypeptide to major mature polypeptides of 29 kDa and 25 kDa (reviewed in 14). Although the significance of the proteolytic processing of lysosomal enzymes remains something of a mystery (14), the fact that virtually all soluble lysosomal enzymes are subject to such limited proteolysis suggests a function for this processing.

In the study reported here, we demonstrate the impaired proteolytic processing of lysosomal Hex α-polypeptide chain in fibroblasts derived from patients suffering from the infantile variant of NeuAc storage disease. Although the relationship between impaired proteolytic processing of lysosomal enzymes and intralysosomal NeuAc accumulation is not yet clear, this finding may provide an important basis for further studies involving the diagnosis, genetic analysis, and biochemical characterization of the NeuAc storage diseases.

Materials and Methods

Cell culture and metabolic labeling. Fibroblasts from control individuals and patients (Patient 1, whom we originally described (1,2); Patient 2, described by Stevenson, et al. as their Case 2 (4); and Patient 3, GM 5520 from the Human Genetic Mutant Cell Repository, Camden, NJ) were maintained in Matalon's Modified Eagle Medium (EMS, 15) (GIBCO) supplemented with 10% heat-inactivated fetal calf serum, penicillin/streptomycin, and gentamicin (GIBCO), and passaged by trypsinization as previously described (2). For metabolic labeling, monolayers in 100 mm plastic culture dishes (Falcon) were washed twice with Hank's Balanced Salt Solution, preincubated for 1-2 h in methionine-deficient medium (Irvine Scientific) supplmented with 4% dialyzed fetal calf serum (GIBCO), and labeled for 14-16 h with [^{35}S]methionine (>800 Ci/mmol, Amersham) at a final concentration of 50-100 μCi/ml. After labeling, cells were immediately harvested (pulse) or incubated for an additional 48 h in EMS/10% fetal calf serum before harvest-

ing (pulse/chase). Incubation times for the pulse/extended chase experiment were adjusted as described in Figure 5.

Subcellular fractionation. Subcellular fractionation of labeled fibroblasts on Percoll gradients was carried out exactly as previously described (3); as previously noted, 15% Percoll was utilized to allow for more efficient separation of the "buoyant" lysosomes of affected fibroblasts from the soluble fraction at the top of the gradient. Prior to immunoprecipitation, Percoll was removed from pooled subcellular fractions by centrifugation at 100,000 X g for at least 3 h; immunoprecipitation was carried out on combined supernatant and membrane pellets.

Immunoprecipitation and SDS-PAGE. Cell homogenates or pooled Percoll fractions (0.8 ml) were brought to a final concentration of 10 mM Tris/0.125 M NaCl/ 0.02% NP-40/1% Triton X-100/0.1% SDS/0.1% sodium desoxycholate, pH 7.4 by the addition of 5X concentrated buffer. After an overnight incubation with 20 µl of normal rabbit serum, samples were preadsorbed with 100 µl of a 10% suspension of Pansorbin (Calbiochem) in the same buffer. After the removal of Pansorbin by centrifugation, samples were incubated overnight with polyclonal antiserum to Hex B (provided by Drs. E.F. Neufeld and R.L. Proia and prepared by Dr. A. Hasilik), and immune complexes were precipitated after the addition of 30 µl of a 10% suspension of Pansorbin. After extensive washing (six times with 700 µl of 10 mM Tris/0.5% NP-40/0.1% SDS/0.6 M NaCl, pH 8.6, and a final wash with 700 µl of 10 mM Tris/50 mM NaCl, pH 7.4), the immunoprecipitated polypeptides were solubilized in SDS-PAGE loading buffer (containing 2-mercaptoethanol) and analyzed by SDS-PAGE according to Laemmli (16) on 3% stacking/9% separating gels, followed by fluorography (Amplify, Amersham).

Enzyme digestions. After immunoprecipitation and washing as described above, Pansorbin-associated immunoprecipitated polypeptides were solubilized by boiling for 5 min in 1% SDS/1% 2-mercaptoethanol. After centrifugation, the supernatant was adjusted to 20 mM potassium phosphate/50 mM EDTA/0.3% NP-40/1% 2-mercaptoethanol, pH 7.0 (endo-β-glucosaminidase F), 10 mM sodium phosphate/0.1 M NaCl/ 1% 2-mercaptoethanol, pH 6.0 (endo-β-N-acetylglucosaminidase H), or 0.02 M acetate, pH 5.0 (neuraminidase), and protease inhibitors (17) and human serum albumin (50 µg) were added. Finally, after the addition of enzyme (25 mU of Endo H, 250 mU of Endo F, 100 mU of Arthrobacter ureafaciens neuraminidase, all from Boehringer-Mannheim), digestion was continued for 16-20 h at 37°C under toluene. Digestion mixtures were lyophilized prior to dissolution in SDS-PAGE loading buffer and analysis by SDS-PAGE.

Miscellaneous methods. Cell homogenates were analyzed for protein according to Lowry, et al. (18) using bovine serum albumin as a standard. Lysosomal enzyme activities were determined as previously described (19), using the appropriate 4-methylumbelliferyl glycosidses as substrates.

Results

We previously demonstrated the occurrence of "buoyant" lysosomes in fibroblasts derived from patients suffering from infantile generalized N-acetylneuraminic acid (NeuAc) storage disease, and the quantitative intralysosomal accumulation of NeuAc in affected fibroblasts; the distribution of Hex activity (typical for lysosomal enzymes) in normal and affected fibroblasts is shown in Figure 1. As previously reported, the mean density of affected lysosomes is 1.035, while that of normal lysosomes is >1.07; the distribution of other subcellular marker enzyme activities is unaltered in affected fibroblasts (3).

In order to demonstrate the association of newly synthesized lysosomal enzymes with the "lysosomal" subcellular fractions defined by the distribution of lysosomal enzyme activities, Hex in subcellular fractions of normal and affected fibroblasts was immunoprecipitated after pulse or pulse/chase labeling. As shown

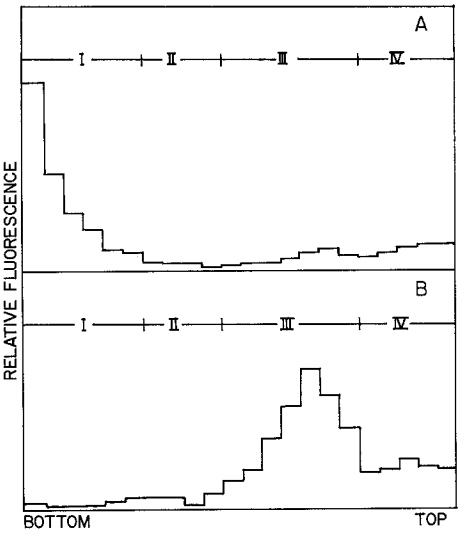

Figure 1. Distribution of Hex activity on Percoll gradients. Hex activity was measured using the 4-MU-β-GlcNAc substrate. Panel A, normal fibroblasts; Panel B, Patient 3 fibroblasts. Pooled fractions I-IV were subjected to immunoprecipitation.

in Figure 2, after overnight labeling of normal fibroblasts (Panel A), precursor polypeptides were associated with buoyant fractions (reflective of their localization in the endoplasmic reticulum and Golgi), while mature α and β polypeptide chains were detected predominantly in dense lysosomes; the mature polypeptides present in Fraction IV probably reflect lysosomal breakage and consequent release of mature polypeptides into the soluble fraction. Similar analysis of pulse/chase subcellular fractions from normal fibroblasts (Panel B) revealed the presence of only mature α and β polypeptides in dense lysosomal fractions. In contrast, analysis of affected fibroblasts showed the co-localization of both precursor and processed polypeptides in buoyant fractions after overnight labeling (Panel C), and the continued association of processed polypeptides with buoyant fractions even after a 48 h chase (Panel D). Similar results were obtained with fibroblasts derived from all three patients, and immunoprecipitation analyses were in all cases consistent with the observed distribution of lysosomal enzyme activity on the Percoll gradients. These results further define the "buoyant" lysosomes of affected fibroblasts as mature lysosomes, on the basis of their content of newly synthesized lysosomal enzymes and the presence of processed polypeptide chains of newly synthesized lysosomal enzymes.

Careful examination of pulse/chase samples, shown in Figure 3, revealed a small but significant difference in the molecular weight of mature 54 kDa α polypeptide chain of normal cells, in comparison to the molecular weight of "mature" α-chain of fibroblasts derived from all three patients; the "mature" α-chain of affected fibroblasts was 1.5-2 kDa (55.5-56 kDa) larger than the mature α-chain of normal cells.

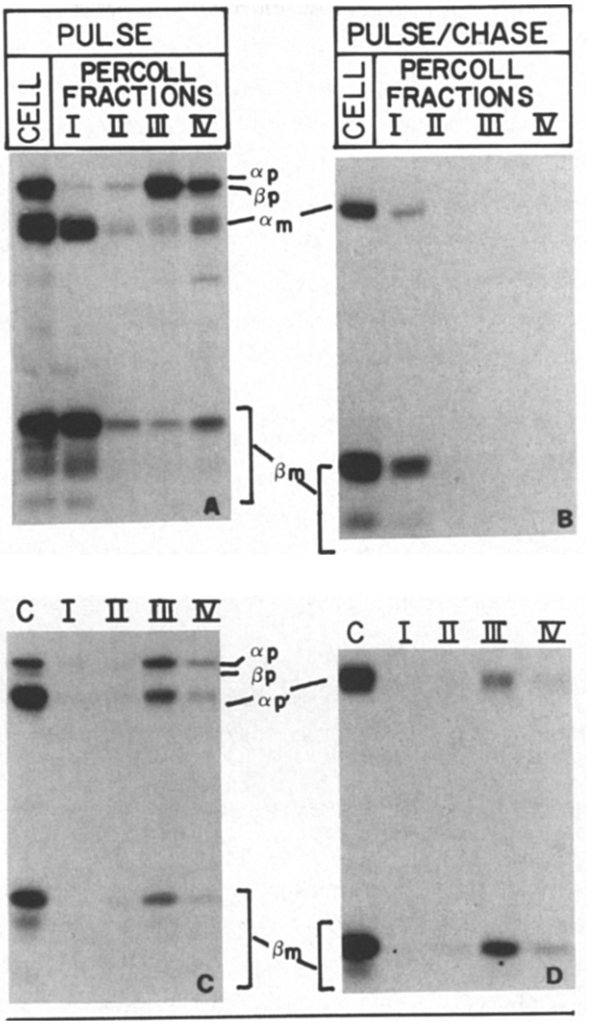

Figure 2. Immunoprecipitation of Hex polypeptides from subcellular fractions. Aliquots of cell homogenates (CELLS) or subcellular fractions (I-IV, Figure 1) were immunoprecipitated and analyzed by SDS-PAGE. Panels A & B, normal fibroblasts; Panels C & D, Patient 3 fibroblasts. Panels A & C, [35S]Met pulse; Panels B & D, [35S]Met pulse/chase. p=precursor; p'=partially processed precursor; m=mature.

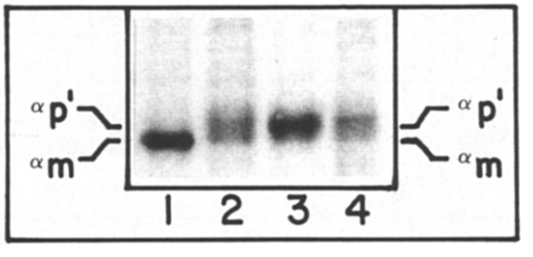

Figure 3. Immunoprecipitation of mature Hex α-chain from cell homogenates. Aliquots of cell homogenates were immunoprecipitated and analyzed by SDS-PAGE after [35S]Met pulse/chase. Lane 1, normal fibroblasts; Lane 2, Patient 1 fibroblasts; Lane 3, Patient 2 fibroblasts; Lane 4, Patient 3 fibroblasts. p'=partially processed precursor; m=mature.

To determine the molecular basis of the observed molecular weight difference, immunoprecipitated α-chain from normal and affected fibroblasts (after pulse/chase labeling) was subjected to treatment with exo- and endoglycosidases, and the products of digestion were analyzed by SDS-PAGE. As shown in Figure 4, after digestion with neuraminidase the molecular weight difference between α-chain from normal and affected fibroblasts was still observed; in fact, there was negligable effect on the apparent molecular weight of α-chain from either normal or affected fibroblasts, suggesting the virtual absence of NeuAc in both normal and affected α-chain. The molecular weight difference was also retained after Endo H digestion, although the loss of molecular weight from both normal and affected α-chain indicates the removal of Endo-H-susceptible N-linked oligosaccharide chains; treatment of normal and affected α-chain with Endo F (not shown) gave a pattern identical to that observed after Endo H digestion. Based on these results, impaired proteolytic processing in affected fibroblasts, rather than differential N-glycosylation or oligosaccharide processing, apparently gives rise to "mature" Hex α-chain 1.5-2 kDa larger than that observed in normal fibroblasts.

To determine the effect of impaired proteolytic processing on the activity of Hex, cell homogenates from a number of control and affected fibroblast strains were assayed for Hex activity, as well as other lysosomal glycosidase activities. As shown in Table I, activities in affected fibroblasts were generally within the range of values obtained with normal cells, or slightly elevated. These results, coupled with the absence of evidence for the lysosomal accumulation of any storage products in addition to free NeuAc in affected fibroblasts or tissue (2,3), suggests that lysosomal Hex (as well as other lysosomal enzymes) are not impaired functionally as a result of incomplete processing.

Finally, to assess both the kinetics of Hex processing and the turnover of Hex polypeptides in normal and affected fibroblasts, cells were subjected to

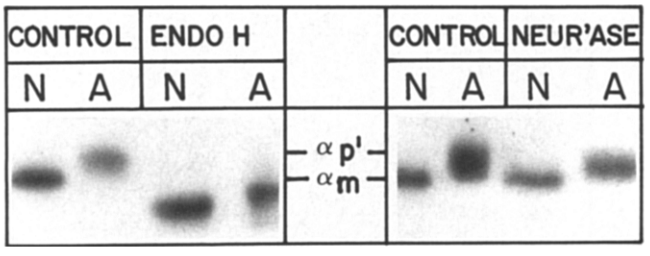

Figure 4. Glycosidase digestions of Hex α-polypeptide chain. Immunoprecipitated Hex polypeptides from cell homogenates after [35S]Met pulse/chase were digested with Endo H or neuraminidase and the digestion products were analyzed by SDS-PAGE. N=normal fibroblasts; A=affected fibroblasts (Patient 1). p'=partially processed precursor; m=mature.

Table I. Lysosomal enzyme activities in cultured fibroblasts

	Enzyme activity (nmol/mg protein/hr)[a]			
	α-fucosidase	α-mannosidase	β-galactosidase	β-hexosaminidase
Control (n=3)[b]	31 (13-43)	13 (13-14)	19 (17-23)	997 (558-1226)
Patient 1	20	15	17	1369
Patient 2	86	36	27	1045
Patient 3	14	19	28	972
α-fucosidosis	--	11	19	714
α-mannosidosis	10	1	26	645

[a]Activity is based on the hydrolysis of the appropriate 4-methylumbelliferyl substrates; values represent the average of two determinations

[b]Values are the average of three different control fibroblast strains, with the range in parentheses

pulse/chase labeling and cell homogenates were immunoprecipitated at various time points (up to a 200 h chase). As shown in Figure 5, there was no significant alteration in the rate of disappearance of Hex α-chain in affected fibroblasts as compared to normal cells, although the difference in molecular weight of α-chain was persistent throughout the course of the experiment. The appearance, even in normal cells, of an intermediate similar in molecular weight to the "mature" α-chain of affected fibroblasts is consistent with the inhibition of a normal event in proteolytic processing of Hex in affected fibroblasts; in addition, the "sharpening" of immunoprecipitable α-polypeptide bands during the course of the extended chase suggests the absence of non-enzymatic glycosylation (glycation) of Hex polypeptides in affected cells. The latter suggestion is also supported by the comparable isoenzyme elution profiles obtained after DEAE ion exchange chromatography of normal and affected fibroblast extracts (not shown).

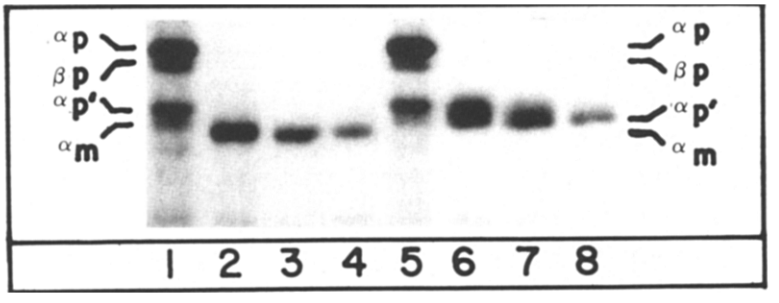

Figure 5. Immunoprecipitation of Hex α-polypeptide chain after extended [35S]Met pulse/chase. Hex polypeptides were immunoprecipitated from cell homogenates and analyzed by SDS-PAGE.
Lanes 1-4, normal fibroblasts; Lanes 5-8, Patient 1 fibroblasts. Lanes 1 & 5, 4 h pulse; Lanes 2 & 6, 4 h pulse/48 h chase; Lanes 3 & 7, 4 h pulse/120 h chase; Lanes 4 & 8, 4 h pulse/200 h chase.

Discussion

The studies reported here demonstrate that, in cultured fibroblasts derived from patients suffering from infantile NeuAc storage disease, Hex α-chain is subject to incomplete proteolytic processing, giving a "mature" polypeptide of molecular weight 56 kDa, in contrast to the 54 kDa mature polypeptide found in normal cells. The existence of intermediates of similar molecular weight in normal fibroblasts (observed after a short pulse) suggests an inhibition of one of a series of proteolytic processing events in affected fibroblasts; since intralysosomal NeuAc accumulation has previously been demonstrated in affected fibroblasts, one might infer that the impaired proteolytic processing cleavage would normally occur in the lysosome. Interestingly, partially processed α-chains of approximately the same molecular weight have been observed in I-Cell Disease (Mucolipidosis II) fibroblasts after the introduction of normal Hex precursor polypeptides via mannose-6-phosphate receptor-mediated endocytosis (20), and after the exposure of Hex precursor polypeptides to lysosomal extracts in vitro (21); the present study marks the first description of such intermediates in metabolically labeled cells. These results are consistent with the existence of a lysosomal processing proteinase or family of proteinases which may be particularly susceptible to inactivation upon disruption of the normal intralysosomal environment (by lysosomal lysis or swelling secondary to the accumulation of certain storage products). Although it is clear that all processing events are not inhibited in affected fibroblasts (e.g., the internal cleavage of Hex β-chain and the conversion of α-chain precursor to the 56 kDa intermediate), preliminary experiments indicate that the processing of at least one other lysosomal enzyme, α-fucosidase (which undergoes processing from a 53 kDa propolypeptide to a 50 kDa mature polypeptide) is completely inhibited in affected fibroblasts (22), while the maturation of cathepsin D is slowed. These preliminary studies raise the intriguing possibility that a number of lysosomal enzymes may undergo a common proteolytic processing event, although they do not exclude the possibility of multiple impaired processing events.

It remains to be determined whether, in infantile NeuAc storage disease fibroblasts, NeuAc accumulation (the result of impaired lysosomal NeuAc transport) leads to impaired proteolytic processing of lysosomal enzymes by disrupting the intralysosomal environment, or whether a primary deficiency in the proteinases responsible for the processing of lysosomal enzymes (and perhaps lysosomal membrane components) may result in impaired lysosomal NeuAc transport and consequent NeuAc accumulation; the impaired processing of Hex α-chain observed in lysosomal extracts and in I-Cell Disease fibroblasts (20,21) would support the former mechanism. The definition of this cause-and-effect relationship may well be critical in establishing the biochemical mechanisms involved in the pathogenesis of the NeuAc storage diseases.

In conclusion, three biochemical markers for infantile NeuAc storage disease have now been established, including: i) intralysosomal NeuAc accumulation, ii) lysosomal buoyancy, and iii) impaired proteolytic processing of lysosomal enzymes. Evaluation of these biochemical criteria in cultured cells may be useful in the differential diagnosis of the variants of the NeuAc storage diseases. Current studies are also directed toward the genetic analysis of the variants of the NeuAc storage diseases by somatic cell hybridization, using these biochemical parameters as a means of assessing complementation.

Acknowledgements

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