Molecular mechanism of lysosomal sialidase deficiency in galactosialidosis involves its rapid degradation

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Galactosialidosis is an inherited lysosomal storage disease caused by the combined deficiency of lysosomal sialidase and β -galactosidase secondary to the deficiency of cathepsin A/protective protein, which is associated with sialidase and β -galactosidase in a high-molecular weight (1.27 MDa) complex. Clinical phenotypes of patients as well as the composition of compounds which are stored in patient's tissues implicate sialidase deficiency as the underlying pathogenic defect. The recent cloning and sequencing of lysosomal sialidase [Pshezhetsky, Richard, Michaud, Igdoura, Wang, Elsiger, Qu, Leclerc, Gravel, Dallaire and Potier (1997), *Nature Genet.* **15**, 316–320] allowed us to study the molecular mechanism of sialidase deficiency in galactosialidosis. By Western blotting, using antibodies against the recombinant human enzyme, and by NH_2 -terminal sequencing, we showed that sialidase is synthesized as a 45.5 kDa precursor and after the cleavage of the 47-amino

acid signal peptide and glycosylation becomes a 48.3 kDa mature active enzyme present in the 1.27 kDa complex. Transgenic expression of sialidase in cultured skin fibroblasts from normal controls and from galactosialidosis patients, followed by immunofluorescent and immunoelectron microscopy showed that in both normal and affected cells the expressed sialidase was localized on lysosomal and plasma membranes, but the amount of sialidase found in galactosialidosis cells was ~ 5 -fold reduced. Metabolic labelling studies demonstrated that the 48.3 kDa mature active form of sialidase was stable in normal fibroblasts (half-life ~ 2.7 h), whereas in galactosialidosis fibroblasts the enzyme was rapidly converted (half-life ~ 30 min) into 38.7 and 24 kDa catalytically inactive forms. Altogether our data provide evidence that the molecular mechanism of sialidase deficiency in galactosialidosis is associated with abnormal proteolytic cleavage and fast degradation.

INTRODUCTION

Sialidase (acetylneuraminyl hydrolase or neuraminidase, EC 3.2.1.18) catalyses the hydrolysis of terminal sialic acid residues of oligosaccharides, glycoproteins and glycolipids. Lysosomal sialidase is associated with cathepsin A (CathA, EC 3.4.16.1, also named 'protective protein'), β -galactosidase (GAL, EC 3.2.1.23) and *N*-acetylgalactosamine-6-sulphate sulphotase (GALNS, EC 3.1.6.4) in a 1.27 MDa multienzyme complex [1–5]. This complex is essential for the expression of sialidase and GAL activities in the lysosome [1–3] as confirmed by an autosomal recessive disease, galactosialidosis, characterized by the combined deficiency of GAL and sialidase secondary to CathA deficiency [6,7]. Clinical phenotypes are different for the patients with early infantile, late infantile and juvenile/adult onsets of galactosialidosis, but generally involve cherry-red macular spots, corneal clouding, skeletal dysplasia, hepatosplenomegaly, growth retardation, neurologic and mental deteriorations. These features as well as the composition of the lysosomal storage compounds (mostly sialylated gangliosides, oligosaccharides and glycopeptides), which are accumulated in patient's tissues and excreted in urine resemble those of patients with the primary sialidase genetic defect, sialidosis [8], and implicate sialidase deficiency as the major pathogenic defect underlying galactosialidosis.

Previous studies showed that in galactosialidosis cells the 85 kDa GAL precursor is incorrectly processed and rapidly

degraded [2,9]. The characterization of various galactosialidosis mutations in CathA [10–13] as well as inactivation of CathA by site-directed mutagenesis [14] demonstrated that rapid degradation of GAL in galactosialidosis results from the absence of its interaction with CathA. On the basis of the structural modelling we suggested that in the complex, CathA monomers cover most of the GAL surface thus preventing the intralysosomal proteolysis of GAL [15]. Recently we showed that in the lysosome CathA may also protect GALNS, another component of the 1.27 MDa complex, since both GALNS activity and cross-reacting material were reduced in the fibroblasts of patients affected with galactosialidosis [5].

The molecular mechanism of sialidase deficiency in galactosialidosis has never been characterized since until recently the lysosomal sialidase has not been cloned or purified. The sialidase activity has always been associated with the 1.27 MDa complex in the process of purification from tissues [16–20]. The dissociation of the complex resulted in the complete inactivation of sialidase. However, the activity could be restored after the reassociation of the complex *in vitro* [3]. These results led to the hypothesis that the association with the complex is required for sialidase to adopt the catalytically active conformation [3] but direct experimental evidence supporting this mechanism was not obtained.

Recently we have identified a cDNA coding for human lysosomal sialidase and characterized molecular defects in several

Abbreviations used: CathA, cathepsin A/protective protein; GAL, β -galactosidase; HEX, β -hexosaminidase; GALNS, *N*-acetyl-galactosamine-6-sulphate sulphotase; PATGAL, *p*-aminophenyl- β -D-thiogalactopyranoside; FPLC, fast protein liquid chromatography; EDTA, ethylenediamine-tetraacetic acid; DTT, dithiothreitol; GST, glutathione transferase; HBSS, Hank's Balanced Salt Solution; BSA, bovine serum albumin; PBS, phosphate-buffered saline; TBS, Tris-HCl-buffered saline; LAMP, lysosomal integral membrane protein; PAGE, polyacrylamide gel electrophoresis; EMEM, Eagle's Minimal Essential Medium, 4 MU α -neur, 2'-(4-methylumbelliferyl)- α -D-*N*-acetylneuraminic acid.

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sialidosis patients [21]. In the present work in order to understand the molecular mechanism of sialidase deficiency in galactosialidosis we studied the biogenesis and intracellular localization of the enzyme in cultured skin fibroblasts of normal controls and of galactosialidosis patients, using the antibodies raised against the recombinant human sialidase. We demonstrated that in both types of cells the sialidase is targeted to lysosomes, but in galactosialidosis fibroblasts it is abnormally processed and further rapidly degraded.

MATERIALS AND METHODS

Cell culturing

Human skin fibroblasts from galactosialidosis and sialidosis patients were obtained from NIGMS Human Genetic Mutant Cell Repository (GM05076, GM02438A), the Montreal Children's Hospital Cell Repository (WG544) and Sainte-Justine Hospital Cell Repository (89.40.11, 83.29.34). Cells were cultured to confluency in Eagle's Minimal Essential Medium (EMEM, Mediatech, Washington DC), supplemented with 10% (v/v) fetal calf serum (MultiCell) and antibiotics.

Purification of lysosomal high-molecular-weight complex

The 1.27 MDa lysosomal complex was purified from human placenta extract by affinity chromatography on a concanavalin A-Sepharose column and a *p*-aminophenyl- β -D-thiogalactopyranoside (PATGAL)-agarose column as previously described [22]. Prior to the PATGAL-agarose chromatography, the crude glycoprotein fraction from human placenta extract was concentrated to 40 mg/ml of protein, dialysed against 20 mM sodium acetate buffer, pH 4.75, containing 50 mM EDTA, and incubated for 120 min at 37 °C to activate and stabilize lysosomal sialidase [3,17]. After affinity purification the preparation was dialysed against 20 mM sodium acetate buffer, pH 5.2, containing 0.15 M NaCl, 0.02% (w/v) NaN₃ and 50 mM EDTA, concentrated to 3 mg/ml of protein, applied to a FPLC Superose™ 6 column (Pharmacia) and eluted with the same buffer at a flow rate of 0.4 ml/min. Collected fractions were analysed for sialidase, GAL, and CathA activities [5] as well as by SDS/PAGE and Western blotting as described below. The M_r values of the eluted enzymes were determined using the calibration curve obtained with the following M_r standards (Pharmacia): blue dextran (M_r ~ 2000 kDa), thyroglobulin (M_r 669 kDa), apo-ferritin (M_r 440 kDa), catalase (M_r 232 kDa), aldolase (M_r 158 kDa), chymotrypsinogen (M_r 25 kDa), and ribonuclease A (M_r 13.7 kDa). The deglycosylation of sialidase was performed by treatment of 1.27 MDa complex (20 μ g) by 2 U of endoglycosidase F (Sigma) in a 20 mM sodium phosphate buffer, pH 7.0, containing 0.1% (w/v) SDS, 0.2% (w/v) DTT and 0.5% (v/v) Triton X-100 for 17 h at 37 °C.

Antibodies

Rabbit polyclonal antiserum against human lysosomal hexosaminidase A was a generous gift of Dr. Roy A. Gravel (McGill University, Montreal). Rabbit polyclonal antibodies against recombinant human sialidase were prepared as follows. A 755 kb *Bst*II/*Tag*I fragment of sialidase cDNA, encoding amino acids 58–308 of the sialidase protein, was treated with Klenow DNA polymerase and inserted into pGEX-2T vector (Pharmacia), restricted with *Eco*RI and treated with Klenow DNA polymerase. The plasmid was expressed in *E. coli* to produce glutathione transferase (GST)-sialidase fusion protein. The fusion protein was purified from the bacteria homogenate by affinity chromatography using glutathione-Sepharose (Pharmacia) followed

Table 1 Immunoprecipitation of lysosomal, cytosolic and plasma membrane sialidase, GAL and HEX in human liver extract by anti-recombinant human lysosomal sialidase antibodies

Antibodies added (μ g)	Enzyme activity (% to control)				
	Lysosomal sialidase	Cytosolic sialidase	Plasma membrane sialidase	HEX	GAL
9	100	100	100	100	100
2	65	98.2	98	105	97.6
5	13.4	98.5	98	105	96.9
10	4.1	95.5	95	102	95.1
20	1.0	94	103	105	92.0
50	2.0	96	98	105	92.1

by the FPLC anion-exchange chromatography on Mono Q column (Pharmacia). Purified fusion protein (1 mg), homogeneous by SDS/PAGE analysis, was used to immunize a rabbit. IgG fraction purified from the obtained antiserum by ammonium sulfate fractionation was passed through a recombinant GST-Sepharose column to absorb the anti-GST-specific antibodies. The resulting antibody preparation was used in a dilution of 1:2500 or 1:5000 for Western blotting, 1:500 – for immunoelectron and 1:200 – for immunofluorescent microscopy.

Immunotitration

1 g of human liver tissue obtained from autopsy materials was homogenized in 50 mM sodium phosphate buffer, pH 6.0, containing 100 mM NaCl and 0.5% (w/v) of sodium deoxycholate. The homogenate was centrifuged at 35 000 *g* for 30 min. 200 μ l of homogenate supernatant were then mixed with 100 μ l of 50 mM sodium phosphate buffer, pH 6.0, containing 100 mM NaCl and 10 mg/ml of BSA and increasing amounts (1–50 μ g) of anti-human sialidase antibodies or of IgG fraction from preimmune rabbit serum. In control experiments instead of liver extract we used 10 μ g of 680 kDa placental GAL-CathA complex [22] dissolved in 200 μ l of phosphate buffer mentioned above. After 2 h of incubation at 4 °C, the pellet from 300 μ l of Pansorbin Cells (Calbiochem) was added. The samples were incubated for 2 h at 4 °C with constant shaking and then centrifuged at 13 000 *g* for 10 min. The supernatants were assayed for GAL and β -hexosaminidase (HEX) activities as described [5]. Activity of lysosomal and cytosolic sialidase was assayed with 4 MU α -neur at pH 4.2 and 6.5, respectively [23]. Plasma membrane sialidase was assayed with GM2-ganglioside in the presence of 0.2% Triton-X100 as described [23,24]. The concentration of released sialic acid was measured by phenobarbituric method [25].

The immunotitration results (Table 1) showed that antibodies raised against recombinant lysosomal sialidase precipitated all activity of lysosomal sialidase in human liver extract, but not of cytosolic or plasma membrane sialidases, thus confirming that the antibodies are specific against the lysosomal enzyme and that the lysosomal, plasma membrane and cytosolic sialidases do not have common antigenic determinants, as has been suggested [26]. The anti-sialidase antibodies did not precipitate purified GAL (not shown) but precipitated up to 8% of GAL activity in liver extract, which probably represents the fraction of GAL associated with the lysosomal sialidase in the 1.27 MDa complex. The activity of HEX which is not part of the complex was not changed by anti-sialidase antibodies.

Figure 1 FPLC gel-filtration (a), Western blotting using anti-sialidase antibodies (b) and SDS-PAGE analysis (c) of the 1.27 MDa complex from human placenta

The complex (3 mg) was analysed by FPLC gel-filtration on Superose 6 HR column. (---), Absorbance at 280 nm; (□), GAL activity; (△), CathA activity ($\times 10^{-1}$); (○), sialidase activity ($\times 10^2$). The positions of the elution peaks of the M_r standards and void volume (V_0) are shown by arrows. The indicated gel-filtration fractions were analysed by SDS/PAGE and Western blot, as described. The protein bands are identified on the right side of the gel as follows: GLUCOR, β -glucuronidase; GAL, 64 kDa subunit of β -galactosidase; GAL46, a 46 kDa product of C-terminal processing of β -galactosidase; SIAL, sialidase; GALNS, 40 kDa subunit of *N*-acetylgalactosamine-6-sulphate sulphatase; CathA32 and CathA20 are 32 kDa and 20 kDa subunits of cathepsin A; Ig κ Ch, immunoglobulin κ -chain.

Western blotting

FPLC gel-filtration fractions (25 μ l), 1.27 MDa complex preparation (5 μ g), treated or not treated with endoglycosidase F, concentrated crude glycoprotein fraction from human placenta (150 μ g) and fibroblast homogenates (50 μ g) were subjected to SDS/PAGE and electrotransferred to NITRO ME nitrocellulose membrane (Micron Separations Inc, Westboro, MA) as described [4]. The detection of protein bands, cross-reacting with anti-sialidase antibodies was performed using the BM Chemiluminescence kit (Boehringer Mannheim) in accordance with the manufacturer's protocol.

Expression of sialidase cDNA in sialidosis fibroblasts

Full-length human sialidase cDNA was obtained as an *Xma*III fragment from pCRII vector and cloned into NotI site of pCMV expression vector [27] kindly provided by Dr. S. G. MacGregor and Dr. T. Caskey (Baylor College of Medicine, Houston).

Skin fibroblasts of galactosialidosis patients and of normal controls were transfected with pCMV-sialidase expression vector using Lipofectamine reagent (Life Technologies Inc, Gaithersburg, MD) in accordance with the manufacturer's protocol. 24 h after the transfection, sialidase and hexosaminidase activities were assayed in cell homogenates using the corresponding fluorogenic 4-methylumbelliferyl glycoside substrates [28,29]. One unit of enzyme activity (U) was defined as the conversion of 1 μ mol of substrate per min. Proteins were assayed according to Bradford [30] with BSA (Sigma) as a standard.

Immunofluorescent microscopy

24 h after the transfection, fibroblasts were fixed on the glass slides with acetone/methanol (4:1) at -20°C , washed in PBS, blocked for 1 h with 2% (w/v) BSA in PBS, incubated with anti-sialidase or anti-hexosaminidase A antibodies at a final dilution

of 1/200 for 1 h at room temperature, washed with PBS and further incubated for 30 min with rhodamine-conjugated goat anti-rabbit IgG at a dilution of 1/100 [81]. Epifluorescent microscopy was performed using Zeiss Axioskop microscope.

Immunoelectron microscopy

24 h after transfection with pCMV-sialidase or pCMV expression vectors cultured fibroblasts were detached from the culture dishes with a rubber policeman, washed with Hank's Balanced Salt Solution (HBSS), and fixed in 4% paraformaldehyde/0.5% glutaraldehyde in 50 mM phosphate buffer, pH 7.5. The cell pellets were dehydrated in methanol and embedded in Lowicryl K4M as described previously [32,33].

Ultrathin Lowicryl sections were mounted on 300-mesh Formvar-coated nickel grids (Polysciences, Inc., Warrington, PA). Each section was incubated for 15 min in 20 mM Tris/HCl-buffered saline (TBS), containing 0.1% (v/v) Tween 20 and 15% (v/v) goat serum and for 30 min in anti-sialidase antibodies diluted 1:500 in TBS. The sections were then washed four times with TBS containing 0.05% (v/v) Tween 20, incubated for 15 min in TBS containing 15% (v/v) goat serum, and for 30 min in colloidal gold (10 nm)-conjugated goat anti-rabbit IgG (Zollinger Incorporated, Montréal, Québec, Canada). The sections were washed twice with TBS containing 0.05% (v/v) Tween 20, twice with distilled water and counterstained with uranyl acetate followed by lead citrate [32,33]. Normal rabbit serum was used as a control. Electron micrographs were taken on a Philips 400 electron microscope (Philips Electronics, Toronto, Ontario). The number of colloidal gold particles per μm^2 was counted in the lysosomes of five normal and five galactosialidosis fibroblasts both transfected with the sialidase expression vector. The area of the lysosomes (vesicles with a diameter of 250–300 nm containing intraluminal electrondense

Figure 2 Immunoblotting of sialidase

(a) 1.27 MDa complex before (lane 1) or after treatment with endoglycosidase F (lane 2). (b) Sialidosis (cell line WG544) fibroblast homogenates: non transfected (lane 1), transfected with pCMV-sialidase (lanes 2 and 3) or with pCMV (lane 3) expression vectors. (c) Concentrated crude glycoprotein fraction from human placenta: non-treated (lane 1) or incubated for 30 (lane 2), 60 (lane 3), 90 (lane 4) and 120 (lane 5) min at 37 °C. Protein samples were subjected to SDS/PAGE, transferred to a nitrocellulose membrane and stained with rabbit anti-sialidase antibodies as described. (d) Activation of sialidase in concentrated crude glycoprotein fraction during the 120 min incubation at 37 °C. The steady state rate of 4 MU α -neur hydrolysis was measured at 0.2 mM substrate concentration. (e) Lineweaver-Burk plot of the substrate dependence of the initial rate of 4 MU α -neur hydrolysis catalysed by sialidase in concentrated crude glycoprotein fraction before (○) or after (●) the 120 min incubation at 37 °C. K_m values and the r values for the regression lines are shown.

material) was measured with a digital analyser MOP-3 (Carl Zeiss, Berlin, Germany).

Metabolic labelling

Human fibroblasts, grown to confluency in 75 cm² culture flasks (~10⁶ cells), were washed twice with HBSS, then incubated for 2 h in methionine-free Dulbecco's Modified Eagles Medium (D-MEM, Gibco-BRL) supplemented with L-glutamine and sodium pyruvate, and for 40 min – in 5 ml of the same medium supplemented with [³⁵S]methionine (Dupont), 0.1 mCi/ml. The radioactive medium was then removed, and the cells were washed twice with HBSS and chased at 37 °C in EMEM supplemented with 20 % (v/v) fetal calf serum.

At the time indicated in the figures, the cells were placed on ice, washed twice with ice-cold PBS then lysed for 30 min on ice in 1 ml of radioimmunoprecipitation assay (RIPA) buffer, containing 50 mM Tris/HCl, pH 8.0; 150 mM NaCl; 1 % (v/v) NP-

40; 0.5 % (w/v) sodium deoxycholate; 0.1 % (w/v) SDS; 5 μ g/ml leupeptin and 0.1 mM PMSF. The lysate was collected and centrifuged at 13000 g for 10 min to remove the cell debris.

Immunoprecipitation, electrophoresis and quantitation of sialidase

1.0 ml of lysate was incubated for 4 h with preimmune serum at a final dilution of 1/20. Then the pellet obtained from 300 μ l of Pansorbin Cells (Calbiochem) was added and the resulting suspension was incubated for 2 h at 4 °C, followed by centrifugation for 10 min at 13000 g. Supernatants were incubated overnight with the anti-sialidase antibodies in a 1/100 final dilution, then for 2 h at 4 °C with the pellet from 100 μ l of Pansorbin Cells and precipitated as above. The pellet was washed three times with 1 ml of RIPA buffer. The antigens were eluted from the pellet by the addition of 100 μ l of a buffer containing 0.1 M Tris/HCl, pH 6.8, 4 % (w/v) SDS, 20 % (v/v) glycerol, 0.2 M DTT and 0.02 % (w/v) bromophenol blue. The proteins

were denatured by boiling for 5 min and 50 μ l of each sample were subjected to SDS/PAGE according to Laemmli [34]. The molecular weights were determined with [14 C]-labelled protein markers (Amersham). The gels were fixed in acetic acid/isopropanol/water (10/50/40), soaked for 30 min in AmplifyTM solution (Amersham), vacuum dried at 60 °C and analysed either by autoradiography and scanning densitometry or by quantitative fluorometry on a PhosphorImager SI analysis screen (Molecular Dynamics) using the software supplied by the manufacturer. The half-lives of sialidase in normal and galactosialidosis cells were calculated from the slopes of regression lines in semilogarithmic plots (e.g. Figure 6c).

RESULTS

Previous studies [3,5] demonstrated that active sialidase can be purified from human tissues only as a part of the 1.27 MDa complex, which has multiple protein components. Therefore to provide the basis for studies of sialidase biogenesis we first had to ensure which protein component of the 1.27 MDa complex represents the mature active sialidase. The 1.27 MDa multi-enzyme lysosomal complex was purified from human placenta as described previously [5] using PATGAL-agarose affinity column followed by FPLC gel-filtration on Superose 6 column (Figure 1a). On the last step the 1.27 MDa complex [4,5] containing all sialidase and about 2% of total GAL and CathA activities (peak I in Figure 1a) was separated from a 680 kDa GAL-CathA

complex which contained most of GAL and CathA but no sialidase activity (peak II in Figure 1a). On Western blots of the gel-filtration fractions anti-sialidase antibodies labelled only the 48.3 kDa band which elution profile matched that of sialidase activity (Figure 1b). The observed molecular mass also corresponded to that predicted from the deduced amino acid sequence of sialidase, in which the potential signal peptide (amino acids 1–47) is removed and three putative sites [21] are *N*-glycosylated. Indeed, the treatment of the preparation with endoglycosidase F prior to Western blotting (Figure 2a) reduced the molecular mass of the enzyme to 43 kDa, close to that predicted for the mature deglycosylated enzyme, 42.5 kDa, and similar to that reported by Bonten et al. [35] and Milner et al. [36]. The comparison of the Western blot with the silver-stained SDS/PAGE gel (Figure 1c vs. Figure 1b) indicated that anti-sialidase antibodies reacted with a component of 46–48 kDa diffuse protein band. Sequencing of the NH₂-terminal of the 46–48 kDa protein yielded both the major amino acid sequence, QRMFEIDYSRD, corresponding to that of human GAL [37] and also a minor sequence, ENDFXLVQ, corresponding to the amino acids 48 to 55 of sialidase sequence [21]. Therefore, the 46–48 kDa diffuse band contained 48.3 kDa mature sialidase which co-migrated on gel with the previously described 46 kDa product of GAL proteolytic digestion [38]. These data also confirmed that the signal peptide (amino acids 1–47) is cleaved in the active sialidase.

Previously [21] we have described the sialidosis fibroblast line, WG544, characterized by 10-fold reduced mRNA level and negligible activity of sialidase. In the homogenates of these cells

Figure 3 Immunofluorescence of cultured human skin fibroblasts of normal controls (a, c) and galactosialidosis patient (b, d)

24 h after the transfection with pCMV-sialidase (a, b, c) or with pCMV (d) expression vectors fibroblasts were fixed with cold acetone/methanol (4:1) and stained with anti-sialidase (a, b, d) or anti-lysosomal hexosaminidase A (c) rabbit antibodies, followed by staining with rhodamine-conjugated goat anti-rabbit IgG antibodies. Magnification $\times 400$.

Figure 4 Electron micrograph of normal fibroblasts transfected with pCMV (a) or with pCMV-sialidase (b–d) expression vectors, immunogold-labelled with anti-sialidase antibodies

(a) Except for the presence of a few grains (arrowheads) in a lysosome (L), the cytoplasm (C) and plasma membrane (PM) of this cell is unlabelled. (b–d) Transfection induced a strong immunogold labelling (arrowheads) of the plasma membrane, vesicles (V) and lysosomes (L). Magnification: $\times 60\,000$ (a–c) or $\times 65\,000$ (d).

transfected with pCMV-sialidase expression vector, which increased 15-fold the specific sialidase activity [21], two 48.3 and 46 kDa bands were detected (Figure 2b, lanes 2 and 3). Only the major, 48.3 kDa band was found to be associated with the 1.27 MDa complex (Figure 1b) or present in the crude glycoprotein fraction of human placenta extract (Figure 2c). Since this fraction contained all sialidase activity in tissue, we proposed that the 48.3 kDa form corresponded to the active sialidase, whereas the 46 kDa form represented a catalytically inactive, partially degraded or alternatively glycosylated sialidase. In a control experiment we did not detect any bands cross-reacting with lysosomal sialidase in untransfected cells or in the cells transfected with pCMV vector (Figure 2b, lanes 1 and 4).

Verheijen et al. [17] reported that sialidase can be activated by 90–120 min incubation of the concentrated crude glycoprotein fraction at 37 °C and suggested that the activation of sialidase

results from the formation of the sialidase-CathA complex. In contrast other researchers hypothesized that activation of sialidase *in vitro* involves its proteolytic processing [39,40]. To understand the molecular mechanism associated with the activation process we performed the Western blotting of sialidase in the samples obtained in the process of the 120 min incubation of the concentrated glycoprotein fraction at 37 °C (Figure 2c), which increased the specific activity of sialidase from 0.94 mU/mg to 3.5 mU/mg (Figure 2d). We could not detect any changes in the molecular mass of sialidase, which indicated that the activation process cannot be attributed to any detectable proteolytic modification of the protein. Besides, kinetic studies (Figure 2e) demonstrated that the ‘activation procedure’ increased the affinity of the enzyme towards the substrate ($K_m = 1.8 \pm 0.7$ mM after the activation versus $K_m = 10 \pm 1.3$ before the activation) but did not change the maximal reaction

Figure 5 Fibroblasts from galactosialidosis patient transfected with pCMV (a) or with pCMV-sialidase (b–c) expression vectors, immunogold-labelled with anti-sialidase antibodies

(a) Plasma membrane (PM), cytoplasm (C) and vesicles (V) are unlabelled. (b–c) Plasma membrane (PM) and vesicles (arrowheads) are moderately labelled. Large vesicles reminiscent of lysosomes (L) with a dispersed electron dense content also show a moderate labelling. Magnification: $\times 60\,000$.

rate. These results, similar to those previously described for β -galactosidase [15], suggest that activation of sialidase in the complex may result from the conformational change of the enzyme. The studies of the tertiary structure of sialidase necessary to understand the molecular mechanism of this phenomenon are in progress in our laboratory.

To study the biogenesis of the lysosomal sialidase we expressed it in cultured human fibroblasts from normal controls and galactosialidosis patients using the previously described pCMV-sialidase vector [21] and characterized the activity, intracellular localization and processing of the expressed enzyme. We found a three-fold increase of sialidase activity in normal fibroblasts transfected with pCMV-sialidase (2.3 ± 0.03 mU/mg) as compared to untransfected cells or to the cells transfected with

pCMV vector which did not contain the sialidase insert (0.82 ± 0.02 mU/mg). In contrast the sialidase activity in all four fibroblast lines of galactosialidosis patients transfected with pCMV-sialidase vector (0.04 ± 0.01 mU/mg) did not differ from that of untransfected cells or of the cells transfected with pCMV (0.05 ± 0.02 mU/mg) which again confirmed that sialidase requires an association with CathA for the expression of enzymatic activity [3].

To understand if in both normal control and galactosialidosis cells the expressed sialidase is targeted to lysosomes we studied its intracellular localization by immunofluorescent and immunoelectron microscopy. Fluorescent microscopy (Figure 3a,b) revealed in both control and galactosialidosis fibroblasts transfected with pCMV-sialidase a perinuclear punctate pattern

Figure 6 Metabolic labelling of sialidase in normal and galactosialidosis fibroblasts

(a, b) Fluorographs of pulse-labelling and chase of sialidase in normal and galactosialidosis (line 83.29.34) fibroblasts 24 h after the transfection with pCMV-sialidase expression vector. Lanes 1, pulse; lanes 2–5, chase for 1, 4, 6 and 22 h, respectively. M_r values of two forms of mature sialidase (48.3 and 46 kDa) and its degradation products (38.7 and 24 kDa) are shown. (c) Semilogarithmic plots of the relative amount of mature sialidase remaining in the normal (○) and galactosialidosis (●) fibroblasts versus the chase time. The half-life ($t_{1/2}$) values and the r values for the regression lines are shown. (d) Fluorograph of the sialidase labelled for 4 h in galactosialidosis (lane 1, cell line GM05076; lane 2, cell line GM02438A) and normal control (lane 3) fibroblasts. The patterns similar to those presented in (a), (b) and (d) were observed in three experiments performed on different occasions.

similar to that obtained with the anti-lysosomal hexosaminidase A antibodies (Figure 3c) and consistent with the localization of the expressed sialidase in a lysosomal compartment. However, the galactosialidosis fibroblasts showed less intense fluorescent staining, suggesting the instability of the expressed sialidase.

Immunoelectron microscopy (Figures 4 and 5) confirmed the intralysosomal localization of the expressed sialidase. Non-transfected normal control fibroblasts incubated with the anti-sialidase antibodies showed occasional immunogold labelling of lysosomes (Figure 4a). Normal fibroblasts transfected with the pCMV-sialidase expression vector demonstrated an intense immunogold labelling of lysosomes, the plasma membrane and some intracellular vesicles (Figure 4b–d). Control serum did not yield any immunogold labelling in either type of cells, indicating that the observed immunoreactivity was specific (not shown).

Non-transfected fibroblasts obtained from the galactosialidosis patients incubated with anti-sialidase antibodies did not yield immunogold labelling of the plasma membrane or of any type of

intracellular vesicles including the lysosomes (Figure 5a), whereas the fibroblasts transfected with the pCMV-sialidase expression vector showed an immunogold labelling of the plasma membrane and of intracellular vesicles (Figure 5b). Large vacuoles reminiscent of lysosomes containing the storage materials were moderately labelled (Figure 5c). The number of gold particles per μm^2 was five-fold higher in lysosomes of the normal cells (59 ± 9) than in those of galactosialidosis fibroblasts (10 ± 5).

Figure 6 shows the results of 24-h pulse-chase experiments with normal and galactosialidosis fibroblasts. Two fragments ($M_r \sim 48.3$ and ~ 46 kDa) similar to those previously observed by Western blotting (Figure 2) were precipitated by anti-sialidase antibodies in normal fibroblast homogenates transfected with pCMV-sialidase vector (Figure 6a). The ratio between the 48.3 and 46 kDa forms of mature sialidase appeared to be constant and the intensities of both fragments decreased proportionally with chase time. In contrast, in galactosialidosis fibroblasts (Figure 6b), the intense abnormal 38.7 kDa sialidase fragment

became detectable after 40 min of pulse, whereas the amounts of both the 48.3 and 46 kDa forms of mature sialidase were remarkably reduced as compared to normal cells. During the chase in galactosialidosis fibroblasts both 38.7 kDa fragment and mature sialidase were rapidly degraded into a 24 kDa product. In Figure 6c these results are shown quantitatively for both control and galactosialidosis cells. In normal fibroblasts mature sialidase is reduced to 5–10 % of total by ~ 15 h, whereas in galactosialidosis fibroblasts, sialidase is nearly undetectable after only 4 h of chase. The kinetic curve for mature sialidase in normal human fibroblasts represents a slow monophasic decline with a half-life of ~ 2.7 h. The same plot for galactosialidosis fibroblasts is characterized by a much lower half-life of ~ 30 min (Figure 6c). Fast degradation of mature sialidase and the presence of the abnormal 38.7 and 24 kDa sialidase fragments were also observed in the three other galactosialidosis cell lines both when transfected with pCMV-sialidase (not shown) and when the metabolic labelling and immunoprecipitation were performed for the endogenous sialidase (Figure 6d).

DISCUSSION

Numerous attempts to purify and characterize the lysosomal sialidase from human, porcine and bovine tissues resulted in the isolation of the 1.27 MDa lysosomal complex [3,5,20], but further purification of sialidase was complicated by its low tissue content, instability and complete inactivation after the dissociation from the 1.27 MDa complex [3]. The 1.27 MDa complex in addition to 64 kDa GAL, 20 and 30 kDa CathA subunits contains five other proteins as demonstrated by SDS/PAGE analysis (see Figure 1c). These proteins with M_r ranging from 78 to 40 kDa were suggested as the candidate sialidase peptides on the basis of immunotitration of sialidase activity and photoaffinity labelling experiments [3,20,41,42]. However, further analysis of these peptides by the NH_2 -terminal sequencing demonstrated that they represented other previously identified proteins [42]. This discrepancy is explained by the results obtained in this work. Using antibodies against recombinant sialidase peptide, we mapped a 46–48 kDa protein band on Western blots of the 1.27 MDa complex. This band contained a 46 kDa fragment of GAL as a major component and a 48.3 kDa sialidase peptide as a minor component which yielded a weak signal probably undetected or ignored in previous studies in the process of NH_2 -terminal sequence analysis. Our data are consistent with the hypothesis that sialidase is synthesized as a 45.5 kDa precursor which after the cleavage of the signal peptide (first 47 amino acid) and glycosylation becomes a 48.3 kDa active mature enzyme. An alternatively glycosylated or processed 46 kDa form, which can be found in fibroblast homogenates is not present in the 1.27 MDa complex and probably has no enzymatic activity.

The immunofluorescent and immunoelectron microscopy studies clearly demonstrated the intralysosomal localization of sialidase in both normal and galactosialidosis cells, suggesting that the intralysosomal targeting of sialidase is independent of the presence of CathA, and that the deficiency of sialidase in galactosialidosis cannot be explained by its mistargeting. Previously [21] we reported that sialidase contains a C-terminal 'Tyr-X-X-hydrophobic residue' motif, common to several lysosomal integral membrane proteins [43–47], which are first targeted to the plasma membrane and then internalized by endocytosis. The hypothesis that sialidase is transported to the lysosome by a similar mechanism is supported by the results of our immunoelectron microscopy experiments. In all cells studied we observed not only the labelling of the inner side of the lysosomal membrane and lysosomal content but also of the

plasma membrane and intracellular (possibly endocytic) vesicles. Alternatively, sialidase can be targeted to the lysosome by the mannose-6-phosphate receptors, as for most of the soluble lysosomal enzymes, and then anchored to the lysosomal membrane via a second protein. The mechanism of sialidase lysosomal targeting is currently under investigation in our laboratory.

The unique feature that distinguishes lysosomal sialidase from other sialidases is its activation by the formation of the 1.27 MDa complex with CathA. We found that two molecular mechanisms underlay this process. First, the association of sialidase with CathA in 1.27 MDa complex is required for this enzyme to obtain the catalytically active conformation. This hypothesis was supported by our finding that *in vitro* the activation of sialidase in the concentrated crude glycoprotein fraction did not involve any change of the enzyme molecular mass but increased the affinity of the enzyme for the substrate. Second, metabolic labelling of sialidase in the fibroblasts of galactosialidosis patients demonstrated that sialidase is not stable in the absence of CathA and undergoes abnormal processing as indicated by the rapid disappearance of active mature sialidase (half-time ~ 30 min vs. ~ 2.7 h in control cells) which is degraded into a 38.7 kDa and further into a 24 kDa protein. Therefore, we conclude that the molecular mechanism of sialidase deficiency in galactosialidosis is associated with its abnormal intralysosomal processing and rapid proteolytic degradation.

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