Association of N-Acetylglactosamine-6-sulfate Sulfatase with the Multienzyme Lysosomal Complex of β-Galactosidase, Cathepsin A, and Neuraminidase

POSSIBLE IMPLICATION FOR INTRALYSOSOMAL CATABOLISM OF KERATAN SULFATE*

From the Service de Génétique Médicale, Hôpital Sainte-Justine, and Département de Pédiatrie, Faculté de Médecine, Université de Montréal, Montréal, Québec, Canada H3T 1C5

N-Acetylglactosamine-6-sulfate sulfatase (GALNS) catalyzes the first step of intralysosomal keratan sulfate (KS) catabolism. In Morquio type A syndrome GALNS deficiency causes the accumulation of KS in tissues and results in generalized skeletal dysplasia in affected patients. We show that in normal cells GALNS is in a 1.27-MDa complex with three other lysosomal hydrolases: β-galactosidase, α-neuraminidase, and cathepsin A (protective protein). GALNS copurifies with the complex by different chromatography techniques: affinity chromatography on both cathepsin A-binding and β-galactosidase-binding columns, gel filtration, and chromatofocusing. Anti-human cathepsin A rabbit antiserum coprecipitates with galactosialidosis, indicating that the complex with cathepsin A may protect GALNS in the lysosome. We suggest that the 1.27-MDa complex of lysosomal hydrolases is essential for KS catabolism and that the disruption of this complex may be responsible for the KS accumulation in β-galactosidosis and galactosialidosis patients.

N-Acetylglactosamine-6-sulfate sulfatase (GALNS, EC 3.1.6.4) is involved in the lysosome in the first step of the catabolism of keratan sulfate (KS), a glycosaminoglycan that consists of β-galactose residues, mostly sulfated, alternating with N-acetylgalactosamine 6-sulfate (1). KS is degraded further by sequential action of β-galactosidase (GAL, EC 3.2.1.23), N-acetylgalactosamine-6-sulfate sulfatase and β-hexosaminidases (HEX) A and B (1). In Morquio type A syndrome GALNS deficiency causes the accumulation of KS in tissues and results in generalized skeletal dysplasia in affected patients (2–4).

Recent studies indicated the importance of the supramolecular association of enzymes catalyzing subsequent metabolic reactions (5, 6), which may lead to an acceleration of the whole process resulting from the directed transport of intermediates. In lysosomes GAL and α-neuraminidase (NEUR, EC 3.2.1.18), the enzymes catalyzing the two first steps of ganglioside hydrolysis are associated with cathepsin A, also named protective protein (CathA, E.C. 3.4.16.1), into a lysosomal multienzyme complex necessary for both the activation of NEUR and stabilization of GAL within the lysosome (7–10). Recently we proposed the molecular mechanism of GAL protection by CathA and demonstrated that the complex purified from human placenta has a molecular mass of about 1.27 MDa and contains multiple protein components (11, 12). In the present work we demonstrate that GALNS is the component of a 1.27-MDa complex, that this association protects GALNS in lysosome, and we explore the hypothesis that the supramolecular organization of lysosomal hydrolases is important for the catabolism of KS.

MATERIALS AND METHODS

Purification of Lysosomal High Molecular Weight Complex—The 1.27-MDa lysosomal complex was purified from human placenta using affinity chromatography on a concanavalin A-Sepharose column followed by affinity chromatography on a p-aminophenyl-β-D-thiogalactopyranoside (PATGAL)-agarose column, which binds GAL, or on a Phenyl-Sepharose column, which binds CathA (12, 13). After affinity purification the preparations were dialyzed against 20 mM sodium acetate buffer, pH 5.2, containing 0.15 M NaCl and 0.02% (w/v) NaN₃, adjusted to pH 4.0 with 1 M HCl in accordance with the manufacturer’s protocol. Fractions containing NEUR, GAL, CathA, and GALNS activities as well as by SDS-polyacrylamide gel electrophoresis (PAGE) as described below. Fractions from the peak corresponding to a molecular mass of 1.27 MDa and containing NEUR, GAL, and CathA activities, were pooled, dialyzed against 25 mM Tris–HCl, pH 7.2, and applied to a Mono Q column equilibrated with the same buffer. The column was eluted at a flow rate of 1 ml/min with a linear梯度 from 0 to 0.5 M NaCl. Fractions containing NEUR, GAL, and CathA activities were collected.

Alexey V. Pshezhetsky‡ and Michel Potier

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‡To whom correspondence should be addressed: Service de Génétique Médicale, Hôpital Sainte-Justine, and Département de Pédiatrie, Faculté de Médecine, Université de Montréal, Montréal, Québec, Canada H3T 1C5.

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pooled, dialyzed against buffer A, and concentrated to 0.5 ml. The preparation of purified 1.27-MDa complex was frozen and stored at −70 °C until used.

**Enzyme Assays**—GAL, NEUR, and HEXA were assayed using the corresponding fluorogenic 4-methylumbelliferyl (Muf)-glycoside derivatives as substrates according to published methods (14–16). CathA activity was measured using CBZ-Phe-Leu as a substrate (10). GALNS activity was then inhibited by addition of 25 μl of 1 M sodium acetate buffer and 25 μl of 1 mM Muf-Gal-6S to the 1.27-MDa complex from human placenta (13). This reaction mixture was incubated further for 2 h at 37 °C to convert all the Muf-Gal-6S in the same buffer was incubated overnight at 37 °C. The positions of the elution peaks of the molecular mass standards described under “Materials and Methods” and the void volume (V0) are shown by arrows. The 25-μl indicated gel filtration fractions, corresponding to the 1.27-MDa complex (peak 1 on both gel filtration profiles) were analyzed by SDS-PAGE. The protein bands are identified on the 280-nm fraction number.

**Fraction number**

![Fraction number](image)

**Fig. 1.** FPLC gel filtration (left panels) and SDS-PAGE analysis (right panels) of the GAL-NEUR-CathA complex preparations from human placenta. 3 mg of the preparations affinity purified using (a) a PATGAL-agarose column or (b) a Phe-Leu-agarose column were analyzed by FPLC gel filtration on a Superose 6 HR column as described under “Materials and Methods.” Broken line, A280 nm; □, GAL activity; ◦, CathA activity (× 10−1); ○, NEUR activity (× 103); ●, GALNS activity (× 103). The positions of the elution peaks of the molecular mass standards described under “Materials and Methods” and the void volume (V0) are shown by arrows. The 25-μl indicated gel filtration fractions, corresponding to the 1.27-MDa complex (peak 1 on both gel filtration profiles) were analyzed by SDS-PAGE. The protein bands are identified on the right side of gels as follows: GAL, 64-kDa subunit of β-galactosidase; GAL46, a 46-kDa product of COOH-terminal processing of β-galactosidase; GALNS, 40-kDa subunit of N-acetylgalactosamine-6-sulfate sulfatase; P35, unidentified NH2-terminally blocked 35-kDa protein; CathA32 and CathA20 are 32- and 20-kDa subunits of cathepsin A.
buffered saline, 0.05% (w/v) MgCl₂, and 3% (w/v) BSA. The membrane was washed completely with 5% (v/v) acetic acid, and nonspecific binding was blocked, since no amino acid sequence was obtained. Molecular mass and NH₂-terminal amino acid sequence (APQPPNI-LLLLMDDM) of the 40-kDa protein were identical to those (23) of the recently reported 46-kDa product of GAL intralysosomal proteolytic digestion (22). The NH₂-terminal residue of the 35-kDa-33-kDa doublet protein (P35 in Fig. 1) was probably blocked, which suggests that GALNS binds to the complex specifically. In agreement with our data it has been reported that a 40-kDa protein with an NH₂-terminal sequence homologous to that of human GALNS copurifies with the protein bands are identified on the right side of gel as in Fig. 1.

Western Blotting of GALNS in Fibroblast Homogenates—Cultured skin fibroblast homogenates (50 µg of protein) were subjected to SDS-PAGE and electrotransferred to Immobilon-P membranes as described above. To visualize protein bands, the membrane was stained with 0.1% (w/v) Ponceau S (Sigma) in 5% (v/v) acetic acid. The dye was then washed completely with 5% (w/v) acetic acid, and nonspecific binding was blocked, since no amino acid sequence was obtained. Molecular mass and NH₂-terminal amino acid sequence (APQPPNI-LLLLMDDM) of the 40-kDa protein were identical to those (23) of the recently reported 46-kDa product of GAL intralysosomal proteolytic digestion (22). The NH₂-terminal residue of the 35-kDa-33-kDa doublet protein (P35 in Fig. 1) was probably blocked, which suggests that GALNS binds to the complex specifically. In agreement with our data it has been reported that a 40-kDa protein with an NH₂-terminal sequence homologous to that of human GALNS copurifies with the protein bands are identified on the right side of gel as in Fig. 1.

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GALNEUR-CathA complex from bovine testis (24).

GALNS activity assay in FPLC gel filtration fractions demonstrated that 50% of total GALNS in human placenta was copurified with the 1.27-MDa complex (peak I in Fig. 1, a and b). The other 50% of GALNS activity eluted as a second peak (peak III in Fig. 1, a and b), consistent with the reported 120-kDa homodimeric form of this enzyme (25).

Purified by gel filtration 1.27-MDa complex was subjected to an additional purification step, FPLC chromatofocusing (Fig. 2). GALNS, GAL, CathA, and NEUR coeluted from the column at a pH 4.8 that corresponds to the pI of the whole complex. Under these conditions, only 5–10% of GALNS and CathA are unbound as estimated from the assay of their activities in the fractions eluting at a pH 6.0 and 5.4, corresponding to the pI values of free GALNS and CathA, respectively.

The presence of GALNS within the 1.27-MDa complex was also confirmed by immunoprecipitation of the purified 1.27-MDa complex using antibodies against human CathA. The titration curves (Fig. 3a) for GALNS, CathA, GAL, and NEUR showed a proportional decrease of the activity of each of the four enzymes. Similar immunotitration curves were obtained with the crude glycoprotein fraction purified by concanavalin A-Sepharose affinity chromatography from human placenta extract (Fig. 3b).

In the lysosome GALNS and GAL catalyze the subsequent reactions of KS hydrolysis; therefore, transfer of the hydrolysis intermediates between the active sites of these enzymes in the 1.27-MDa complex may be an important step in the KS catabolic process. To explore the hypothesis that the association of GALNS with the complex is necessary for the proper intralysosomal KS catabolism we verified whether the complex exists in the cells of patients which accumulate KS. Two autosomal recessive diseases in which KS accumulates in abnormal amounts are known, both of which are associated with a characteristic skeletal dysplasia and corneal clouding in affected patients. The first, Morquio type A syndrome (MPS IV type A), is caused by the primary deficiency of GALNS (2–4). The second, Morquio type B syndrome (MPS IV type B) (26), clinically similar to MPS IV type A, results from GAL deficiency and is also named β-galactosidosis (27). Interestingly, a different set

![Figure 3](https://example.com/fig3.png)

**Fig. 3.** Immunoprecipitation of CathA, GAL, NEUR, and GALNS in the purified preparation of the 1.27-MDa lysosomal complex (panels a and c) and in concentrated glycoprotein preparation from human placenta (panels b and d). The immunoprecipitation was performed with an increasing amounts (1–50 µl) of anti-human CathA rabbit polyclonal antibodies (panels a and b), anti-human HEXA rabbit polyclonal antibodies (panels c and d), or control preimmune rabbit serum (dashed line in panel a) as described under “Materials and Methods.” □, GAL activity; Δ, CathA activity; ◦, NEUR activity; ●, GALNS activity; ●, HEXA activity.
of mutations in GAL results in another clinical condition of β-galactosidosis, GM1-gangliosidosis, in which lipid substrate of GAL, GM1-ganglioside, accumulates in brain and viscera, resulting in mental retardation and hepatosplenomegaly. KS accumulation, which occurs in both clinical forms of β-galactosidosis (27), is presently explained by the primary deficiency of GAL. However, this hypothesis still does not explain the accumulation of both undersulfated and normally sulfated forms of KS, since normal or low normal levels of GALNS activity are detectable in cultured cells from patients (26, 27).

Using FPLC gel filtration on Superose 6 column we analyzed fibroblast extracts of β-galactosidosis patients (both GM1-gangliosidosis and MPS IV type B clinical forms) as well as extracts of control fibroblasts. About 30–40% of the GALNS activity and all of the NEUR activity of normal fibroblasts (Fig. 4a) are associated with the 1.27-MDa complex (peak I). Under these conditions, the 1.27-MDa complex exists in equilibrium with a 680-kDa GAL-CathA binary complex (peak II), GAL tetramers (peak III), and CathA and GALNS dimers (peak IV). This distribution of oligomeric forms is similar to that reported previously for human placenta (12). In contrast, in fibroblasts from β-galactosidosis patients (MPS IV type B) the 1.27-MDa complex and 680-kDa GAL-CathA complex are not detectable, and GALNS is present only in the 120-kDa homodimeric form (Fig. 4b, peak IV). The elution profiles of the enzyme activities of fibroblast extracts from GM1-gangliosidosis patients (not shown) were similar to those of MPS IV type B fibroblasts.

Using the same method, we also analyzed fibroblast extracts from CathA-deficient (galactosialidosis) and NEUR-deficient (sialidosis) patients because in both diseases a deficiency of a single component of the 1.27-MDa complex could result in the disruption of the complex. Gel filtration of galactosialidosis fibroblast extracts (Fig. 4c) demonstrated the absence of the 1.27-MDa complex (previously (8) also shown by the ultracentrifugation method) suggesting that patients with galactosialidosis may excrete higher than normal amounts of KS in urine, similar to those described for MPS IV type A and β-galactosidosis patients. Indeed, the KS urinary excretion of a 16-year-old female galactosialidosis patient was elevated at the level of 2.1 mg/24 h, similar to that of MPS IV type A patients (1.8–2.4 mg/24 h; normal < 0.8 mg/24 h). On the other hand, in fibroblasts of NEUR-deficient patients, which do not accumulate KS (28), the 1.27-MDa complex was present, and the amount of complex-bound GALNS corresponded to those in the control fibroblasts (Fig. 4d, peak I). These results also suggest that the presence of NEUR is not essential for the association of the other enzymes in the 1.27-MDa complex.

Interestingly, the disruption of the 1.27-MDa complex in galactosialidosis is also associated with a decreased level of GALNS activity (Fig. 4c). Further assay of GALNS activity in three other fibroblast lines of galactosialidosis patients (Table 2 Results of the analysis (n = 6) performed over several years at the Service de Génétique Médicale, Hôpital Sainte-Justine.)

![Fig. 4. FPLC gel filtration of human fibroblast extracts. Extracts of human fibroblasts from normal individuals (panel a), β-galactosidosis (MPS IV type B), cell line GM01259 (panel b), galactosialidosis, cell line GM02934 (panel c), and sialidosis, cell line GM02685 (panel d) patients were analyzed on a FPLC Superose 6 column as described in under “Materials and Methods.” 0.5-ml fractions were collected and assayed for enzyme activities. □, GAL activity (× 10); △, CathA activity; ○, NEUR activity (× 103); ●, GALNS activity (× 104). HEXA activity in fractions (not shown) was used as an internal control. Each curve represents the average of three independent experiments. The positions of the elution peaks of the molecular mass standards described under “Experimental Procedures” and void volume (V0) are shown by arrows.](image-url)
GalNAc-6-sulfatase and Multienzyme Lysosomal Complex

Table I

| Fibroblast line | HEX | GAL | CathA | NEUR | GALNS (GalNAc-6S) | GALNS (Muf-Gal-6S) |
|-----------------|-----|-----|-------|------|-------------------|-------------------|
| Sialidosis      |     |     |       |      |                   |                   |
| GM02921         | 109 | 12  | 2     | 70   | 0.03 ± 0.01 (1.8) | 0.034 ± 0.008 (100) |
| GM02685         | 68  | 18  | 4     | 67   | ND                | 0.026 ± 0.003 (76)  |

β-Galactosidosis (Gm-galactosidosis)

| (GM05653)       | 144 | 1.2 | 0.05 (4.6) | 394 | 1.3 | 0.2 (81) | 0.027 ± 0.03 (79) |
| (GM0405535)     | 61  | 0.3 | 0.03 (1.1) | 336 | 2.1 | 0.2 (131)| 0.012 ± 0.001 (35) |
| (Morquio type B) | 84  | 1.6 | 0.8 (5.9)  | 253 | 1.7 | 0.1 (106)| 0.027 ± 0.015 (79) |

GALNS residual activity did not exceed 1–2% of normal (Table I).

The enzyme activities as the percents of control values are presented in parentheses.

Table II

Correction of GALNS activity in galactosidosis cells

| Correcting factor | HEX | GAL | NEUR | GALNS (Muf-Gal-6S) | GALNS (GalNAc-6S) |
|-------------------|-----|-----|------|-------------------|-------------------|
| No addition       | 80  | 1.4 | 0.1 (100) | 0.04 ± 0.003 (100) | 0.006 ± 0.001 (100) |
| Leupeptin         | 95  | 8.1 | 0.3 (578) | 0.04 ± 0.003 (100) | 0.022 ± 0.001 (266) |
| CathA             | 71  | 3.9 | 0.2 (278) | 0.20 ± 0.001 (500) | 0.032 ± 0.001 (533) |
| GAL • CathA complex | 116 | 4.8 | 0.2 (343) | 0.23 ± 0.001 (575) | 0.040 ± 0.002 (667) |

The enzyme activities as the percents of control values (no additions to the cell culture media) are presented in parentheses.

I) showed that the activity is reduced to 3–21% of normal. Of note, in MPS IV type A fibroblasts, used as a negative control, GALNS residual activity did not exceed 1–2% of normal (Table I). We found that the activity of GALNS, as well as that of GAL and NEUR, could be restored in galactosidosis fibroblasts (cell line 83.29.34) by the addition of purified CathA or GAL-CathA 680-kDa complex to the culture medium (Table II). GALNS and GAL activities were also increased 3–4-fold by leupeptin inhibition of lysosomal proteases (Table II). These data are consistent with the rapid proteolytic degradation of unbound GALNS within lysosomes similar to that demonstrated for GAL (7, 8). Indeed, the reduced amount of GALNS cross-reacting material was detected by Western blotting in fibroblast homogenate of galactosidosis patient (Fig. 5, lane 4), compared with control (lane 1), MPS IV type B (lane 2), and sialidosis (lane 3) patients. The leupeptin inhibition of lysosomal proteases resulted in reappearance of GALNS cross-reacting band on gel (lane 5).

On the other hand, GALNS activity is low normal (~50% of control), and NEUR activity is normal in β-galactosidosis fibroblasts (Table I), in which the complex does not exist. Gel filtration analysis of β-galactosidosis fibroblast extracts (Fig. 4b) demonstrated that NEUR is present as large (>3 MDA) aggregates with CathA which elute in the void volume (V0) of the column (Fig. 4b, peak V). As was demonstrated previously (9), the association with CathA only is sufficient for the expression of NEUR activity. It is tempting to speculate that a similar protective mechanism may also apply to GALNS in β-galactosidosis cells, although a GALNS•CathA binary complex has not yet been directly demonstrated. The normal level of GALNS activity in sialidosis fibroblasts (Table I) is in agreement with the presence of the 1.27-MDA complex in these cells (Fig. 4d, peak I).

Altogether, our results demonstrate that the majority of GALNS in the lysosome is associated with the 1.27-MDA multienzyme complex, which also includes GAL, CathA, and NEUR. The complex is not present in the cells of β-galactosidosis and galactosidosis patients which accumulate KS. We suggest that 1.27-MDA multienzyme complex is necessary for protein KS catabolism, which can reflect the importance of supramolecular structure for the hydrolytic function of the lysosome.
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Note Added in Proof—The recent assays performed with two other fibroblast lines from galactosialidosis patients demonstrated that residual GALNS activity could be higher than that reported in the paper, up to 40% of normal control level.

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