Analysis of the multiple forms of Gaucher spleen sphingolipid activator protein 2

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Gaucher spleen sphingolipid activator protein 2 was fractionated into concanavalin A binding- and non-binding fractions. These fractions each contained several bands on non-denaturing polyacrylamide gel electrophoresis (PAGE). The two fractions were further fractionated by electroblotting the proteins from preparative gels onto nitrocellulose, staining with Ponceau S to locate the bands of protein and then eluting the protein components from the nitrocellulose. A total of ten fractions, each containing only one or two major components, was collected. All of these subfractions activated β -glucocerebrosidase and sphingomyelinase and most subfractions also activated β -galactocerebrosidase. The structural relationship of the bands was investigated using endoglycosidase digestions. The results indicated that the two bands with the fastest mobility on non-denaturing PAGE did not contain any carbohydrate. The remaining bands showed only limited or partial digestion with endoglycosidase H and endoglycosidase D, but were readily hydrolysed with endoglycosidase F. The products of these digestions included bands with similar mobilities to the non-carbohydrate containing bands.

INTRODUCTION

An activator protein for β -glucocerebrosidase was first isolated from a patient with Gaucher's disease type I (Ho & O'Brien, 1971). Since then, similar preparations [variously referred to as factor P, Gaucher factor/activator, heat stable factor, sphingolipid activator protein 2 (SAP-2), co- β -glucosidase and cohydrolase sphingolipid-1] have been isolated from normal human spleen tissue (Peters *et al.*, 1977), bovine spleen (Berent & Radin, 1981a) and control and Gaucher's disease brain (Wenger & Roth, 1982). In addition, immunologically cross-reacting material has been identified in mouse brain and liver (Datta & Radin, 1986) and human skin fibroblasts (Fujibayashi & Wenger, 1985; Ranieri *et al.*, 1986). The activator activates sphingomyelinase and β -galactocerebrosidase in addition to β -glucocerebrosidase (Wenger *et al.*, 1982; Poulos *et al.*, 1984a). In the case of β -glucocerebrosidase, the activator has been shown to bind to the enzyme (Ho, 1975; Berent & Radin, 1981b; Aerts *et al.*, 1987) and there is also a requirement for acidic phospholipids (Ho & Rigby, 1975; Berent & Radin, 1981b; Prentice *et al.*, 1985; Basu *et al.*, 1985; Prentice *et al.*, 1986). Activation of β -galactocerebrosidase by the activator also requires acidic phospholipids (Wenger *et al.*, 1982).

The various activator preparations have been shown to consist of a group of low molecular mass acidic glycoproteins. Iyer *et al.* (1983) noted four major bands in their preparations after non-denaturing gel electrophoresis and staining with Stains-All. Using a more sensitive immunological staining procedure, we were able to detect many more bands in a similar preparation from Gaucher spleen (Ranieri *et al.*, 1986). Multiple bands of immunologically cross-reacting material have

also been detected in skin fibroblasts (Fujibayashi & Wenger, 1985; Ranieri *et al.*, 1986). Some differences in the mobility of the activator components between control human, Gaucher and bovine spleen activator preparations (Iyer *et al.*, 1983) and also between human skin fibroblasts and Gaucher spleen activator (Ranieri *et al.*, 1986) have been noted. Christomanou & Kleinschmidt (1985) and Christomanou *et al.* (1986) also reported heterogeneity in their low molecular mass activator preparation for sphingomyelinase. However, their activator components, while showing many similarities, also differ in some respects from the activator preparations already mentioned and may represent a different class of activator proteins. An apparently distinct β -glucocerebrosidase activator has also been isolated from human placenta (Vaccaro *et al.*, 1985). In addition, apolipoprotein C III, which is present as two low molecular mass components, stimulates sphingomyelin hydrolysis (Alpert & Beaudet, 1981; Ahmad *et al.*, 1986).

In 1980, it was proposed that Niemann-Pick disease type C resulted from a deficiency in an activator for β -glucocerebrosidase (Christomanou, 1980). However, neither Fujibayashi & Wenger (1985) nor our own laboratory (Ranieri *et al.*, 1986) was able to confirm a deficiency of SAP-2 in this disorder. However, it is conceivable that the discrepancy results from heterogeneity in the Niemann-Pick type C genotype. More recently, Christomanou *et al.* (1986) have reported a deficiency of one of their activator components in a patient with a variant form of Gaucher's disease and this clearly warrants further investigation. Evidence for the physiological importance of SAP-2 comes from studies on the effect of the activator on the residual sphingomyelinase activity in different types of Niemann-Pick disease. The residual activity in fibroblasts from the more

Abbreviations used: Con A, concanavalin A; Endo D, F and H, endo- β -N-acetylglucosaminidase D, F and H, respectively; N-glycanase, peptide:N-glycosidase F; PAGE, polyacrylamide gel electrophoresis; PSA, *Pisum sativum* agglutinin; RCA₁₂₀, *Ricinus communis* agglutinin I; SAP-2, sphingolipid activator protein 2.

severely affected type A patients does not respond to activator, while the residual activity in Niemann-Pick types B and C is responsive to the activator (Poulos *et al.*, 1984b). In addition, the activator is induced in mouse tissues after injecting mice with a β -glucocerebrosidase inhibitor or with glucocerebroside, galactocerebroside, or sphingomyelin (Datta & Radin, 1986).

In this paper we examine the multiple activator components present in Gaucher spleen extract. We were interested to know whether the heterogeneity of the preparation could be attributed to differences in carbohydrate content and/or whether the individual components have any specificity towards the three different sphingolipid hydrolases.

MATERIALS AND METHODS

Materials

The DEAE-cellulose anion exchanger (DE 52) was from Whatman Ltd. (Maidstone, Kent, U.K.) and the Fractogel TSK HW 55S from Merck (Darmstadt, Germany). Acrylamide, *NN'*-methylenebisacrylamide, *NNN'*-tetramethylethylenediamine, ammonium persulphate, 4-chloro-1-naphthol, Bio-Gel HPHT hydroxylapatite column with hydrophilic guard column and the Bio-Gel phenyl-5PW hydrophobic interaction column were obtained from Bio-Rad Laboratories (Richmond, CA, U.S.A.). Pharmacia (Uppsala, Sweden) supplied the Mono Q HR 5/5 anion exchange column. Schleicher und Schull (Dassel, Germany) provided the 0.1 μ m pore nitrocellulose membrane and the Vectastain ABC kit, biotinylated lectin kits I and II, and the agarose-bound *Ricinus communis* agglutinin I (RCA₁₂₀) (4 mg/ml packed gel) and *Pisum sativum* agglutinin (PSA) (3 mg/ml packed gel) were from Vector Laboratories (Burlingame, CA, U.S.A.). Endo- β -*N*-acetylglucosaminidase D (Endo D) was from Seikagaku Kogyo Co. Ltd. (Tokyo, Japan), while endo- β -*N*-acetylglucosaminidase H (Endo H) came from Miles Scientific (Naperville, IL, U.S.A.). Peptide:*N*-glycosidase F (*N*-glycanase) was obtained from Genzyme Corp. (Boston, MA, U.S.A.) and endo- β -*N*-acetylglucosaminidase F (Endo F) and [*choline-methyl*-¹⁴C]sphingomyelin were obtained from New England Nuclear (Boston MA, U.S.A.). Sigma Chemical Co. (St Louis, MO, U.S.A.) supplied the concanavalin A (Con A)-Sephrose 4B (10 mg/ml packed gel), methyl α -D-mannopyranoside, methyl β -D-galactopyranoside, Ponceau S, bovine brain sphingomyelin, glucocerebrosides from Gaucher spleen, *L*- α -phosphatidylcholine type X1-E from fresh egg yolk, galactocerebrosides type 11 from bovine brain, *L*- α -phosphatidylserine from bovine brain, phosphorylcholine chloride and galactose oxidase type V. Sodium boro[³H]hydride was from Amersham International plc (Amersham, U.K.). All other reagents were of analytical grade.

Purification of total activator preparation

Activator protein was isolated from the spleen of a patient with Gaucher's disease type 1, essentially as previously described (Poulos *et al.*, 1984a), using a modification of the method of Peters *et al.* (1977). Briefly, the tissue (stored at -80°C for up to 6 years) was homogenized in nine volumes of ice-cold water, freeze-thawed and re-homogenized twice, and then centrifuged (48 000 g_{max} for 90 min) to remove particulate matter. The extract was then heated to 80°C , filtered

through eight layers of gauze and centrifuged for 15 min at 15 000 g_{max} . The resulting supernatant was adjusted to pH 4.4 with 1 M-acetic acid, the precipitate was removed by centrifugation (15 000 g_{max} for 15 min) and the supernatant concentrated by freeze-drying. The precipitate formed after adjustment to 90% ethanol (v/v) was then treated with trichloroacetic acid (Peters *et al.*, 1977) and the resulting soluble fraction applied to a DEAE-cellulose DE 52 anion exchange column (Poulos *et al.*, 1984a). The activator was eluted with a NaCl gradient. After this step the activator was either fractionated or further purification of the total preparation was achieved using a Fractogel TSK HW 55S gel filtration column followed by preparative polyacrylamide gel electrophoresis (PAGE) (Ranieri *et al.*, 1986). During purification the activator was monitored by activation of fibroblast sphingomyelinase activity. The activator was stored in silanized glassware at -20°C for all steps after the trichloroacetic acid precipitation.

When stained for the presence of protein, with either Coomassie Blue R250 or silver, the post-preparative PAGE-purified SAP-2 contains only two closely-migrating, diffusely-staining bands after either SDS or urea/SDS/PAGE (Ranieri *et al.*, 1986). On a non-denaturing gel, only two diffuse bands are visualized with Coomassie Blue R250, but with Stains-All four major bands are visible (Ranieri *et al.*, 1986). Three of the Stains-All staining bands correspond to the slower migrating Coomassie staining band. Additional bands, within the region corresponding to protein staining on the original gel, are detected if the gel is electroblotted and immunostained. Activator activity is associated with all of the protein staining region of a non-denaturing gel of SAP-2 (Ranieri *et al.*, 1986).

Separation of activator components

A portion (13 mg of protein) of the DE 52 column purified activator was first separated into Con A binding- and non-binding fractions using a 2.5 cm $\times$ 2.5 cm Con A-Sephrose 4B column. Unbound components were eluted with 50 ml of 20 mM-Tris/HCl/0.5 M-NaCl buffer, pH 7.0, while bound fractions were eluted with 50 ml of the same buffer containing 0.5 M-methyl α -D-mannopyranoside. The fractions were extensively dialysed against water, freeze-dried and redissolved in 10 mM-Tris/HCl, pH 7.0. Aliquots ($\frac{1}{3}$) of each fraction were applied independently to non-denaturing polyacrylamide gels. The gels were prepared as described previously (Ranieri *et al.*, 1986), but with 15% (w/v) acrylamide in the resolving gel and 4% (w/v) acrylamide in the stacking gel. After electrophoresis, the protein bands were transferred to 0.1 μ m pore nitrocellulose by electroblotting (Burnette, 1981) for 60 min at 0.2 A. The nitrocellulose was then stained for 10 min with Ponceau S (Salinovich & Montelaro, 1986), rinsed with water to remove background staining and then strips, corresponding to bands of activator, were removed by cutting the nitrocellulose. Where possible individual bands were separated, but, in the slower mobility region, it was not possible to discern individual components. The strips were cut into pieces and placed in silanized vials and rinsed with 10 ml of water with intermittent shaking for several minutes. The water was replaced with a fresh 10 ml, and the samples left at 4°C overnight. The next day the water was removed and the proteins eluted with 1 ml of 20% (v/v) acetonitrile/0.1 M-ammonium acetate,

pH 8.9, with gentle shaking for 3 h (Parekh *et al.*, 1985). The samples were collected with a further 0.5 ml rinse and centrifuged (15000 *g* for 5 min) in an Eppendorf 5413 centrifuge to remove any particulate nitrocellulose. The samples were then freeze-dried and dissolved in 100 μ l of 10 mM-Tris/HCl buffer, pH 7.0. To remove residual nitrocellulose and Ponceau S, the samples were then run on a high pressure 100 mm $\times$ 7.8 mm Bio-Gel HPHT hydroxylapatite column with a 50 mm $\times$ 4.0 mm hydrophilic guard column. The column was equilibrated with 10 mM- NaH_2PO_4 /0.3 mM- CaCl_2 , pH 6.8, buffer. The sample was injected and the column washed for a few minutes with the same buffer and then with a linear 0–100% gradient of 0.3 M- NaH_2PO_4 /0.01 mM- CaCl_2 , pH 6.8 buffer. Residual nitrocellulose and Ponceau S eluted at low phosphate concentrations, while the activator components eluted at high phosphate concentrations. The presence of activator components was confirmed by immunoblotting. The relevant fractions were then pooled, dialysed extensively against water, freeze-dried and dissolved in water.

Other chromatographic procedures

The lectin binding studies indicated that some of the activator components bind to Con A, RCA₁₂₀ and PSA. The Con A column chromatography is described above. For the RCA₁₂₀–agarose column, the equilibration buffer was 20 mM-Tris/HCl, 0.15 M-NaCl, pH 7.0 and the activator was eluted with the same buffer containing 0.5 M-methyl β -D-galactopyranoside. With the PSA–agarose column, the same equilibration buffer was used and the elution buffer contained 0.5 M-methyl α -D-mannopyranoside.

As the activator protein contains a hydrophobic region, aliquots of the total activator were applied to an h.p.l.c. phenyl-5PW hydrophobic interaction column. The column was equilibrated with a 1.7 M-(NH_4)₂ SO_4 , 0.1 M- NaH_2PO_4 buffer, pH 7.0. After injection, the activator was eluted with a linear gradient of decreasing (NH_4)₂ SO_4 concentration.

Total activator, or Con A bound and unbound fractions, were also applied to a high performance Mono Q anion exchange column, to see if this column gave better resolution than the low pressure DE 52 anion exchanger. The starting buffer was 20 mM-Tris/HCl, pH 7.5 and elution was with a gradient of the same buffer containing 0.3 M-NaCl.

Protein assays

The protein content of activator samples was determined by the Lowry method (Lowry *et al.*, 1951) or, where greater sensitivity was required, by Sargent's modification of this procedure (Sargent, 1987). For the fibroblast extracts used in the enzyme assays, protein was determined by the method of Bradford (1976). In each case human albumin was used as the standard.

Immunostaining

Immunostaining of spots of activator or of electroblotted gels was performed as previously described (Ranieri *et al.*, 1986) using the sensitive Vectastain ABC kit method which utilizes a biotinylated goat anti-(rabbit IgG) serum and an avidin bridge to biotinylated horseradish peroxidase. The primary antibody was raised in rabbits (Ranieri *et al.*, 1986) using 0.4 mg of post-preparative PAGE-purified activator for the pri-

mary injection and 0.1 mg of activator protein for the booster injection 10 weeks later.

Lectin staining

Nitrocellulose-bound Gaucher SAP-2, either spots or from electroblotted gels, was stained using biotinylated lectins (Vector kits I and II) in combination with the ABC reagent from the Vectastain ABC kit (as used for immunostaining). Although highly purified gelatin or bovine serum albumin was used for the blocking solution, problems with background staining occurred with a few of these lectins. It should be noted that approx. 20-fold more protein was required for lectin staining than for immunostaining.

The lectin staining kits contained the following biotinylated lectins: Con A, soybean agglutinin, wheat germ agglutinin, *Dolichos biflorus* agglutinin, *Ulex europaeus* agglutinin I, RCA₁₂₀, peanut agglutinin, *Bandeiraea simplicifolia* lectin I, PSA, *Lens culinaris* agglutinin, *Phaseolus vulgaris* erythroagglutinin, *Phaseolus vulgaris* leucoagglutinin, *Sophora japonica* agglutinin and succinylated wheat germ agglutinin.

Analysis of activator components by PAGE

Activator protein fractions were analysed on non-denaturing Triton polyacrylamide gels. These gels were prepared as described for the preparative gels, but included 0.2% (w/v) Triton X-100 in the stacking gel, resolving gel and reservoir buffer. The samples were applied to the wells in 0.1 M-Tris/HCl, pH 6.8, 10% (w/v) sucrose, 0.2% (w/v) Triton X-100 and 0.02% (w/v) Bromophenol Blue. After electrophoresis, the gels were electroblotted onto nitrocellulose and then immunostained.

Endoglycosidase digestions

Aliquots of activator fractions were digested with various endoglycosidases as indicated. For Endo D digestions, the buffer was 25 mM- NaH_2PO_4 , pH 6.5 and 5 munits of Endo D was added per assay. For Endo F digestions, the buffer was 25 mM- NaH_2PO_4 /2.5 mM-EDTA, pH 6.1 and 0.5 units of Endo F was used per assay. For Endo H digestions, the buffer was 25 mM- NaH_2PO_4 , pH 5.5, and 6 munits of Endo H was added to each incubation. In some preliminary studies, Con A-bound and unbound fractions were digested with *N*-glycanase. The buffer for these incubations was 10 mM- NaH_2PO_4 /0.1 mM-EDTA, pH 8.6, and 0.3 units of *N*-glycanase was used per assay tube. In each case the incubations also contained Triton X-100 at a final concentration of 0.6% (w/v), and the final incubation volume was 30 μ l. The assays were set up in sealed microfuge tubes and incubated at 37 °C for 24 h (72 h for the earlier studies with *N*-glycanase). Units of activity for the endoglycosidases were as defined by the suppliers.

Sphingolipid hydrolase assays

Normal human skin fibroblast cell lines were used as a source of sphingomyelinase, β -glucocerebrosidase and β -galactocerebrosidase. The cells were cultured as described (Ranieri *et al.*, 1986), harvested by trypsinization and resuspended in 0.25 M-sucrose. The cells were then disrupted by repeated freeze-thawing and sonication. The substrates for the enzyme assays were added in the liposomal form (Poulos *et al.*, 1983) with 10 μ l of the appropriate liposomal substrate added to each assay in a

Fig. 1. Fractionation of Gaucher SAP-2

Subfractions of Gaucher SAP-2 were isolated after Ponceau S staining of electroblots from non-denaturing PAGE of Con A-unbound or bound material. Aliquots of each fraction were then run on a non-denaturing Triton polyacrylamide gel, electroblotted and immunostained. Lanes A and N, 0.74 μ g of post DE 52 column Gaucher activator; lane B, 0.68 μ g of Con A-unbound activator; lanes C–H, subfractions from Con A-unbound activator with decreasing mobility; lanes I–L, subfractions from Con A-bound activator with decreasing mobility; and lane M, 0.44 μ g of Con A bound activator. Gaucher activator fractions in lanes C–L are designated fractions 1–10, respectively. The protein applied to the gel for the subfractions varied between 0.12 and 0.36 μ g.

final volume of 100 μ l. The incubations contained 20–40 μ g of fibroblast protein as enzyme source. The basal activity was measured in the absence of added activator protein. Appropriate blank incubations of activator samples without added enzyme had no hydrolase activity. For the sphingomyelinase assay, the liposomes contained 7.5 μ g of bovine brain sphingomyelin and 50000 d.p.m. of bovine brain [*choline-methyl- 14 C*]sphingomyelin (51 mCi/mmol) per 10 μ l and each incubation contained 50 mM-acetate buffer, pH 4.2. The assay tubes were incubated at 37 °C for 2 h. With the β -glucocerebrosidase assay, the liposomes contained 10 μ g of egg yolk L- α -phosphatidylcholine, 16 μ g of glucocerebroside from Gaucher spleen and 60000 d.p.m. of [*glucosyl-6- 3 H*]ceramide per 10 μ l. The labelled substrate was prepared by the method of McMaster & Radin (1977) with boro[3 H]hydride reduction at the 6-position of the glucose moiety. The incubations contained 50 mM-acetate buffer, pH 4.5 and were carried out at 37 °C for 1 h. The liposomes for the β -galactocerebrosidase assay contained 50 μ g of bovine brain phosphatidylserine, 0.8 μ g of galactocerebroside from bovine brain and 120000 d.p.m. of [*galactosyl-6- 3 H*]ceramide per 10 μ l. The labelled substrate was prepared by treating galactocerebroside with galactose oxidase and then labelling with sodium boro[3 H]hydride (Suzuki & Suzuki, 1972). The assays contained 50 mM-acetate buffer, pH 4.5, and the incubations were for 2 h at 37 °C. All three assays were terminated as previously described and the radioactivity in the aqueous phase determined (Poulos *et al.*, 1984a).

Table 1. Activation of sphingomyelinase, β -galactocerebrosidase and β -glucocerebrosidase enzyme activities

The same fibroblast preparation was used for all of these assays. The fraction numbers are as indicated in Fig. 1. The protein values refer to the amount of activator protein added to each enzyme assay. The activator fractions were added as equal proportions of the total amount recovered in each fraction. A fold activation of 1.0 indicates no activation of the enzyme.

Activator preparation	Protein (μ g)	Fold activation over basal activity		
		Sphingomyelinase	β -Galactocerebrosidase	β -Glucocerebrosidase
Post DE 52 column	1.20	5.1	3.5	5.9
Con A-unbound	1.71	5.8	3.6	4.7
Con A-bound	1.10	4.7	6.6	4.5
Fraction				
1	0.51	3.3	1.1	3.1
2	0.18	2.8	1.0	3.0
3	0.33	3.3	1.3	2.6
4	0.24	2.9	1.4	1.7
5	0.42	4.2	1.5	3.5
6	0.33	5.5	3.0	3.3
7	0.48	4.0	4.1	3.6
8	0.45	3.1	1.8	3.2
9	0.54	4.8	3.3	2.8
10	0.30	3.0	2.4	1.7

RESULTS

Separation of activator components

Our preparative PAGE-purified Gaucher SAP-2 preparation had previously been shown to contain a number of immunoreactive components on non-denaturing polyacrylamide gels. As the antibody used for the staining was polyclonal, we were interested to check if all the components had activator activity. A number of chromatography procedures were used to attempt to separate the bands. We were already aware that the preparation could be divided into Con A binding- and non-binding fractions (Ranieri *et al.*, 1986). Likewise, separation into binding- and non-binding fractions could also be attained on PSA and RCA₁₂₀ lectin columns. A combination of Con A and RCA₁₂₀ chromatography gave some additional separation, but the recoveries were low (results not shown). Only partial separation of the activator components was obtained with an h.p.l.c. anion exchange column (results not shown), while all the bands co-eluted from either an h.p.l.c.-hydrophobic interaction column or an h.p.l.c.-hydroxylapatite column (results not shown).

As several bands could be identified on polyacrylamide gels, we opted to cut out bands of activator protein after electroblotting the gels onto nitrocellulose and staining with Ponceau S. Because of the considerable number of upper bands, prior separation into Con A binding- and non-binding fractions was beneficial. After eluting the bands from the nitrocellulose, contaminants were re-

moved by h.p.l.c-hydroxylapatite column chromatography and dialysis. The final extracts were dissolved in 40 μ l of water and 2 μ l of each was then applied to analytical gels (Fig. 1). It was not possible to estimate recovery of activator protein in the fractions because the Con A bound and unbound material applied to the gels contains contaminating Con A (the Con A leeches from the column in spite of extensive washing and the use of a high salt buffer). The Con A is separated from the activator components during electrophoresis, so is not present in the final activator fractions.

Activation of sphingolipid hydrolase

The ten fractions of Gaucher activator were tested for activation of β -glucocerebrosidase, sphingomyelinase and β -galactocerebrosidase (Table 1). The fraction numbers are as designated in Fig. 1. Two concentrations (1 μ l and 3 μ l) of each fraction were tested against each enzyme, but only results for 3 μ l are shown. All the fractions activated β -glucocerebrosidase and sphingomyelinase and most fractions stimulated β -galactocerebrosidase. The lack of response of β -galactocerebrosidase activity to fractions 1 and 2, and the only slight response to fractions 3, 4 and 5, may result from using non-optimal concentrations of these fractions. Unfortunately, there was not enough material to test an extensive range of concentrations. The results indicate that individual fractions do not have specific activator activity towards a single hydrolase. It was not possible to estimate the per cent recovery of activator activity in the different fractions, because the activity of the activator is not linearly related to the amount of activator added. In addition, the response to each activator preparation varies with the batch of fibroblasts used in the assays.

Endoglycosidase digestions

Each activator fraction was digested with Endo D, F and H. Blank incubations of the endoglycosidase enzymes, without added activator fractions, did not produce any cross-reacting material to Gaucher SAP-2 (results not shown). In addition, activator proteins incubated without any endoglycosidase enzymes remained unchanged (results not shown). It should be noted that a different set of isolated fractions were used for these studies than in Fig. 1. Slight differences in recovery and banding pattern were found between different batches of fractions, particularly for those fractions with slower mobility on PAGE. This arose because of the poorer resolution of the bands and the diffuseness of Ponceau S staining for bands in this region. The actual fractions used for the incubations are indicated (Fig. 2 and 3). Fractions 1 and 2 showed no change after digestion with the three endoglycosidases (Fig. 2a), indicating that they did not contain any *N*-linked oligosaccharide. This observation was supported by lectin staining studies. Neither of these fractions stained with any of the 14 lectins tested (results not shown). The remaining eight fractions showed only limited or partial hydrolysis with Endo D and Endo H (Fig. 2 and 3), presumably because of lack of appropriate specificity or because of the presence of blocking terminal sugar residues. In general the Con A-binding fractions were more susceptible to Endo D and H than the Con A-

non-binding fractions. In contrast, all eight fractions were readily digested with Endo F (Fig. 2 and 3).

On digestion with endoglycosidase, bands with mobility similar to either fraction 1 and/or 2 were produced. Where bands similar to both 1 and 2 (that is with digests of fractions 4, 5, 6, 8 and 9) were produced from the one fraction, this was probably accounted for by heterogeneity in the original sample. In some instances the mobility of the resulting band(s) was a little slower than either fraction 1 (as with fraction 3) and/or 2 (as with fractions 4, 5 and 6), or there was heterogeneous staining in the region of either fraction 1 and/or 2 (as with fractions 7, 8 and 9). This may result from heterogeneity in the starting fractions. However, it might also be caused by the presence of small amounts of contaminating enzymes in the endoglycosidase preparations. When Endo D, F and H cleave an oligosaccharide(s) from glycoproteins, an *N*-acetylglucosamine residue(s) remains linked to the asparagine residue(s) on the protein. Since the molecular mass of the activator components is quite low [3000–6500 by urea/SDS/PAGE (Ranieri *et al.*, 1986)], the presence of a sugar residue on the peptides may noticeably retard their mobility. However, the presence of small contaminating amounts of *N*-acetylglucosaminidase in the Endo D and H preparations, or of *N*-glycanase in the Endo F, may remove the sugar from some or all of these molecules to yield products with the same mobility as either fraction 1 or 2. Interestingly, in earlier studies using *N*-glycanase digestions of Con A-unbound and bound fractions, the products ran in line with fraction 1 and/or 2 (results not shown). Unlike Endo D, F and H, *N*-glycanase removes all of the oligosaccharide from the protein. Unfortunately, the cost of this enzyme prevented more extensive investigation.

Where digestion of the fractions with Endo F did occur, bands running ahead of fraction 1 were observed on the gels. The identity of this material is not known, but it may represent the oligosaccharides released from the glycopeptides. Similar bands had also been seen with the *N*-glycanase digestions. The faster moving of these bands appeared to be generated when a product with mobility similar to fraction 1 was formed (digests of fraction 3, 4, 5, 6, 7 and 8), while the slower moving of these bands was associated with digestions which yielded fraction 2-like material (digests of fraction 4, 5, 6 and 10). It would have been interesting to compare the lectin staining of activator components before and after endoglycosidase digestion. However, the relatively low sensitivity of the lectin staining compared to immunostaining would not allow detection of the small amounts of activator used for the endoglycosidase digestions.

DISCUSSION

Our Gaucher SAP-2 preparation was separated into ten fractions, all of which had activator activity. In addition, there did not appear to be any specificity of any fraction towards a single sphingolipid hydrolase. Two of the fractions appeared to be non-glycosylated, implying that carbohydrate is not required for activator activity. Endoglycosidase digestions indicated that the other glycosylated components are probably related to one or other of these peptides. We are currently investigating whether or not the two non-glycosylated peptides are

Fig. 2. Endoglycosidase digests of Con A-unbound fractions

Activator fractions were incubated with endoglycosidase enzymes. The incubations were then run on non-denaturing Triton gels, electroblotted and immunostained. (a): Lanes A–D are fraction 1; A, untreated; B, +Endo D; C, +Endo F; and D, +Endo H. Lanes E–H are fraction 2; E, untreated; F, +Endo D; G, +Endo F; and H, +Endo H. Lanes I–L are fraction 3; I, untreated; J, +Endo D; K, +Endo F; and L, +Endo H. Lanes M, Con A-unbound untreated; N, Con A-bound untreated; and O, total activator preparation untreated. (b): Lanes A–D are fraction 4; A, untreated; B, +Endo D; C, +Endo F; and D, +Endo H. Lanes E–H are fraction 5; E, untreated; F, +Endo D; G, +Endo F; and H, +Endo H. Lanes I–L are fraction 6; I, untreated; J, +Endo D; K, +Endo F; and L, +Endo H. Lanes M, N and O are as in Fig. 2(a).

Fig. 3. Endoglycosidase digests of Con A-bound fractions

Activator fractions were incubated with endoglycosidase enzymes. The incubations were then run on non-denaturing Triton gels, electroblotted and immunostained. (a): Lanes A–D are fraction 7; A, untreated; B, +Endo D; C, +Endo F; and D, +Endo H. Lanes E–H are fraction 8; E, untreated; F, +Endo D; G, +Endo F; and H, +Endo H. Lanes I–L are fraction 9; I, untreated; J, +Endo D; K, +Endo F; and L, +Endo H. Lanes M, and O are as in Fig. 2(a). (b): Lanes A–D are fraction 10; A, untreated; B, +Endo D; C, +Endo F; and D, +Endo H. Lanes E, F and G are as in lanes M, N and O in Fig. 2(a), respectively.

related proteins or not. The fact that all the fractions co-eluted from either an h.p.l.c.-hydrophobic or an h.p.l.c.-hydroxylapatite column supports the contention that they are related proteins.

Because of the size limitation of the activator components, it seems likely that there are only one or two oligosaccharides on each of the glycopeptides. Thus, some of the heterogeneity of the banding pattern may

result from processing of terminal sugars on core oligosaccharides. After treatment with Endo F, bands with faster mobility than the non-glycosylated peptides were produced. We presume these are the oligosaccharides which have been removed from the glycopeptides, as the antigenicity of oligosaccharides is well documented (Feizi & Childs, 1987). Preliminary evidence indicates that there are probably only two major oligosaccharide products, but clearly the staining pattern would depend on whether all the possible oligosaccharide variations are recognized by our polyclonal antiserum, whether or not the different released oligosaccharides co-migrate on electrophoresis, and to what extent each oligosaccharide binds to the nitrocellulose. Results with fraction 1 and 2 clearly indicate that our polyclonal antibody also recognizes the peptide structure(s) of the Gaucher SAP-2 components. In theory, some indication of an oligosaccharide's structure can be deduced from its affinity to different lectins and its susceptibility to hydrolysis by certain endoglycosidases. However, in the present case the overall pattern was too confusing to draw any firm conclusions.

Fujibayashi & Wenger (1986) demonstrated that the SAP-2 is first synthesized as 68000 and 73000 Da forms and is then processed to 12000 and 9000 Da forms (molecular mass estimated by SDS/PAGE). In our earlier studies using SDS/PAGE (Poulos *et al.*, 1984a), we found that our activator migrated in a similar molecular mass region to the processed forms, but a lower molecular mass range (3000–6500) was estimated by urea/SDS/PAGE. Similar lower molecular masses were obtained on urea/SDS gels by Berent & Radin (1981a) for bovine spleen activator and also by Christomanou & Kleinschmidt (1985) for their activator preparation. (Of course, it should be noted that the glycosylation of the activator components may affect the relationship between molecular mass and mobility of the bands on SDS and urea/SDS gels.) Fujibayashi & Wenger (1986) showed that their 9000 molecular mass form could be deglycosylated with Endo F to give a 7600 Da form. Better resolution of activator components is found on non-denaturing gels (Iyer *et al.*, 1983; Ranieri *et al.*, 1986), and, when immunostaining is used, additional components are detected. Thus, we were able to detect more lower molecular mass components than Fujibayashi & Wenger (1986). Presumably their 7600 Da form corresponds to one or other or both of our non-glycosylated activator components.

Our results indicate that, if an SAP-2 deficiency disease state exists, patients would be expected to accumulate β -glucocerebroside, sphingomyelin and β -galactocerebroside in their tissues. In addition, the hydrolysis of the cytotoxic compound galactosylsphingosine (psychosine) is carried out by β -galactocerebrosidease and this hydrolase activity is also stimulated by the same activator (Goda *et al.*, 1987). Thus, psychosine would also be expected to accumulate if there was a deficiency in activator. It may be that, if there was a total deficiency in Gaucher activator, the accumulation of so many storage products would be lethal during fetal life.

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REFERENCES

- Aerts, J. M. F. G., Donker-Koopman, W. E., van Laar, C., Brul, S., Murray, G. J., Wenger, D. A., Barranger, J. A., Tager, J. M. & Schram, A. W. (1987) *Eur. J. Biochem.* **163**, 583–589
- Ahmad, T. Y., Beaudet, A. L., Sparrow, J. T. & Morrisett, J. D. (1986) *Biochemistry* **25**, 4415–4420
- Alpert, A. J. & Beaudet, A. L. (1981) *J. Clin. Invest.* **68**, 1592–1596
- Basu, A., Prence, E., Garrett, K., Glew, R. H. & Ellingson, J. S. (1985) *Arch. Biochem. Biophys.* **243**, 28–34
- Berent, S. L. & Radin, N. S. (1981a) *Arch. Biochem. Biophys.* **208**, 248–260
- Berent, S. L. & Radin, N. S. (1981b) *Biochim. Biophys. Acta* **664**, 572–582
- Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
- Burnette, W. N. (1981) *Anal. Biochem.* **112**, 195–203
- Christomanou, H. (1980) *Hoppe-Seyler's Z. Physiol. Chem.* **361**, 1489–1502
- Christomanou, H. & Kleinschmidt, T. (1985) *Biol. Chem. Hoppe-Seyler* **366**, 245–256
- Christomanou, H., Aignesberger, A. & Linke, R. P. (1986) *Biol. Chem. Hoppe-Seyler* **367**, 879–890
- Datta, S. C. & Radin, N. S. (1986) *Lipids* **21**, 702–709
- Feizi, T. & Childs, R. A. (1987) *Biochem. J.* **245**, 1–11
- Fujibayashi, S. & Wenger, D. A. (1985) *Clin. Chim. Acta* **146**, 147–156
- Fujibayashi, S. & Wenger, D. A. (1986) *J. Biol. Chem.* **261**, 15339–15343
- Goda, S., Kobayashi, T. & Goto, I. (1987) *Biochim. Biophys. Acta* **920**, 259–265
- Ho, M. W. (1975) *FEBS Lett.* **53**, 243–247
- Ho, M. W. & O'Brien, J. S. (1971) *Proc. Natl. Acad. Sci. U.S.A.* **68**, 2810–2813
- Ho, M. W. & Rigby, M. (1975) *Biochim. Biophys. Acta* **397**, 267–273
- Iyer, S. S., Berent, S. L. & Radin, N. S. (1983) *Biochim. Biophys. Acta* **748**, 1–7
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- McMaster, M. C. & Radin, N. S. (1977) *J. Labelled Compd. Radiopharm.* **13**, 353–357
- Parekh, B. S., Mehta, H. B., West, M. D. & Montelaro, R. C. (1985) *Anal. Biochem.* **148**, 87–92
- Peters, S. P., Coyle, P., Coffee, C. J., Glew, R. H., Kuhlen-schmidt, M. S., Rosenfeld, L. & Lee, Y. C. (1977) *J. Biol. Chem.* **252**, 563–573
- Poulos, A., Shankaran, P., Jones, C. S. & Callahan, J. W. (1983) *Biochim. Biophys. Acta* **751**, 428–431
- Poulos, A., Ranieri, E., Shankaran, P. & Callahan, J. W. (1984a) *Biochim. Biophys. Acta* **793**, 141–148
- Poulos, A., Ranieri, E., Shankaran, P. & Callahan, J. W. (1984b) *Pediatr. Res.* **18**, 1088–1093
- Prence, E., Chakravorti, S., Basu, A., Clark, L. S., Glew, R. H. & Chambers, J. A. (1985) *Arch. Biochem. Biophys.* **236**, 98–109
- Prence, E. M., Garrett, K. O. & Glew, R. H. (1986) *Biochem. J.* **237**, 655–662
- Ranieri, E., Paton, B. & Poulos, A. (1986) *Biochem. J.* **233**, 763–772
- Salinovich, O. & Montelaro, R. C. (1986) *Anal. Biochem.* **156**, 341–347
- Sargent, M. G. (1987) *Anal. Biochem.* **163**, 476–481

Suzuki, Y. & Suzuki, K. (1972) *J. Lipid Res.* **13**, 687–690
Vaccaro, A. M., Muscillo, M., Gallozzi, E., Salvioli, R., Tatti,
M. & Suzuki, K. (1985) *Biochim. Biophys. Acta* **836**,
157–166

Wenger, D. A. & Roth, S. (1982) *Biochem. Int.* **5**, 705–
710

Wenger, D. A., Sattler, M. & Roth, S. (1982) *Biochim. Biophys.*
Acta **712**, 639–649

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