Preliminary evidence for a processing error in the biosynthesis of Gaucher activator in mucopolipidosis disease types II and III

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Activator protein (AP), which stimulated fibroblast sphingomyelinase activity, was isolated from the spleen of a patient with Gaucher's disease type I by the combined techniques of heat and alcohol denaturation, DEAE-cellulose column chromatography, gel filtration, preparative polyacrylamide-gel electrophoresis and decyl-agarose chromatography. Urea/sodium dodecyl sulphate (SDS)/polyacrylamide-gel electrophoresis showed two bands, one with an M_r of approx. 3000 and the other with an M_r of 5000–6500. Similarly, SDS/polyacrylamide-gel electrophoresis performed in the absence of urea revealed the presence of two components, one of which adsorbed to a concanavalin A (Con A) column. Both components stimulated sphingomyelinase activity. On a non-denaturing polyacrylamide gel containing Triton X-100, four major components, two of which bound to Con A, were detected with the dye Stains-All. Cross-reacting material (CRM) to polyclonal Gaucher spleen AP antibodies was detected in normal fibroblasts and in fibroblasts from patients with sphingomyelinase and β -glucocerebrosidase deficiency states (Niemann–Pick and Gaucher's diseases respectively). CRM in normal fibroblasts adsorbed to Con A columns and had the same mobility on SDS/polyacrylamide-gel electrophoresis as Con A-adsorbing Gaucher spleen AP. Normal AP was not observed in mucopolipidosis type II (I-cell disease) fibroblasts; instead, extracts from these cells revealed the presence of two closely migrating bands with higher M_r values than normal fibroblast CRM. Furthermore, extracts of media from I-cell fibroblast cultures, but not from control or Gaucher fibroblast cultures, contained AP activity towards sphingomyelinase and β -glucocerebrosidase. Fibroblasts from a patient with mucopolipidosis type III (pseudo-Hurler polydystrophy) showed an intermediate pattern consisting of normal as well as the higher- M_r CRM. Our data provide evidence for the existence of AP in cultured skin fibroblasts and suggest that these proteins may be targetted to the lysosome by post-translational modification in a similar manner to that reported for lysosomal enzymes.

INTRODUCTION

Patients with Gaucher's disease suffer from a genetic deficiency of the lysosomal enzyme β -glucocerebrosidase, and so elevated levels of the substrate are found in their tissues, especially liver and spleen (Brady, 1978). A low- M_r protein that activates β -glucocerebrosidase also accumulates in the spleen of these patients (Ho & O'Brien, 1971; Ho, 1973). Iyer *et al.* (1983) provided evidence that the activators isolated from Gaucher and normal human spleens are similar, differing primarily in concentration, hydrophobicity and binding to Con A–Sephadex. The activator protein from bovine spleen has been shown to have an M_r of approx. 6200 and contains a carbohydrate moiety (Berent & Radin, 1981). It has been shown by using purified enzymes that the activator protein binds to the catalytic protein, unlike the activators of cerebrosidase sulphate sulphatase (Fischer & Jatzkewitz, 1978), G_{M_2} ganglioside β -hexosaminidase (Conzelmann & Sandhoff, 1979) and G_{M_1} ganglioside β -galactosidase (Li & Li, 1976), which appear to bind the sphingolipid substrates in a 1:1 ratio and thereby facilitate hydrolysis by the respective enzyme (Fischer & Jatzkewitz, 1978; Conzelmann & Sandhoff, 1979). The Gaucher activator does not appear to be specific for β -glucocerebrosidase, since it stimulates two other lysosomal enzymes, β -galacto-

cerebrosidase and sphingomyelinase (Wenger *et al.*, 1982; Christomanou, 1980; Poulos *et al.*, 1984).

Although activator deficiencies have been described for some lysosomal storage disorders such as the variant forms of metachromatic leucodystrophy (Shapiro *et al.*, 1979; Inui *et al.*, 1983), G_{M_1} and G_{M_2} gangliosidosis (Conzelmann & Sandhoff, 1978; Hechtman *et al.*, 1982; Li *et al.*, 1981; Inui & Wenger, 1983), there have been no reports of a deficiency of the Gaucher activator in skin fibroblasts. However, it has been recently reported that β -glucosidase activities in ML II (I-cell disease) fibroblasts are deficient when assayed in the absence of detergents, although normal activities are found when the same cells are tested in the presence of detergents (Varon *et al.*, 1982). It was proposed that the deficiency of β -glucosidase activity was due to a lack of activator protein required for the *in vivo* activation of the enzyme activity. The primary defect in ML II is an error in post-translational glycosylation of some lysosomal enzymes, which leads to their excretion as higher- M_r forms (Hasilik & Neufeld, 1980; Hasilik *et al.*, 1980). Activator proteins have been shown to be located in the lysosome and, like lysosomal enzymes (Chiao *et al.*, 1978; Reitman & Kornfeld, 1981), may undergo similar post-translational modifications that allow them to be targetted to this particular site. Thus those lysosomal

Abbreviations used: Con A, concanavalin A; ML II (III), mucopolipidosis type II (III); AP, activator protein; CRM, cross-reacting material; APA, activator-protein activity; R_{BPB} , electrophoretic mobility relative to that of Bromophenol Blue.

enzymes that are not affected by the primary ML-II defect may show lowered enzyme activities if there is an inappropriate targetting of their activator protein(s). Inui *et al.* (1983) and Inui & Wenger (1984) provided immunological evidence for a partial deficiency of activator protein for the hydrolysis of G_{M1} ganglioside and sulphatide in skin fibroblasts, liver and brain in ML-II-disease patients.

In the present study we describe investigations on AP in fibroblasts by using specific polyclonal antibodies to purified Gaucher spleen AP. Using the antibody we have been able to identify CRM to Gaucher AP in fibroblasts from normal individuals and from patients with Niemann–Pick and Gaucher's diseases. However, ML-II fibroblasts were deficient in Gaucher AP but contained CRM with a higher M_r than that of normal fibroblast AP. These findings support the view that APs may be targetted to the lysosome in a similar manner to that observed for lysosomal enzymes.

MATERIALS AND METHODS

Materials

Chemical reagents. Palmitoyldihydrogalactocerebroside, glucosylceramide (from Gaucher spleen), bovine brain sphingomyelin, phosphatidylcholine (palmitoyl), bovine brain phosphatidylserine, decyl-agarose, Coomassie Blue R250 and Protease Type XIV were obtained from Sigma (St. Louis, MO, U.S.A.). Fractogel TSK HW 55S was from Merck (Darmstadt, Germany). Basal Eagle's Medium (BME) and MCDB-104 medium were obtained from Flow laboratories (Irvine, Ayrshire, Scotland). SDS, nitrocellulose paper, goat anti-(rabbit IgG)-conjugated horseradish peroxidase [goat anti-(rabbit IgG)–HRP], Stains-All, silver-staining kit, acrylamide, NN' -methylenebisacrylamide, NNN' -tetramethylethylenediamine, ammonium persulphate and 4-chloro-1-naphthol were obtained from Bio-Rad Laboratories (Richmond, CA, U.S.A.). The nitrocellulose paper of pore size $0.1\ \mu\text{m}$ came from Schleicher and Schüll (Dassel, Germany) and the Vectastain ABC kit from Vector Laboratories (Burlingame, CA, U.S.A.). Protein A–Sepharose 4B, Con A–Sepharose 4B and Sephacryl S-200 were purchased from Pharmacia (Uppsala, Sweden). Glucosyl[^3H]ceramide was prepared from Gaucher spleen glucocerebroside by catalytic tritiation (Poulos & Pollard, 1978) and [choline-methyl- ^{14}C] sphingomyelin ($51.0\ \text{mCi/mmol}$) was obtained from New England Nuclear Corp. (Boston, MA, U.S.A.). All other reagents were of analytical grade.

Tissues. Post-mortem spleen from a patient with Gaucher's disease type I had been stored at -80°C for approx. 1 year before use. The diagnosis was based on clinical and biochemical evidence, the latter including lipid and enzymic analysis of tissue. Skin fibroblast cell lines from patients with Gaucher's disease types I and II, Niemann–Pick disease types A, B and C and mucopolidiosis types II and III were obtained from the Mutant Human Cell Repository (Camden, NJ, U.S.A.).

Methods

Cell culture. Human skin fibroblasts of normal and mutant cell lines were grown to confluence in BME containing 10% (v/v) foetal-calf serum, supplemented

with glutamine (2 mM), NaHCO_3 (0.15%) and benzylpenicillin (320 units/litre) and maintained in an atmosphere of CO_2/air (1:19) at 37°C . Fibroblast cells were maintained in MCDB-104 medium when AP was to be isolated from the culture medium.

Enzyme assays. Sphingomyelinase and β -glucocerebrosidase assays were performed with liposomes of [choline-methyl- ^{14}C]sphingomyelin and β -glucosyl[^3H]ceramide respectively as substrates, as described by Poulos *et al.* (1984). APA was assessed by measuring the stimulation of fibroblast sphingomyelinase activity as described by Poulos *et al.* (1984). Protein determinations were made by the methods of Lowry *et al.* (1951) and Bradford (1976), with human albumin as standard.

Purification of AP from Gaucher's-disease spleen. Partially purified AP (preparation B) was isolated from a Gaucher type I spleen as described by Poulos *et al.* (1984). This preparation was further purified by preparative polyacrylamide-gel electrophoresis (preparation C) followed by decyl-agarose chromatography. The former was carried out in the following manner: pooled active material from Sephacryl S-200 or Fractogel TSK HW 55S chromatography was concentrated by freeze-drying to a protein concentration of 4–8 mg/ml and dialysed against 10 mM-Tris/HCl, pH 7.0 (Buffer A). This material was loaded on to a 12.5%-(w/v)-polyacrylamide gel and subjected to electrophoresis until the tracking dye, Bromophenol Blue, reached the anode end of the gel. The gel was then sliced into 0.5 cm zones and each slice was eluted with 5 ml of Buffer A for 24 h, after which samples (20–50 μl) were taken and APA estimated. The active fractions were pooled, dialysed against distilled water and concentrated by freeze-drying. The AP eluted from the gel was next applied at room temperature to a column (1 cm $\times$ 5 cm) of decyl-agarose in Buffer A. The column was washed with 20 ml of Buffer A, then eluted with a step gradient of 10 ml each of solutions made up of ethanediol/water in the ratios of 2:3, 3:2 and 9:1 (v/v) respectively. Each of the 10 ml fractions were dialysed against distilled water and assayed for APA. Most of the activity was eluted with ethanediol/water at a ratio of 3:2. This solution was then dialysed against distilled water, concentrated by freeze-drying and reconstituted in Buffer A (activator preparation D) to give a protein concentration of 1–2 mg/ml and stored at -20°C .

Electrophoresis. All gel slabs were prepared by using an acrylamide/bisacrylamide ratio of 75:2 (w/w). The gels were 1.5 mm thick with 10 cm $\times$ 14 cm resolving gels and 3.5 cm $\times$ 14 cm stacking gels. The method of Laemmli (1970) was used for SDS/polyacrylamide-gel electrophoresis with 15% (w/v) acrylamide in the resolving gel and 4% (w/v) acrylamide in the stacking gel. The same procedure, except for the omission of SDS, was used for preparative polyacrylamide-gel electrophoresis, where the resolving gel was 12.5% (w/v) acrylamide and the stacking gel 7.5% (w/v) acrylamide. Non-denaturing Triton/polyacrylamide-gel electrophoresis was run as for the preparative polyacrylamide-gel electrophoresis, but with the inclusion of 0.1% (w/v) Triton X-100. The resolving gel for this system contained 15% (w/v) acrylamide with 4% (w/v) acrylamide in the stacking gel. Urea/SDS/polyacrylamide-gel electrophoresis was

modified from the method of Swank & Munkres (1971) to run at pH 7.2 with 6 M-urea and with a 15%-polyacrylamide resolving gel and 7.5%-polyacrylamide stacking gel. The urea/SDS/polyacrylamide gels were run at a constant voltage of 50 V for 20 h. All the other gels were run at a constant current of 30 mA for 4–5 h.

Staining of polyacrylamide gels. Gels were stained with Coomassie Blue R250 by a modification of the method of Fairbanks *et al.* (1971), whereas staining with Stains-All was by the method of Green *et al.* (1973). A Bio-Rad kit employing the method of Merrill *et al.* (1981) was used for silver staining.

Antibody preparation. Female rabbits (3–4 kg) were immunized subcutaneously in the shoulder region at several sites with Gaucher AP (preparation D, 0.25 mg of protein) prepared in complete Freund's adjuvant mixture (1:1, v/v) in a final volume of 1 ml. Booster injections made up in incomplete Freund's adjuvant (1:1, v/v) were administered at 8- and 10-week intervals (0.25 and 0.15 mg of protein respectively) after primary immunization. Blood was drawn and serum tested for antibody against Gaucher AP either by immunoprecipitation using the Ouchterlony double-immunodiffusion technique on 0.9%-agarose plates (Hjelm *et al.*, 1972) or by the double-antibody enzyme-linked method using conjugated goat anti-(rabbit IgG)-HRP on nitrocellulose paper (Burnette, 1981).

Immunological identification. The antiserum raised against Gaucher AP was tested for specificity by the technique of immunoblotting (Burnette, 1981). Partially purified and purified AP were subjected to SDS/polyacrylamide-gel electrophoresis. After electrophoresis the proteins on the gels were transferred to nitrocellulose paper by electroblotting. The gel layers were placed in a chamber containing 25 mM-Tris/192 mM-glycine/20% (v/v) methanol, pH 8.3. Electrophoretic transfer was carried out towards the anode at 0.17 A for 4 h. The nitrocellulose paper was then incubated with 3% (w/v) gelatin for 1 h to block all residual binding sites. After removal of the blocking solution the paper was covered with primary rabbit antiserum at appropriate dilutions (1:250, 1:500) in 20 mM-Tris/HCl buffer/0.15 M-NaCl, pH 7.5 (Buffer B), and incubated at room temperature overnight. It was then washed three times for 15 min with 20 mM-Tris/HCl (pH 7.5)/0.5 M-NaCl (Buffer C), the second wash containing 0.05% (v/v) Tween 20, then incubated for 1 h with goat anti-(rabbit IgG)-HRP (1:2000 dilution in Buffer B). The paper was then subjected to the same washing procedure and developed in 100 ml of Buffer C containing 0.01% (v/v) H₂O₂ mixed with 20 ml of methanol containing 60 mg of 4-chloro-1-naphthol. The reaction was terminated by the addition of 0.01% (w/v) NaN₃ in water.

Con A-Sepharose chromatography. Gaucher AP (300 µg of protein, preparation C) was applied to a column (0.5 cm × 1.0 cm) of Con A-Sepharose 4B equilibrated in Buffer A. The column was washed with 5 ml of equilibration buffer followed by elution with 5 ml of 0.5 M- α -methyl mannoside. Fractions (1 ml each) were collected and 25 µl aliquots were used from each fraction

for the estimation of APA. Active fractions (2–4 and 7–9) were pooled, dialysed against distilled water, concentrated by freeze-drying and reconstituted in buffer for electrophoreses.

Proteinase treatment. Gaucher AP (10 µg of protein, preparation D) was incubated with 500 µg of Protease Type XIV for 4, 8 and 18 h at 50 °C in 10 mM-Tris/HCl, pH 8.2, in a final volume of 250 µl. At each time point, 25 µl aliquots were withdrawn and APA estimated. The activity was compared with a control containing all reagents except Protease Type XIV.

Immunoaffinity binding of Gaucher AP. Pre-immune and immune serum (2.6 mg and 3.2 mg of protein respectively) were loaded on to two separate Protein A-Sepharose 4B columns (0.5 cm × 1.0 cm) and were washed with equilibration buffer [0.1 M-Tris/HCl (pH 7.0)/0.15 M-NaCl]. Gaucher AP (250 µg of protein, preparation C) was applied to each column, then washed with 8 ml of equilibration buffer. Fractions (1 ml) were collected and a 50 µl aliquot was taken from each fraction for estimating APA.

Immunoprecipitation. Gaucher AP (preparation D, 4 µg of protein) was incubated with various concentrations of pre-immune or immune serum at 4 °C for 18 h in 0.1 M-Tris/HCl (pH 7.5)/0.15 M-NaCl in a final volume of 100 µl. Immunoprecipitation was carried out by adding 20 µl of pre-titred goat anti-(rabbit IgG) serum and the mixture was left at 4 °C for 30 min, after which it was centrifuged at 1000 g for 15 min. The pellet was treated with 0.125 M-Tris/HCl (pH 6.8)/4% (w/v) SDS/20% (v/v) glycerol/50 mM-dithiothreitol and subjected to SDS/polyacrylamide-gel electrophoresis followed by immunoblotting as described above.

Processing of skin fibroblasts for AP analysis. Confluent skin fibroblast cell lines were harvested by treatment with trypsin and homogenized in 1.0 ml of distilled water by using a sonicator probe (Ystrom model; setting 6; three 10 s bursts). The homogenate (1.5–2 mg of protein/ml) was spun at 5000 g for 15 min and the supernatant heated at 70–100 °C for 3 min and centrifuged at 4 °C at 5000 g for 15 min. The pH of the resulting supernatant was adjusted to 4.4 by treatment with 1 M-acetic acid and left at 4 °C for 15 min, after which the sample was clarified by centrifugation (5000 g for 15 min) and freeze-dried. The samples were taken up in 10 mM-Tris/HCl, pH 8.3, to give a protein concentration of 2–3 mg/ml. The sample (100 µg of protein) was subjected to non-denaturing Triton or SDS/polyacrylamide-gel electrophoresis followed by immunoblotting for identification of antigen. In more recent experiments the sensitivity of the immunostaining was improved by using a biotin/avidin system [Vectastain ABC kit with biotinylated goat anti-(rabbit IgG) serum and an avidin bridge to biotinylated horseradish peroxidase]. When electroblotting from the non-denaturing Triton gels it was essential to use 0.1 µm-pore-size nitrocellulose because AP passed through membranes with larger pore sizes.

Processing of media from cell cultures for AP analysis. Skin fibroblasts that had been grown to confluency on BME containing 10% foetal-calf serum were washed with

Fig. 1. Preparative gel electrophoresis of Gaucher AP

Gaucher AP (preparation B; 8 mg of protein) was applied to a preparative polyacrylamide slab gel, subjected to electrophoresis, and APA estimated (Δ) on individual gel slices as described in the text. The end of the gel was sliced and stained for protein with a silver stain. The stained gel (a) is aligned to correspond with the graph of APA migration (b).

iso-osmotic phosphate-buffered saline, pH 7.3, then cultured in 10 ml of MCDB-104 medium. The MCDB-104 medium was collected every 5 days and stored at -20°C until 100 ml was accumulated. The medium was then dialysed against water, freeze-dried and reconstituted in Buffer A to give a protein concentration of 10–20 mg/ml. The concentrate was heated at $70\text{--}100^{\circ}\text{C}$ for 3 min, then centrifuged at 7500 g for 20 min. The supernatant was adjusted to pH 4.4 with acetic acid and left at 4°C for 15 min, then centrifuged at 15000 g for 20 min. The supernatant was freeze-dried and reconstituted in Buffer A at a protein concentration of 0.5–1 mg/ml. This material was then used to determine APA towards sphingomyelinase and β -glucocerebrosidase. For electrophoresis, the media extracts were denatured with ethanol, and the precipitated proteins were treated with trichloroacetic acid in a similar manner to Gaucher AP preparations (Poulos *et al.*, 1984). The extracts (150 μg of protein) were subjected to electrophoresis, and the resulting gels immunoblotted as described above.

RESULTS

Purification of the Gaucher AP

Gaucher AP was purified up to the gel-filtration-chromatography step by the method of Poulos *et al.* (1984) and was monitored by its stimulation of fibroblast sphingomyelinase activity. Further purification of AP was achieved by preparative gel electrophoresis (Fig. 1). Activating ability towards sphingomyelinase activity was eluted with an average R_{BPP} of 0.6 on an alkaline 12.5%-polyacrylamide gel. Under alkaline conditions, Gaucher AP migrated rapidly and was well separated from a number of contaminating proteins (Fig. 1), and more than 88% of APA was eluted from preparative gels.

Final purification of AP was achieved by using

Fig. 2. SDS/polyacrylamide-gel electrophoresis of Gaucher AP

Gaucher AP preparations D (50 μg of protein, lane a) and C (100 μg of protein, lane b) were subjected to SDS/polyacrylamide-gel electrophoresis and stained with the silver reagent as described in the text.

Fig. 3. Urea/SDS/polyacrylamide-gel electrophoresis of Gaucher AP

Gaucher AP (preparation C) was subjected to urea/SDS/polyacrylamide-gel electrophoresis as described in the text. Lane (a) shows low- M_r (1360, 2512, 6214, 8159, 14404 and 16949) markers and lane (b) contained 25 μg of preparation C. The gel was stained with Coomassie Brilliant Blue R250.

hydrophobic chromatography. The hydrophobic nature of the activator was suggested by its strong binding to decyl-agarose and the relatively high concentrations of ethanediol in water (60%, v/v) required for its elution, with a greater than 75% recovery of APA. AP bound less efficiently to both phenyl- and octyl-agarose columns, with only 58% and 46% of the total activity binding respectively.

SDS/polyacrylamide-gel electrophoresis of the decyl-agarose-purified Gaucher AP showed two broad bands on silver staining, the slower zone migrating with an average R_{BPP} of 0.5 and the faster zone migrating with

an R_{BPP} of 0.65 (Fig. 2). Urea/SDS/polyacrylamide-gel electrophoresis revealed a similar pattern, with two zones, one with an M_r of approx. 3000 and a broader zone with an M_r of 5000–6500 (Fig. 3). These M_r estimates are lower than those previously estimated by SDS/polyacrylamide-gel electrophoresis (Poulos *et al.*, 1984). Treatment with proteinase resulted in a gradual decline in activity with time, with a total loss in activity occurring after 18 h. Purified Gaucher AP was also able to stimulate β -glucocerebrosidase activity.

Characterization of the immune serum

The specificity of the rabbit antiserum against Gaucher AP was assessed by the immunoblotting technique. After

Fig. 4. Immunoblotting of Gaucher AP

Gaucher AP (preparation D) was subjected to SDS/polyacrylamide-gel electrophoresis, followed by electroblotting on to nitrocellulose paper. The antigen was located on the paper by using the double-antibody enzyme-linked method. The amount of AP applied to the gel was 10 μg in lane (a) and 2.5 μg in lane (b).

Fig. 5. Immunoaffinity binding of Gaucher AP

Gaucher AP (preparation C) was applied to columns of Protein A-Sepharose 4B loaded with either pre-immune (2.6 mg of protein, ▲) or immune serum (3.2 mg of protein, ■). The columns were eluted and the fractions assayed for APA as described in the text. The basal sphingomyelinase activity was 1.2 nmol/h per mg of protein and the activity in the presence of pre-column AP was 13.0 nmol/h per mg of protein (□).

SDS/polyacrylamide-gel electrophoresis of AP (preparation D), and electrophoretic transfer to nitrocellulose paper, two major bands were detected with the antibody. The antiserum reacted towards both bands with equal intensity (Fig. 4). A number of slower-moving, high- M_r , components also reacted weakly with the antiserum when cruder preparations were assessed. By using immunoblotting, 1 μ g of purified Gaucher AP could be detected with a 6000-fold dilution of immune serum.

To show whether APA was removed from solution by antibody binding, Gaucher AP was applied to Protein A-Sepharose columns to which either pre-immune or immune serum had been bound. Fig. 5 shows that APA was bound totally by the immune-serum column, whereas the pre-immune column did not retain any activity. The immune complex formed was not immunoprecipitable, but was precipitated with the use of a second antibody [goat anti-(rabbit IgG)] directed against the primary rabbit antibody. This was determined by identifying AP in the precipitate by SDS/polyacrylamide-gel electrophoresis.

Binding of Gaucher AP to Con A-Sepharose

When Gaucher AP was applied to a Con A-Sepharose column, it was separated into adsorbing and non-adsorbing components, both of which had APA (Fig. 6). On SDS/polyacrylamide-gel electrophoresis, Con A-

adsorbing AP migrated more slowly than the AP that did not bind to Con A (Fig. 7a). AP components were further resolved on a non-denaturing Triton/polyacrylamide gel. In this system, where four major bands are detected with Stains-All, the Con A-adsorbing and non-adsorbing components were both resolved into two bands (Fig. 7b), as reported by Iyer *et al.* (1983).

Identification of Gaucher activator in cultured skin fibroblasts

To determine whether AP could be identified immunologically in cultured human skin fibroblasts, cell extracts were prepared as described in the Materials and methods section and subjected to immunoblotting after SDS/polyacrylamide-gel electrophoresis. By using antibody to Gaucher AP, extracts of fibroblasts from normal individuals and patients with Gaucher's disease types I and II, Niemann-Pick disease types A, B and C and mucopolipidosis types II and III were examined for the presence of antigen. CRM against Gaucher AP antibodies was detected in normal individuals and in patients with Gaucher's and Niemann-Pick diseases (Fig. 8a; Gaucher type I and Niemann-Pick type B results not shown). These fibroblast extracts all showed a distinct pair of bands that migrated in the region of Con A-adsorbing Gaucher AP. A number of other components with $M_r > 20000$ that cross-reacted with the antiserum were also detected. It was also shown that the CRM in normal fibroblasts, which co-migrated with Gaucher AP, was adsorbed to Con A-Sepharose and was eluted with 0.5 M- α -methyl

Fig. 6. Con A-Sepharose 4B chromatography of Gaucher AP

Gaucher AP (preparation C, 300 μ g of protein) was subjected to Con A-Sepharose 4B column chromatography and APA (▲) and the protein concentration (●) were determined as described in the text. The basal sphingomyelinase activity was 4.5 nmol/h per mg of protein and sphingomyelinase activity in the presence of precolumn AP was 24.0 nmol/h per mg of protein (▲).

Fig. 7. Identification of Con A-Sepharose 4B-binding components of Gaucher AP

The α -methyl mannoside-eluted APA from Fig. 6 was pooled and approx. 50 μ g of protein was subjected to SDS/polyacrylamide-gel electrophoresis, followed by immunoblotting (a). On this gel, lane 1 shows pre-column AP and lane 2 shows the material that absorbed to the Con A column and was eluted with α -methyl mannoside. Samples were also applied to a non-denaturing Triton/polyacrylamide gel (b) with pre-column AP in lane 1, non-absorbing AP in lane 2, and absorbing AP in lane 3. After electrophoresis the gel was stained with Stains-All. The material staining at the bottom of this gel in lanes 1 and 2 did not cross-react with our antibody to AP.

mannoside (results not shown). The mobility of this CRM in fibroblasts and its binding to Con A suggest that it may be related to one of the forms of Gaucher AP. When fibroblast extracts from three patients with ML II were examined for the presence of CRM, there were two closely migrating bands with higher M_r than that of the CRM found in controls (Fig. 8). Furthermore, CRM migrating with the same mobility as either the Gaucher spleen or the fibroblast AP was not detected in ML-II fibroblasts (Fig. 8). Fibroblasts from a patient with ML III had an activator electrophoretic pattern intermediate between that shown by ML-II cells and that shown by controls (Fig. 8b), in that they contained easily detectable amounts of normal fibroblast CRM to AP and also the higher- M_r CRM found in ML-II cells.

In view of the absence of normal fibroblast AP in ML-II cells, we examined culture medium from these cells for the presence of APA. Extracts of medium from ML-II fibroblasts contained considerable APA towards sphingomyelinase and β -glucocerebrosidase (Fig. 9). However, no APA was found in similar extracts of culture medium from Gaucher- or normal-cell incubations. Culture medium from ML-II and control fibroblasts was processed further and run on a non-denaturing Triton gel

before immunoblotting. Medium from ML-II-cell cultures contained CRM to Gaucher AP that corresponded to the CRM found in ML-II-cell extracts (Fig. 10).

It should be noted that, as with Gaucher AP, the CRM from control fibroblasts was resolved into more components by the non-denaturing electrophoresis procedure (Fig. 10) than by SDS/polyacrylamide-gel

Fig. 8. Identification of Gaucher-activator CRM in skin fibroblast extracts

Fibroblast extracts (100 μ g of protein) were subjected to SDS/polyacrylamide-gel electrophoresis followed by electrophoretic transfer on to nitrocellulose paper, and the antigen was located on the paper as described in the text. (a): Lanes 1 and 8, Gaucher AP preparation D (2.5 μ g of protein); lane 2, normal fibroblasts; 3, ML-II fibroblasts; 4, Gaucher-type-II fibroblasts; 5, normal fibroblasts spiked with Gaucher AP preparation D; 6, Niemann-Pick-type-A fibroblasts; 7, Niemann-Pick-type-C fibroblasts. (b): Lane 1, Gaucher AP preparation D (1 μ g of protein); 2 and 7, normal fibroblasts; 3, 4 and 5, different cell lines of ML-II fibroblasts; 6, ML-III fibroblasts.

Fig. 9. APA in culture medium from ML-II fibroblasts.

Extracts of medium from ML-II fibroblast cultures were prepared as described in the Materials and methods section. Aliquots were tested for APA towards sphingomyelinase (▲) and β -glucocerebrosidase (■).

electrophoresis (Fig. 8). Some differences in the mobility and the relative staining of different bands between CRM from control fibroblasts and Gaucher spleen AP were observed. Differences between control spleen and Gaucher spleen AP have already been noted (Iyer *et al.*, 1983; Poulos *et al.*, 1984), but there may also be some tissue specificity for AP components.

DISCUSSION

We have already demonstrated that AP isolated from Gaucher's spleen activates skin fibroblast and brain sphingomyelinase activity when sphingomyelin in a liposomal form was used as the substrate. The activation of sphingomyelinase from both tissue sources was carried out in the absence of exogenous phospholipids (Poulos *et al.*, 1984). Under similar conditions we showed that this AP was also able to stimulate the activity of β -glucocerebrosidase and, to a much lesser degree, β -galactocerebrosidase. Our highly purified AP showed four bands on non-denaturing Triton/polyacrylamide-gel electrophoresis, two of which bound to Con A. Both Con A-adsorbing and non-adsorbing AP were active towards sphingomyelinase. However, because our liposomal assay system for APA is sensitive to Triton X-100, we were not able to determine if all four bands are active towards sphingomyelinase. Our Gaucher AP showed a very similar pattern on non-denaturing Triton/polyacrylamide-gel electrophoresis to the co-hydrolase isolated by Iyer *et al.* (1983). They found that all four components could stimulate β -glucosidase in their assay system, which used a synthetic substrate and purified enzyme. It seems likely that our AP for sphingomyelinase and the co-hydrolase isolated by Iyer *et al.* (1983), which were obtained from similar sources, are identical. At this stage the relationship between the four AP components is uncertain. One possibility is that the four components differ in their carbohydrate content. This is supported by the observation that only two of the AP components bind to Con A, suggesting the presence of exposed D-mannose or D-glucopyranose residues (Goldstein *et al.*, 1965) on these, but not on the other two, AP components. However, it is also possible that there are differences in

the protein content of the four components. Urea/SDS/polyacrylamide-gel electrophoresis indicated that our Gaucher AP consisted of components ranging in M_r from 3000 to 6500. This corresponds well with the estimated M_r for the co-hydrolase isolated from bovine spleen (Berent & Radin, 1981).

The physiological role of the AP in sphingomyelin and β -glucocerebroside metabolism is, as yet, uncertain. We have used a polyclonal antibody to detect AP in normal fibroblasts and fibroblasts from patients with deficiencies in sphingomyelin and β -glucocerebroside metabolism (Niemann-Pick and Gaucher diseases respectively). We were able to detect immunoreactive material in normal and mutant fibroblasts which, we presume, is either identical with AP found in Gaucher spleen or some antigenically related protein. Our observation of similar CRM in normal and Niemann-Pick-type-C fibroblasts contrasts with the findings of Christomanou (1980), who reported that there is a deficiency of AP for sphingomyelinase and β -glucocerebrosidase in Niemann-Pick-

Fig. 10. Identification of Gaucher-activator CRM in skin fibroblast cell and culture-medium extracts

Media extracts (150 μ g of protein), fibroblast extracts (100 μ g of protein) and Gaucher AP preparation C (1 μ g of protein) were applied to a non-denaturing Triton/polyacrylamide gel. After electrophoresis the gel was immunoblotted by using a biotin/avidin system. Lane 1, media extract from a control fibroblast cell line; lane 2, cell extract from the same control fibroblasts; lane 3, media extract from an ML-II fibroblast cell line; lane 4, cell extract from the same ML-II fibroblasts; lane 5, Gaucher AP.

type-C spleen. It is possible that the difference in results reflects genetic heterogeneity in this phenotype. Inui *et al.* (1983) and Inui & Wenger (1984), using immunological procedures, have shown that the AP for G_M ganglioside β -galactosidase and cerebroside sulphate sulphatase, which appears to be distinct from the Gaucher activator, is also present in skin fibroblasts, and moreover is either deficient or present as higher- M_r forms in some variants of G_{M1} β -gangliosidosis and metachromatic leucodystrophy.

Our results clearly show that ML-II fibroblasts have an abnormal AP pattern on non-denaturing Triton/polyacrylamide-gel electrophoresis and SDS/polyacrylamide-gel electrophoresis. The pattern observed with cultures of ML-III fibroblasts was also abnormal, but, because of the presence of apparently normal migrating AP, the pattern was clearly differentiated from that for ML II. In fibroblasts from patients with ML II and ML III, the segregation of lysosomal enzymes from the secretory pathway is impaired and the newly synthesized lysosomal enzymes are largely secreted into the medium or remain with the cell as precursors (Hasilik & Neufeld, 1980; Robey & Neufeld, 1982). We have shown that media extracts from ML-II cells contained APA towards both sphingomyelinase and β -glucocerebrosidase, unlike media from control-cell lines. Analysis of its molecular forms in fibroblasts of patients with ML II indicated that AP, like lysosomal enzymes, may only exist as precursor forms. This CRM was also found in the media from ML II cultures. In addition, the same high- M_r CRM was found in urine from patients with ML II, but no CRM to AP was found in normal control urine (E. Ranieri & A. Poulos, unpublished work). This is consistent with our hypothesis of a processing error which results in the secretion of precursor AP in ML II. Moreover, in ML III, where the disorder is less severe, both high- and low- M_r forms, possibly representing precursor and matured AP, were present in fibroblast extracts. If the CRM in ML-II fibroblasts is precursor AP, our results suggest that this precursor also has APA under our assay conditions. Interestingly, Inui & Wenger (1984) were unable to detect any CRM to the antibody of their AP for G_M ganglioside and sulphatide hydrolysis in extracts from ML-II fibroblasts.

Our data on ML-II, ML-III and control cell lines are consistent with the hypothesis that AP for sphingomyelinase and β -glucocerebrosidase is subject to similar post-translational modification to that found for many lysosomal enzymes. This hypothesis needs to be confirmed by showing sequence homology between the CRM in ML-II and control cells. Moreover, further studies are needed to determine whether all protein activators are modified in the same way, and to confirm if the mechanism parallels that found for a number of lysosomal enzymes.

Addendum

Since this work was first submitted for publication, Christomanou & Kleinschmidt (1985) have shown that their Gaucher APs for sphingomyelin degradation have M_r values in the same range as our Gaucher AP components. However, their AP had some properties different from our own. This may relate to differences in the individual components being studied. A deficiency of normal AP for sphingomyelinase in ML-II fibroblasts has

also been reported by Fujibayashi & Wenger (1985). We extend their observation to include a partial deficiency of normal AP in ML-III fibroblasts. In addition we have shown that culture medium from ML-II cells contains APA.

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