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Only sphingolipid activator protein B (SAP-B or saposin B) stimulates the degradation of globotriaosylceramide by recombinant human lysosomal α-galactosidase in a detergent-free liposomal system

Ryoichi Kase[a,*], Uwe Bierfreund[b], Andreas Klein[b], Thomas Kolter[b], Kohji Itoh[a], Minoru Suzuki[c], Yasuhiro Hashimoto[c], Konrad Sandhoff[b], Hitoshi Sakuraba[a]

[a]*Department of Clinical Genetics, The Tokyo Metropolitan Institute of Medical Science, 18–22, Honkomagome-3, Bunkyo-ku, Tokyo 113, Japan*
[b]*Institut für Organische Chemie und Biochemie der Universität Bonn, Gerhard-Domagk-Str. 1, D-53121 Bonn, Germany*
[c]*Department of Membrane Biochemistry, The Tokyo Metropolitan Institute of Medical Science, 18–22, Honkomagome-3, Bunkyo-ku, Tokyo 113, Japan*

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Abstract The degradation of globotriaosylceramide ($GbOse_3Cer$) by insect-cell derived recombinant human α-galactosidase (EC 3.2.1.22) was carried out in a detergent-free liposomal system in order to mimic intralysosomal conditions. $GbOse_3Cer$ incorporated into unilamellar liposomes was used as the substrate, and naturally occurring sphingolipid activator proteins, rather than detergents, were used to stimulate the enzyme reaction. The degradation of $GbOse_3Cer$ was dependent on the presence of both α-galactosidase and sphingolipid activator protein B (SAP-B or saposin B). It proceeded optimally at pH 4.6, and was enhanced by increasing amounts of both α-galactosidase (0.24–24 mU/50 μl assay) and SAP-B (0–5 μg/50 μl assay). The enzyme reaction was not affected by SAP-A, SAP-C, or SAP-D. Therefore, our results indicate that only SAP-B is essential for the degradation of $GbOse_3Cer$ by α-galactosidase.

Key words: Sphingolipid activator protein B; Saposin B; α-Galactosidase; Globotriaosylceramide

1. Introduction

Glycosphingolipids (GSL) are components of the outer leaflet of the plasma membranes of vertebrate cells. After endocytosis their degradation is facilitated in lysosomes, the acidic compartments of the cell. According to a recent hypothesis on the topology of lysosomal digestion, vesicles enriched in plasma membrane components can enter into the lumen of the lysosomes carrying their glycosphingolipids on their outer surface facing the digestive juice of the lysosomes [1]. Here, GSL catabolism is catalyzed by the sequential action of exohydrolases. The inherited deficiency of one of these enzymes results in the accumulation of its lipid substrates in the lysosomes. For example, the genetic defect of α-galactosidase (EC 3.2.1.22) encoded by a gene localized to the X-chromosomal region, Xq22, results in Fabry disease, which is characterized by intralysosomal accumulation of GSL, predominantly $GbOse_3Cer$, in the cells of heart, kidney, and the vascular endothelial system [2].

For their lysosomal degradation by exohydrolases, several GSL with short oligosaccharide headgroups need the assistance of the so-called sphingolipid activator proteins (SAPs), small non-enzymatic glycoproteins [1,3]. Four of the five known SAPs, SAP-A to D, also called saposin A–D, are derived from a common precursor [4–6]. Despite their homology, like the identical locations of cysteine residues and glycosylation sites, SAP-B, C and D have different functions, which comprise lipid binding and enzyme stimulating specificities [3,7]. On the other hand, the physiological function of SAP-A remains unclear because no SAP-A deficiency has been characterized so far [3].

Zschoche and co-workers [8] demonstrated that lactosylceramide (LacCer) can be hydrolyzed by two different lysosomal β-galactosidases, galactosylceramide- and G_{M1}-β-galactosidase, in the presence of the activators, SAP-B or SAP-C. The α-galactosidase and G_{M1}-β-galactosidase are both water-soluble lysosomal enzymes acting on lipid substrates, $GbOse_3Cer$ and LacCer. α-Galactosidase hydrolyzes $GbOse_3Cer$ to yield LacCer which is then degraded by the β-galactosidases. Since an inherited deficiency of SAP-B results in sulfatide storage [3] and mild accumulation of $GbOse_3Cer$, we attempted to clarify the specificities of the four SAPs, especially SAP-B and SAP-C, for the enzymatic hydrolysis of $GbOse_3Cer$ in vitro using a detergent-free liposomal system.

2. Materials and methods

2.1. Materials

Restriction enzymes and T4 DNA ligase were purchased from Nippon Gene (Tokyo, Japan). *Spodoptera frugiperda* clonal isolated 9 (Sf9) cells, vector plasmid pAcYM1 [9], and wild-type *Autgrapha californica* nuclear polyhedrosis virus (AcNPV) were supplied by Dr. A. Takase (National Institute of Neuroscience, N.C.N.P., Tokyo, Japan). Egg phosphatidylcholine and dl-α-tocopherol were from Sigma (Deisenhofen, Germany). Concanavalin A (Con A)-Sepharose and Mono Q HR 5/5 were from Pharmacia LKB Biotechnology (Uppsala, Sweden). 4-Methylumbelliferyl-α-D-galactopyranoside was from Nacalai Tesque (Kyoto, Japan). Lichroprep RP-18 was from Merck (Darmstadt, Germany). TSKgel G3000 SW_{XL} was from Tosoh (Tokyo, Japan). Other reagents were of the highest grade available.

2.2. Expression and purification of recombinant human α-galactosidase

Expression and purification of human α-galactosidase were performed according to methods described previously [10]. In brief, the cDNA for human α-galactosidase (1.3 kb) was subcloned into the *Bam*HI site of pAcYM1, the resultant being designated as pAcGal.

*Corresponding author. Fax: (81) (3) 3823-6008.
E-mail: kase@rinshoken.or.jp

Abbreviations: Cer, ceramide or *N*-acylsphingosine; $GbOse_3Cer$, globotriaosylceramide, Galα1-4Galβ1-4Glcβ1-1Cer; G_{M1}, Galβ1-3GalNAcβ1-4Gal(3-2αNeuAc)β1-4Glcβ1-1Cer; LacCer, lactosylceramide, Galβ1-4Glcβ1-1Cer; MALDI/TOF/MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; SAP-A, -B, -C, and -D, sphingolipid activator protein A, B, C and D or saposin A, B, C and D; SAPs, sphingolipid activator proteins

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After co-transfection with AcNPV DNA and pAcGal into Sf9 cells, the recombinant virus was selected and propagated. Sf9 cells (2×10^7) were seeded in a 75 cm^2 flask, infected with the recombinant virus at a multiplicity of infection of 10, for 18 h, and then cultured in serum-free Grace's medium (10 ml) for 4 days. The expressed recombinant human α-galactosidase (3 mg) was purified from the culture medium (200 ml) by Con A-Sepharose and Mono Q column chromatography.

2.3. Liposomes

$GbOse_3Cer$ was tritiated at the terminal galactose [11] to a specific activity of 7.7 Ci/mol. Large unilamellar liposomes (80.0 ± 25 nm) were prepared using modified methods of Zschoche et al. [8], and MacDonald et al. [12]. Liposomes with a total lipid concentration of 2 μmol/ml were prepared from egg phosphatidylcholine, 93% (mol/mol), dl-α-tocopherol, 2% (mol/mol), and tritiated $GbOse_3Cer$, 5% (mol/mol). The lipids, dissolved in ethanol, were mixed, dried at 37°C under a stream of nitrogen, and then dispersed in an appropriate volume of water. After suspending the lipids by vigorous shaking, the suspension was sonicated for 2 min in a water-cooled cup horn at 100 W (Branson sonifier B12; Branson, Danbury, USA). The sonicated preparation was frozen in liquid nitrogen and thawed at 37°C four times. Then the preparation was extruded 19 times through an extrusion device (Liposo Fast-Basic; Avestin Inc., Ottawa, Canada) using 100 nm pore polycarbonate membranes.

2.4. Enzyme assays

The α-galactosidase assay mixture comprised liposomes (10 μl, 1 nmol of tritiated $GbOse_3Cer$), α-galactosidase (2.4 mU), and appropriate amount of SAPs, 50 mM sodium citrate buffer, pH 4.6, and BSA (5 μg), in a total volume of 50 μl. After incubation for 1 h at 37°C, the reaction was terminated by the addition of 50 μl of methanol and then the reaction mixture was loaded onto a small reverse-phase column of RP-18 (0.25 ml). After washing the column with 100 μl of chloroform/methanol/0.1 M aqueous KCl solution, 3:48:47 (v/v), liberated tritiated galactose was eluted with 2 ml of water, and the collected radioactivity was measured with a scintillation counter. Control radioactivity was measured by adding SAPs and enzyme only after termination of the incubation. SAPs, SAP-A to D, were purified from human tissues [7].

α-Galactosidase activity was measured with 4-methylumbelliferyl-α-D-galactopyranoside as the artificial substrate [10]. One unit of enzyme activity was defined as the amount of the enzyme that hydrolyzed 1 μmol of substrate per min.

2.5. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry

α-Galactosidase was dialyzed against water and then lyophilized. The enzyme was dissolved in 0.1% trifluoroacetic acid to a final concentration of 10 pmol/μl (0.5 μg/μl). The enzyme solution (0.5 μl) was applied onto a stainless-steel target, 2,5-dihydroxybenzoic acid (0.5 μl, 10 mg/ml), which was used as the matrix, was added and then the mixture was dried under an air stream. The molecular weight of α-galactosidase was determined by MALDI/TOF/MS (VISION 2000; Finigan MAT Instruments, Bremen, Germany), in the positive ion mode.

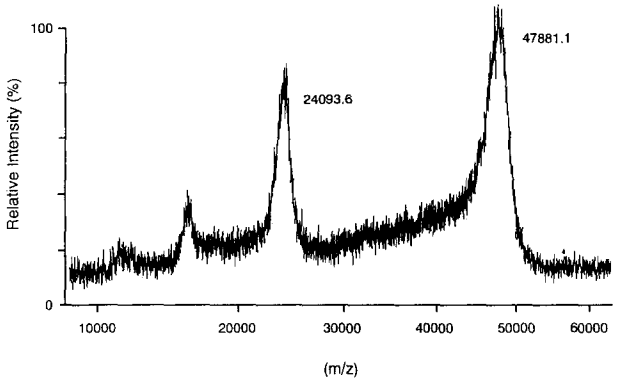

Fig. 1. Mass spectrum of recombinant human α-galactosidase. α-Galactosidase amounting to 0.25 μg was subjected to MALDI/TOF/MAS, as described under Section 2. Characterization of each peak is described in the text.

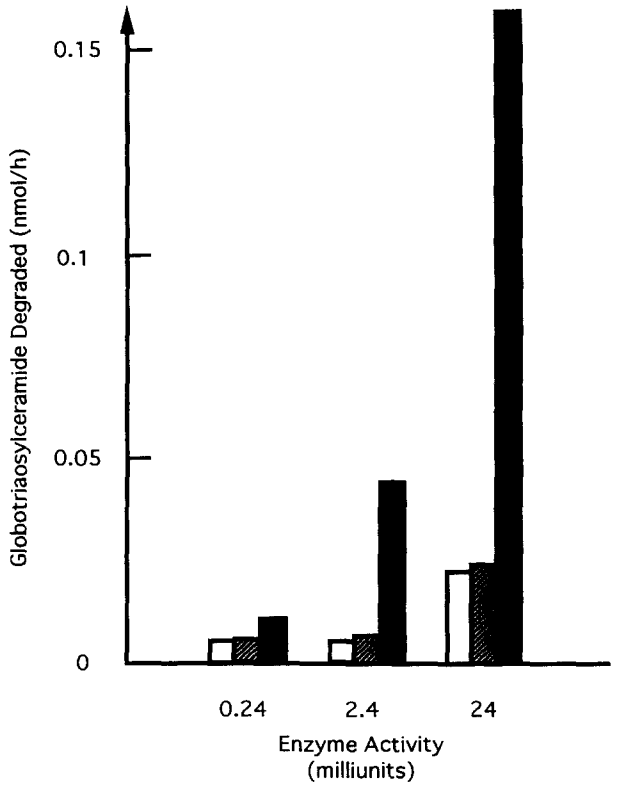

Fig. 2. Stimulation by SAP-B of the enzymatic hydrolysis of liposomal globotriaosylceramide. Assays were carried out in the absence (hatched bars) or presence of 1 μg of SAP-B (closed bars) using increasing α-galactosidase activities (0.24–24 mU), as indicated. Controls (open bars) were run by adding SAP-B and α-galactosidase after termination of the incubation of liposomes. Release of radio labeled galactose was quantified as described under Section 2.

3. Results and discussion

α-Galactosidase expressed in insect cells showed a homogeneous activity peak corresponding to a molecular mass of 48 kDa at pH 4.5 and at 6.0 on gel filtration (TSKgel G3000 SWXL) analysis (data not shown). Gel-filtration analysis data were supported by the result of MALDI/TOF/MS analysis (Fig. 1). Two peaks of *m/z* 47881.1 and 24093.6 appeared which were interpreted as arising from singly and doubly charged recombinant α-galactosidase, respectively. These results indicate that the recombinant α-galactosidase exists as a monomeric form in solution, in contrast to the homodimeric form of the native human enzyme [13,14]. The reason for the structural difference in the molecular assembly of both enzyme preparations remains uncertain. However, it was reported that the kinetic properties, against artificial substrate, of recombinant α-galactosidase closely corresponded to those known for α-galactosidase purified from human spleen [10,14].

We focused our work on the hydrolysis of $GbOse_3Cer$ by α-galactosidase, using recombinant enzyme, in the presence of SAPs. Gärtner and co-workers [15] already described stimulation of the degradation of micellar $GbOse_3Cer$ by α-galactosidase in a detergent-free system by an activator protein. This protein was later shown to be identical with SAP-B [16]. How-

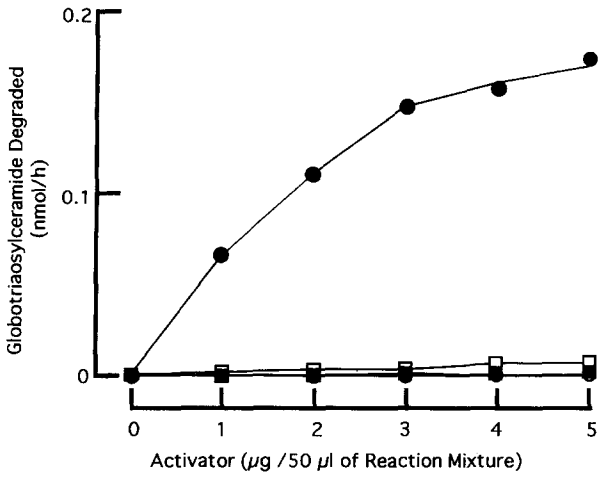

Fig. 3. Dependence of enzymatic globotriaosylceramide hydrolysis on increasing concentrations of the sphingolipid activator proteins, SAP-A to D. Reaction rates for lipid degradation by α-galactosidase, in the detergent-free liposomal system, were determined as described under Section 2. Each value is the mean of duplicate determinations. SAP-A (○), SAP-B (●), SAP-C (■), and SAP-D (□).

ever, it has never been determined whether or not the hydrolysis of liposome-integrated $GbOse_3Cer$ by α-galactosidase is stimulated by SAP-B, or if the other activators, i.e. SAP-A, C and D, are also active.

Liposomal $GbOse_3Cer$ was hydrolyzed by recombinant human α-galactosidase at significant rates only after the addition of SAP-B (Fig. 2). In the presence of 2.4 mU of α-galactosidase and 1 μg of SAP-B, about 7% of $GbOse_3Cer$ present on the outer surface of the liposome was degraded within 1 h. There was a linear relationship between enzyme activity and reaction time until 1 h. Therefore, subsequent experiments were carried out with 2.4 mU of α-galactosidase per assay. As shown in Fig. 3, other activators had almost no effect. Highly purified preparations of SAP-A, SAP-C and SAP-D at concentrations up to 5 μg/assay did not enhance the hydrolysis of $GbOse_3Cer$ by α-galactosidase significantly. These findings demonstrate the importance of the most active stimulator, SAP-B, for the lysosomal degradation of $GbOse_3Cer$ by α-galactosidase, SAP-C not being able to substitute for SAP-B, in contrast to the observations for the degradation of LacCer by G_{M1}-β-galactosidase [8].

With increasing amounts of SAP-B the breakdown of $GbOse_3Cer$ by α-galactosidase increased in hyperbolic fashion (Fig. 3), as would be expected from its proposed mechanism of action as a non-specific glycolipid binding protein lifting glycolipids from lipid bilayers and presenting them as substrates to their water-soluble enzymes [3].

Our in vitro data also support the results of loading studies with cultured mutant cells. Schlote and co-workers [17] reported that the catabolism of externally added $GbOse_3Cer$ in the cultured skin fibroblasts from a patient with a genetic defect of SAP-B is almost as low as that in fibroblasts defective in α-galactosidase.

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References

[1] Sandhoff, K. and Kolter, T. (1996) Trends Cell Biol. 6, 98–103.
[2] Desnick, R.J., Ioannou, Y.A. and Eng, C.M. (1995) Metabolic and Molecular Bases of Inherited Disease, 7th edn., pp. 2741–2784, McGraw-Hill, New York.
[3] Fürst, W. and Sandhoff, K. (1992) Biochim. Biophys. Acta 1126, 1–16.
[4] O'Brien, J.S., Kretz, K.A., Dewji, N., Wenger, D.A., Esch, F. and Fluharty, A.L. (1988) Science 241, 1098–1101.
[5] Nakano, T., Sandhoff, K., Stümper, J., Christomanou, H. and Suzuki, K. (1989) J. Biochem. 105, 152–154.
[6] Fürst, W., Machleidt, W. and Sandhoff, K. (1988) Biol. Chem. Hoppe-Seyler 369, 317–328.
[7] Klein, A., Henseler, M., Klein, C., Suzuki, K., Harzer, K. and Sandhoff, K. (1994) Biochem. Biophys. Res. Commun. 200, 1440–1448.
[8] Zschoche, A., Fürst, W., Schwarzmann, G. and Sandhoff, K. (1994) Eur. J. Biochem. 222, 83–90.
[9] Matsuura, Y., Possee, R.D., Overton, H.A. and Bishop, D.H.L. (1987) J. Gen. Virol. 68, 1233–1250.
[10] Ishii, S., Kase, R., Sakuraba, H., Fujita, S., Sugimoto, M., Tomita, K., Semba, T. and Suzuki, Y. (1994) Biochim. Biophys. Acta 1204, 265–270.
[11] Suzuki, Y. and Suzuki, K. (1972) J. Lipid Res. 13, 680–686.
[12] MacDonald, R.C., MacDonald, R.I., Menco, B.Ph.M., Takeshita, K., Subbarao, N.K. and Hu, L., (1991) Biochim. Biophys. Acta 1061, 297–303.
[13] Kusiak, J.W., Quirk, J.M. and Brady, R.O. (1978) J. Biol. Chem. 253, 184–190.
[14] Bishop, D.F. and Desnick, R.J. (1981) J. Biol. Chem. 256, 1307–1316.
[15] Gärtner, S., Conzelmann, E. and Sandhoff, K. (1983) J. Biol. Chem. 258, 12378–12385.
[16] Vogel, A., Fürst, W., Abo-Hashish, M.A., Lee-Vaupel, M., Conzelmann, E. and Sandhoff, K. (1987) Arch. Biochem. Biophys. 259, 627–638.
[17] Schlote, W., Harzer, K., Christomanou, H., Paton, B.C., Kustermann-Kuhn, B., Schmid, B., Seeger, J., Beudt, U., Schuster, I. and Langenbeck, U. (1991) Eur. J. Pediatr. 150, 584–591.