Am J Hum Genet 36:93–100, 1984

Biosynthesis and Maturation of α-*N*-Acetylglucosaminidase in Normal and Sanfilippo B-Fibroblasts

KURT VON FIGURA,[1] ANDREJ HASILIK,[1] FRIEDHELM STECKEL,[1] AND JACQUES VAN DE KAMP[2]

SUMMARY

The biosynthesis of α-*N*-acetylglucosaminidase in normal and Sanfilippo B fibroblasts was studied by labeling cells with [^{35}S]methionine and isolation of the enzyme by immunoprecipitation. The immunoprecipitated polypeptides were separated by polyacrylamide gel electrophoresis and visualized by fluorography. α-*N*-acetylglucosaminidase is synthesized as a precursor of an apparent mol. wt. of 87,000. Intracellular processing of the precursor yields two polypeptides of apparent mol. wts. of 73,000 and 76,000 via several intermediates. It is accomplished within 3 days after synthesis. Less than 30% of the newly synthesized precursor is secreted. In the presence of 10 mM NH_4Cl, secretion is enhanced to more than 80%. In our study, no α-*N*-acetylglucosaminidase polypeptides could be detected in fibroblasts from patients affected with either the severe or mild form of Sanfilippo disease, type B.

INTRODUCTION

The basic defect in Sanfilippo B disease (mucopolysaccharidosis III B) is a profound deficiency in activity of α-*N*-acetylglucosaminidase [1, 2]. Clinically, the Sanfilippo B disease presents as an entity with great inter- and intrafamiliar heterogeneity [3–5]. On clinical grounds, deficiency in α-*N*-acetylglucosaminidase cannot be distinguished from that in sulfamate sulfatase (MPS III A), acetyl-CoA:α-glucosamine-*N*-acetyltransferase (MPS III C), and α-*N*-acetylglucosamine-6-sulfate sulfatase (MPS III D). In a concerted action, these four enzymes accomplish the

Received April 19, 1983; revised June 17, 1983.

This work was supported by grant SFB-104 from the Deutsche Forschungsgemeinschaft and by the Fonds der Chemischen Industrie.

[1] Physiologisch-Chemisches Institut der Universität Münster, Waldeyerstrasse 15, D 4400, Münster, West Germany.

[2] Department of Pediatrics, University Hospital Leiden, Rijnsburgerweg 10, 2333 AA Leiden, The Netherlands.

removal of the variably substituted α-linked glucosamine residues from heparan sulfate. Deficiency in any of these four enzymes leads to intralysosomal accumulation of heparan sulfate, the principal polymer containing α-linked glucosamine residues.

Lysosomal enzymes are synthesized in the rough endoplasmic reticulum and transported via the Golgi apparatus to the lysosomes. The transport is associated with and dependent on various modifications of the polypeptide chain and its carbohydrate moiety. Segregation into lysosomes is accompanied by limited proteolysis of precursor polypeptides that yield the mature forms of lysosomal enzymes (for review, see [6, 7]).

In most lysosomal storage diseases studied so far, synthesis of the mutant enzyme was not detectable [8–10]. A few diseases or variant forms of diseases have been found, in which a mutant enzyme is synthesized, fails to be transported into lysosomes, or is subjected to rapid degradation. Besides other effects, the mutation may impair catalytic activity of the gene product [11–15]. Here we describe the biosynthesis and processing of α-*N*-acetylglucosaminidase in normal fibroblasts and in fibroblasts from patients affected with mild as well as severe variants of Sanfilippo B disease.

MATERIALS AND METHODS

Cell Culture

Human diploid fibroblasts were maintained at 37°C in 5% CO_2 in Eagle's minimum essential medium supplemented with antibiotics, nonessential amino acids, and 7.5% fetal calf serum (Boehringer, Mannheim, West Germany) as described [16]. The Sanfilippo B fibroblasts were obtained from patients detected during a survey of the Sanfilippo syndrome in the Netherlands [4, 17] and from biopsies submitted to our laboratory for diagnosis. The clinical course of the patients with the mild forms is summarized as follows. *77RD186: male, now 22 years old.* He had normal early development and learned to speak properly, attended primary school, and learned to read, write, and calculate, but lagged behind his peers and had to go to a special school. At present he can speak and help himself rather well. He is of normal height, has normal head circumference, and has no facial anomaly, organomegaly, contractures, or neurological abnormalities. *75RD35: male, now 24 years old.* Early development was normal, but at primary school he lagged behind. At present, he works in a sheltered workshop. He is tall (192 cm) and has normal head circumference and slightly coarse facial features but no other somatic anomalies. *75RD36: female, now 21 years old.* She is the sister of patient 75RD35. She had normal development until she lagged behind at primary school. Her speech ability regressed and movements somewhat stiffened after age 20; she works in a sheltered workshop. She is of normal height and has a normal head circumference. Her face is slightly coarsened, but there are no other physical anomalies.

Fibroblasts from three patients affected with the severe form of Sanfilippo B disease were also studied; one of these (550 LAD) was of Dutch origin.

Labeling of Cells

Confluent cultures in 25-cm^2 flasks were washed twice with 5 ml of serum-free medium ([18], as formulated in the catalog of Gibco, Grand Island, N.Y.) with added antibiotics, but free of methionine, and incubated with 5 ml of this medium for 1 hr.

For labeling, the serum-free medium, 2 ml, was supplemented with 0.1 ml of dialyzed fetal calf serum (Gibco) that had been incubated at pH 10.4 at 37°C for 30 min to inactivate

lysosomal hydrolases, and with 0.05 mCi of [L-^{35}S]methionine, 1,190 Ci/mmol (New England Nuclear, Boston, Mass.). The cells were incubated for 16–24 hrs in the presence of the labeling medium.

Endocytosis of Labeled α-N-Acetylglucosaminidase

Confluent cultures in 75-cm^2 flasks were incubated for 24–30 hrs with 5 ml of labeling medium as described above supplemented with 0.1 mCi [^{35}S]methionine and with 10 mM NH_4Cl. NH_4Cl enhances the secretion of newly synthesized precursors of lysosomal enzymes [8, 10]. The secretions were precipitated with 2.5 g of $(NH_4)_2SO_4$. The precipitate was dissolved in 1 ml of H_2O and dialyzed overnight against Eagle's minimum essential medium. After dialysis, 0.25 ml of fetal calf serum was added, and the volume adjusted to 5 ml with medium. Confluent cultures in 75-cm^2 flasks were incubated for 6–24 hrs with medium containing the labeled secretions. After incubation, cells were either harvested or grown for up to 7 days in the cultivation medium.

Immunoprecipitation

Preparation of extracts of cells and medium, immunoprecipitation of α-*N*-acetylglucosaminidase, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, and fluorography were performed as described [8]. α-*N*-acetylglucosaminidase was adjusted in extracts of cells and media to 3 mU/ml (~1.5 μg/ml) by addition of α-*N*-acetylglucosaminidase partially purified from human urine [19]. The antiserum had been raised in rabbits to purified human urine α-*N*-acetylglucosaminidase [19] and was used in a final dilution of 1:40. Cathepsin D was immunoprecipitated as described [8]. The radioactivity in the polypeptides was visualized by fluorography and quantified by densitometry.

Other Methods.

Activity of α-*N*-acetylglucosaminidase was determined as described [19] at pH 4.5 and 6.0. One unit of enzyme activity is the amount of enzyme hydrolyzing 1 μmol of substrate/min. Protein was determined according to Lowry et al. [20] using bovine serum albumin as standard.

RESULTS AND DISCUSSION

Synthesis and Processing of α-N-*Acetylglucosaminidase in Human Fibroblasts*

Immunoprecipitates from human skin fibroblasts labeled for 16 hrs with [^{35}S]methionine contained a major, rather diffusely appearing, polypeptide of apparent mol. wt. of 82,000 (77,000–86,000). A minor polypeptide of apparent M_r of 73,000 was present in variable amounts. Figure 1 shows the results obtained with two fibroblast lines representing the extremes in amounts of the smaller polypeptide in immunoprecipitates from cells labeled for 16 hrs. After labeling cells for longer periods (up to 36 hrs), a relative increase in the radioactivity in the smaller polypeptide was observed. Independent on the length of the labeling period, only a 87,000 polypeptide was found in the medium. The relationship between the polypeptides immunoprecipitated from the cells and medium was established by endocytosis experiments. Radioactively labeled secretions obtained from normal cultures treated with 10 mM NH_4Cl were added to fibroblasts. Preliminary experiments had established that media of such cells contain greatly enhanced amounts of secreted 87K polypeptides, whereas the intracellularly immunoprecipitable polypeptides are diminished (see below). After incubation for

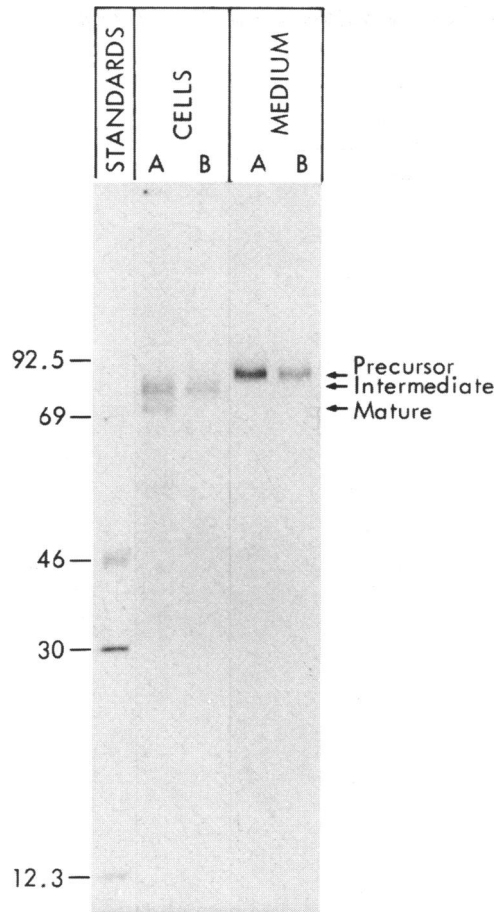

FIG. 1.—α-*N*-acetylglucosaminidase in human skin fibroblasts. Fibroblasts were incubated for 16 hrs in the presence of [^{35}S]methionine. α-*N*-acetylglucosaminidase was immunoprecipitated from extracts of cells and medium. Results obtained with two normal cell lines, *A* and *B*, are shown. The immunoprecipitates were solubilized under reducing conditions [8], separated by gel electrophoresis in the presence of sodium dodecyl sulfate. Labeled polypeptides were visualized by fluorography. The migration of α-*N*-acetylglucosaminidase polypeptides is indicated by *arrows*. Several minor polypeptides were irregularly found as contaminants of the immunoprecipitates. The following ^{14}C-methylated standards (New England Nuclear) were used: phosphorylase B, 92.5 kDa; bovine serum albumin, 69 kDa; ovalbumin, 46 kDa; carbonic anhydrase, 30 kDa; and cytochrome c, 12.3 kDa.

6 hrs and 24 hrs with cells, α-*N*-acetylglucosaminidase was immunoprecipitated from extracts of cells and medium. Three cultures were further incubated for 1–7 days in cultivation medium (fig. 2). After incubation for 6 hrs, the 87K polypeptide was the only radioactive species precipitable from the recipient cells. This polypeptide was progressively shortened during prolonged incubation. In cells incubated for 24 hrs in the presence of the labeled secretions, the internalized polypeptide appeared as a broad band ranging from 74K to 86K. After 2 more days, the

internalized polypeptides were shortened to 73K and 76K forms, which appeared to be stable.

From our experiments (shown in figs. 1 and 2), we conclude that α-*N*-acetylglucosaminidase is synthesized in fibroblasts as an 87K precursor. Part of the precursor (less than 30%) is secreted. In the presence of NH_4Cl, this fraction is enlarged as reported for other lysosomal enzymes [8–10]. The secreted precursor can be taken up by fibroblasts. Intracellularly retained as well as endocytosed, precursor is processed via a series of hardly distinguishable intermediates to two forms of 73K and 76K. Based on 16–24-hr labeling experiments, we mentioned in several reviews that α-*N*-acetylglucosaminidase is synthesized as an 86K precursor and processed to an 80K mature form [7, 21, 22]. The findings presented here show that the 80K form is a processing intermediate rather than a mature polypeptide.

Synthesis of α-N-*Acetylglucosaminidase in Sanfilippo B Fibroblasts*

None of the α-*N*-acetylglucosaminidase-related polypeptides was found in three cell lines from patients affected with the severe form of Sanfilippo B disease. In figure 3 an experiment is shown in which fibroblasts from a control, from three patients with the mild (77RD186, 75RD36, 75RD35), and one patient with the

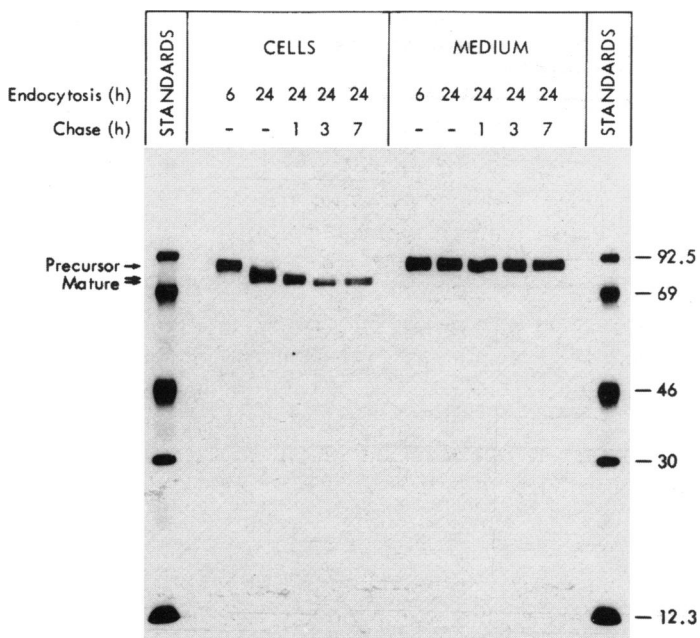

FIG. 2.—Endocytosis of radioactively labeled α-*N*-acetylglucosaminidase. Secretions were prepared from fibroblasts labeled with [^{35}S]methionine in the presence of 10 mM NH_4Cl. The secretions containing labeled α-*N*-acetylglucosaminidase precursor were incubated with confluent fibroblast cultures (14×10^6 cpm/culture). After incubation for 6 hrs and 24 hrs, medium was removed and the cells either harvested or incubated for 1–7 days in cultivation medium. α-*N*-acetylglucosaminidase was immunoprecipitated from the cells and the medium. For standards, see figure 1.

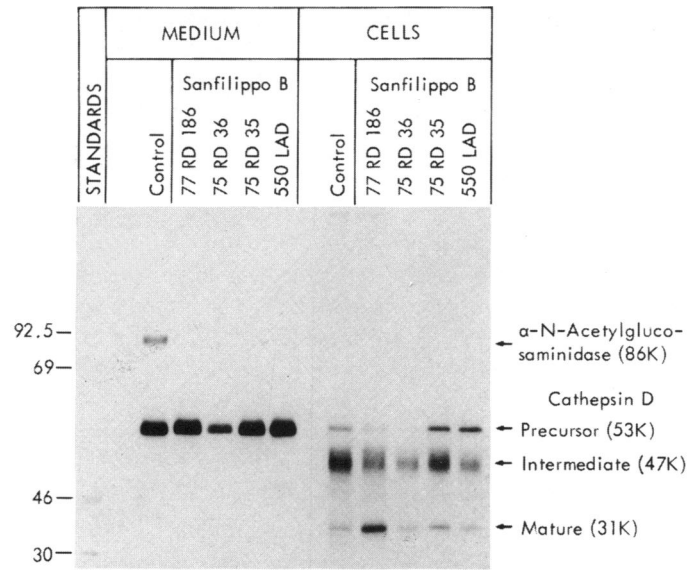

FIG. 3.—Synthesis of α-*N*-acetylglucosaminidase in Sanfilippo B fibroblasts. Fibroblasts from patients with the severe (550 LAD) or mild form (77RD186, 75RD36, 75RD35) of Sanfilippo B disease and a control were incubated for 16 hrs in the presence of [^{35}S]methionine and 10 mM NH_4Cl. α-*N*-acetylglucosaminidase and cathepsin D were immunoprecipitated simultaneously from cells and medium. Cathepsin D is synthesized and secreted as a 53K precursor and intracellularly processed via a 47K intermediate to a major 31K mature form [8]. Activity of α-*N*-acetylglucosaminidase measured at pH 4.5 and 6.0 was in all four Sanfilippo B cell lines below the limit of detection. For standards, see figure 1.

severe form (550 LAD) of Sanfilippo B disease were labeled for 16 hrs with [^{35}S]methionine in the presence of 10 mM NH_4Cl. α-*N*-acetylglucosaminidase and cathepsin D were simultaneously immunoprecipitated from cells and medium. Cathepsin D served as an internal control for lysosomal enzymes not affected in Sanfilippo B disease. Within the control cells, the retention of α-*N*-acetylglucosaminidase and cathepsin D was severely decreased and processing of α-*N*-acetylglucosaminidase was completely and that of cathepsin D extensively inhibited. Even in the presence of NH_4Cl, which induces secretion and inhibits intralysosomal degradation of the newly synthesized lysosomal enzymes, no polypeptides related to α-*N*-acetylglucosaminidase were detectable in fibroblasts from patients with severe and mild forms of Sanfilippo B disease.

The absence of α-*N*-acetylglucosaminidase synthesis in cultured fibroblasts is at variance with the reported presence of cross-reacting material in urine of Sanfilippo B patients [23] and in Sanfilippo B fibroblasts [24]. Since the same antiserum was used in this and the previous studies, the differences in results are probably related to different immunological procedures. In the present experiments, conditions were chosen that eliminate precipitation of weakly bound antigens by inclusion of high concentrations of salt (0.4 M KCl) and detergent (1% Triton X-100), whereas in previous experiments, physiological salt concentrations and buffers free of detergents were used. Earlier studies had indicated that the cross-

reacting material in Sanfilippo B urine has a lower affinity to the antiserum than does normal human urine α-*N*-acetylglucosaminidase [23]. The relation between these weakly interacting antigens in Sanfilippo B disease and α-*N*-acetylglucosaminidase remains to be established.

ACKNOWLEDGMENTS

The help of Drs. M. Niermeijer and W. Kleijer, Rotterdam, in obtaining fibroblast cultures from Sanfilippo B patients and the technical assistance of Mrs. H. Antemann, H. Flentje, and G. Hess are acknowledged.

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