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The binding of glucosylceramidase to glucosylceramide is promoted by its activator protein

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A protein activator of glucosylceramidase (EC 3.2.1.45) has been previously identified by us in human placenta [(1985) Biochim. Biophys. Acta 836, 157–166]. In the present paper we report that its function in vitro is to stimulate the binding of the enzyme to its substrate, glucosylceramide. After the purification step which frees the enzyme of most of its activator protein (octyl-Sepharose 4B chromatography), the capacity of glucosylceramidase to bind to the glucosylceramide micelles is dramatically decreased. The addition of the activator protein to the purified enzyme restores this binding.

Protein activator; Glucosylceramidase; Gaucher's disease

1. INTRODUCTION

Glucosylceramide is hydrolyzed into glucose and ceramide by glucosylceramidase (EC 3.2.1.45), a membrane-bound, lysosomal enzyme [1], which displays a markedly reduced activity in Gaucher disease [2,3].

The catabolism of several glycosphingolipids requires special proteins, known as natural activator proteins or sphingolipid activator proteins (SAP) [4–6]. We have recently identified in human placenta such an activator protein ('placental factor') which enhances several-fold the enzymatic hydrolysis of glucosylceramide and is present in large excess over the glucosylceramidase in the tissue [7]. A comparison between a previously reported glucosylceramidase activator, the 'Gaucher factor' [8–11], and the placental factor indicates that they are different substances [12]; the former is mainly active in the hydrolysis of 4-methylumbelliferyl-β-D-glucopyranoside, while the latter is specifically effective for that of glucosylceramide.

Here, we have investigated the possible mechanism of the placental factor in the activation of the enzymatic hydrolysis of glucosylceramide.

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2. MATERIALS AND METHODS

2.1. *Materials*

Glucosylceramide purified from spleen of patients with Gaucher disease was labelled with tritium in the glucose moiety according to McMaster and Radin [13]. Sodium taurocholate (synthetic, 98% pure), oleic acid, trypsin inhibitor from soybean, bovine serum albumin, ovalbumin and bovine thyroglobulin were from Sigma (St. Louis, MO). [^{14}C]Taurocholate (55 mCi/mmol) and [1-^{14}C]oleic acid (56 mCi/mmol) were from Amersham International (England). Other chemicals and reagents were from standard commercial sources.

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2.2. *Glucosylceramidase and placental factor preparations*

Glucosylceramidase was purified from placenta as in our previous paper [7] for preparation II. In the present work we have used the enzyme at two different steps of purification: the butanol extraction (prep B.E.) and the octyl-Sepharose 4B chromatography (prep II itself). The placental factor was prepared as in [7].

2.3. *Glucosylceramidase assay*

The standard assay mixture contained, in a final volume of 0.2 ml, 40 μg [^{3}H]glucosylceramide (2600 dpm/nmol), 0.25 mg sodium taurocholate, 50 μg oleic acid, 0.1–0.2 mol/l sodium citrate-phosphate buffer, pH 5.6 (buffer A), and an appropriate amount of enzyme source. The reaction time was 1 h at 37°C. The enzymatically released glucose was estimated as described [7]. When present the placental factor was added to the assay mixture immediately before starting the enzymatic reaction. One unit of glucosylceramidase is defined as the amount of enzyme which hydrolyzes 1 nmol glucosylceramide/h under the standard assay conditions.

2.4. *Glucosylceramide micelle preparation and its interaction with glucosylceramidase*

200 μl of 0.2–0.4 mol/l sodium citrate-phosphate buffer (pH 5.6) was added to a dried mixture of 160 μg glucosylceramide, 1 mg taurocholate and 200 μg oleic acid. After sonication for 30 s at 4°C under a stream of nitrogen (Branson B 15 sonifier, equipped with a 3 mm microtip) the lipid micelles were left overnight at room temperature. A solution of 70 μg trypsin inhibitor in 200 μl water was then added. When required the latter solution also contained the enzyme source with or without the placental factor. After 5 min at 37°C under agitation, the samples were mixed with 400 μl of 20% (w/v) sucrose in buffer A and kept in ice for 15 min before centrifugation.

2.5. *Sucrose density gradient centrifugation*

Discontinuous sucrose density gradients were prepared by manually layering the following solutions into Ultra-clear™ centrifuge tubes (14 × 89 mm): 5.8 ml of 50% (w/v) sucrose in buffer A, the lipid micelle suspension prepared as described above (0.8 ml, 10% (w/v) sucrose), 5 ml of 5% (w/v) sucrose in buffer A, and 0.4 ml buffer A. The top buffer A layer contained additionally 2 mg sodium taurocholate. The presence of detergent in the upper layer was necessary to maintain the floating lipids in a dispersed form; without taurocholate in the upper layer the recovery of glucosylceramide and oleic acid was very poor. Centrifugation was performed in a Beckman SW 41 Ti rotor for 15 min at 40000 rpm at 4°C. Fractions of 1 ml were pumped out at a flow rate of 0.5 ml/min through a long needle carefully inserted down to the bottom of the centrifuge tube. The last 1 ml was left in the tube to rinse the lipid film of the meniscus remaining in part attached to the wall of the tube during gradient fractionation.

2.6. *Determination of protein*

Protein was determined by the dye-binding method of Bradford [14] with bovine serum albumin as standard.

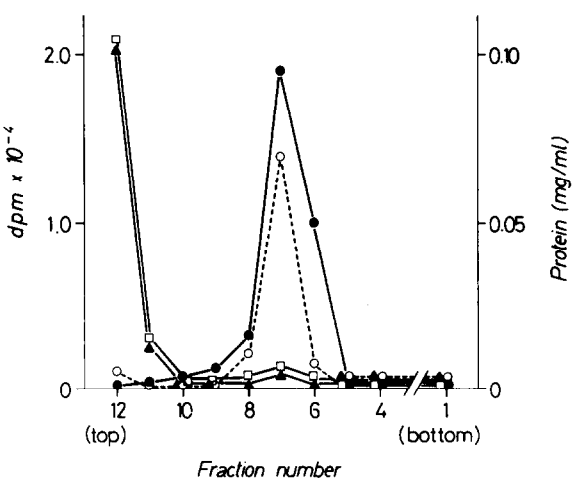

Fig.1. Discontinuous density gradient centrifugation of the glucosylceramide micelles. Two identical samples containing glucosylceramide, taurocholate, oleic acid and trypsin inhibitor, were prepared and centrifuged as reported in section 2. One sample was supplemented with 79 nCi [^{3}H]glucosylceramide and 80 nCi [^{14}C]taurocholate, the other with 79 nCi [^{14}C]oleic acid. The glucosylceramide (□), taurocholate (●) and oleic acid (▲) content of the fractions was determined by their ^{3}H and ^{14}C radioactivity measured on aliquots of 200 μl. The protein content was tested on aliquots of 100 μl (○).

3. RESULTS AND DISCUSSION

When the lipid micelle suspension, containing glucosylceramide, oleic acid and taurocholate at the same ratios as in the glucosylceramidase assay, was layered in the middle of the discontinuous sucrose density gradient and centrifuged, glucosylceramide and oleic acid moved to the upper layer of the gradient, while taurocholate remained at the starting position together with the trypsin inhibitor, which had been added to the sample to observe the behaviour of an unrelated protein

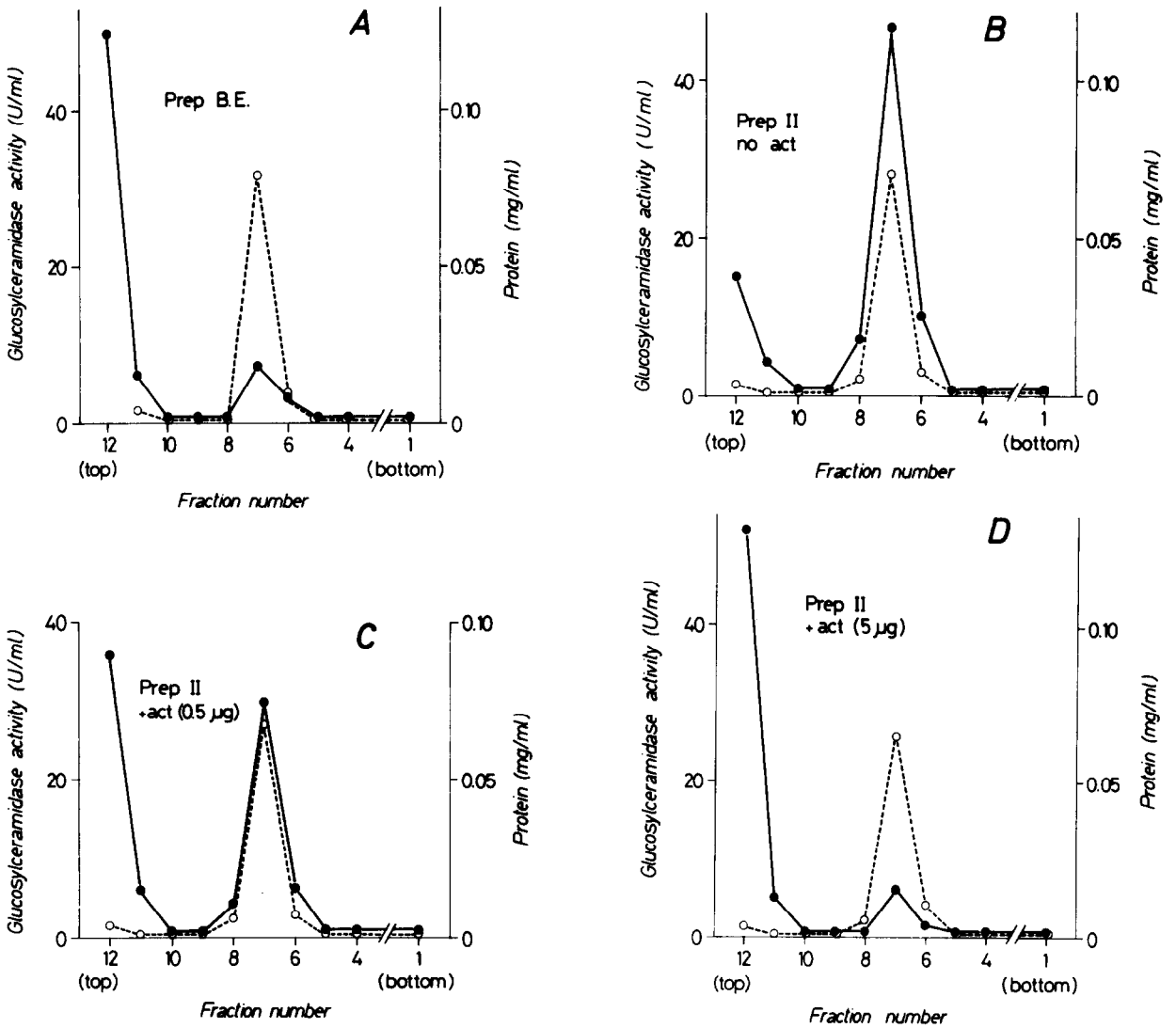

Fig.2. Discontinuous density gradient centrifugation of glucosylceramidase incubated with glucosylceramide micelles. The samples were the same as in fig.1 except for the presence of glucosylceramidase. The enzyme sources were either prep B.E. (A) (70 U when measured without placental factor or 85 U when measured in its presence) or prep II (B–D) (13 U when measured without placental factor or 85 U when measured in its presence). The placental factor was included in the samples of panel C (0.5 μg) and panel D (5 μg). The samples were centrifuged as in fig.1. Aliquots of 50 μl of every fraction were tested for glucosylceramidase activity in the presence of 1 μg/tube of placental factor (●), and aliquots of 100 μl for protein content (○). Glucosylceramide, taurocholate and oleic acid patterns after centrifugation were determined adding, in parallel experiments, the corresponding labelled compounds. The patterns were always identical to that reported in fig.1 and are omitted for clarity.

under these ultracentrifugation conditions (fig.1). The same pattern was observed in the absence of the trypsin inhibitor. The recovery of glucosylceramide and oleic acid after centrifugation was 75–85% and that of trypsin inhibitor and taurocholate 90–100%. A mixture of taurocholate and oleic acid is commonly used to stimulate the enzymatic hydrolysis of glucosylceramide [15]. It has been assumed that one of the functions of taurocholate is to form mixed micelles with the lipid substrate [16]. Our results showed that it is not the case, at least under our glucosylceramidase assay conditions. The ease with which taurocholate and glucosylceramide can be separated indicates that they do not form mixed micelles.

When the same ultracentrifugation procedure was carried out with a sample also containing a water-soluble preparation of glucosylceramidase partially purified by butanol extraction (prep B.E.), most of the glucosylceramidase activity moved to the upper part of the gradient together with oleic acid and glucosylceramide (fig.2A). The latter must be the component responsible for transfer of the enzyme since in its absence the glucosylceramidase activity remained at the sample layer (not shown). The capacity of glucosylceramidase to bind to its substrate was dramatically decreased when the enzyme was further purified by octyl-Sepharose chromatography. In fact, when the ultracentrifugation was performed using prep II as the enzyme source, most of the enzyme activity was found at the starting position and only about 20% of it moved to the top layer (fig.2B). The addition of increasing amounts of placental factor, a protein capable of increasing 6–9-fold the rate of enzymatic hydrolysis of glucosylceramide [7], increased the percentage of the floating glucosylceramidase until almost all the enzyme activity was found in the top layer (fig.2C,D). When comparable amounts of other proteins such as albumin, ovalbumin and thyroglobulin were substituted for the placental factor, they did not promote any binding of glucosylceramidase (not shown). The recovery of enzyme activity was 80–100% in all the reported experiments.

Our previous [7] and present results indicate that the placental factor, which is removed from glucosylceramidase by the octyl-Sepharose chromatography [7], is responsible for the increase of both the enzymatic activity and the binding of the enzyme to its substrate, most probably the first phenomenon being the consequence of the second.

The best characterized mechanism by which activator proteins promote the hydrolysis of sphingolipids is that described for the GM_2-ganglioside activator [17]. It extracts the ganglioside from the micelles to form a water-soluble activator-lipid complex which can interact with its hydrolysing water-soluble enzyme, β-hexosaminidase A. In contrast, the placental factor promotes the binding of the glucosylceramidase to its substrate anchoring the enzyme to the glucosylceramide micelles. This is the first example of such an effect exerted by a glycosphingolipid activator protein.

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