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Additional biochemical findings in a patient and fetal sibling with a genetic defect in the sphingolipid activator protein (SAP) precursor, prosaposin

Evidence for a deficiency in SAP-1 and for a normal lysosomal neuraminidase

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It has been shown that sphingolipid activator proteins (SAPs) 1 and 2 are encoded on the same gene along with two other putative activator proteins [Fürst, Machleidt & Sandhoff (1988) Biol. Chem. Hoppe-Seyler **369**, 317–328 and O'Brien, Kretz, Dewji, Wenger, Esch & Fluharty (1988) Science **241**, 1098–1101]. We have undertaken further biochemical investigations on a patient and fetal sibling, who were previously shown to have a unique sphingolipid storage disorder associated with an SAP-2 deficiency [Harzer, Paton, Poulos, Kustermann-Kuhn, Roggendorf, Grisar & Popp (1989) Eur. J. Pediatr. **149**, 31–39]. The severity of their disorder suggested that other products of the SAP precursor or prosaposin gene may also be deficient. The turnover of cerebroside sulphate and globotriaosylceramide were investigated and were both impaired in fibroblasts from the patient and fetus. However, the activities of cerebroside sulphate sulphatase and globotriaosylceramide α-galactosidase *in vitro* were normal in cells from the fetus and patient respectively. In addition, there was an increase in cerebroside sulphate concentration in the kidney of the affected fetus. These results indicate that, in addition to the SAP-2 deficiency, there was a defect in SAP-1 function in this disorder. Additional increases in the concentration of monohexosyl- and dihexosyl-ceramide in the fetal kidney probably reflect the deficiency of SAP-2 in the case of monohexosylceramides, and the combined activator deficiency in the case of dihexosylceramides. Lactosylceramide-loading studies confirmed that there was a defect in the turnover of this lipid in fibroblasts from the affected patient and fetus but not from a patient with an isolated SAP-1 deficiency, or from patients with Krabbe disease, G_{M1} gangliosidosis or galactosialidosis. It has been suggested [Potier, Lamontagne, Michaud & Tranchemontagne (1990) Biochem. Biophys. Res. Commun. **173**, 449–456] that the prosaposin gene also codes for lysosomal neuraminidase. However, we found normal neuraminidase activity in fibroblasts from our patient, using assay conditions which are diagnostic for sialidosis patients. The role of prosaposin gene products in sphingolipid metabolism is discussed in view of our biochemical findings in this genetic disorder.

INTRODUCTION

The term sphingolipid-activator protein (SAP) was coined by Wenger & Inui (1984) to describe a group of low-molecular mass (glyco)proteins which stimulate the hydrolysis of sphingolipids. To date, the best characterized SAPs are SAP-1, SAP-2 and SAP-3. First described by Mehl & Jatzkewitz (1964) as an activator for cerebroside sulphatase (cerebroside 3-sulphate 3-sulphohydrolase; EC 3.1.6.8), SAP-1 has also been shown to stimulate the hydrolysis of G_{M1} ganglioside and globotriaosylceramide (Li *et al.*, 1985; Vogel *et al.*, 1987) and probably has even broader specificity (Li *et al.*, 1988). A distinct activator, SAP-3, appears to be specific for two substrates (ganglioside G_{M2} and glycolipid G_{A2} of β-hexosaminidase (Conzelmann & Sandhoff, 1979). SAP-2 was first isolated by Ho & O'Brien (1971) as a β-glucocerebrosidase (D-glucosyl-*N*-acylsphingosine glucohydrolase; EC 3.2.1.45) activator and has since been shown also to stimulate the hydrolysis of β-galactocerebroside and sphingomyelin *in vitro* (Wenger *et al.*, 1982; Poulos *et al.*, 1984; Paton & Poulos, 1988). Unlike SAP-3 (Conzelmann & Sandhoff, 1979; Conzelmann *et al.*, 1982) and SAP-1 (Fischer & Jatzkewitz, 1978), which act by solubilizing their lipid substrates, SAP-2 appears to act by interacting with the enzyme (Ho, 1975; Berent & Radin, 1981*a*,*b*; Prence *et al.*, 1985). Immunocytochemical studies have shown that SAP-1 is largely confined to lysosomes in cells from human liver and colon (Tamaru *et al.*, 1986) as is SAP-2 in cultured human skin fibroblasts (Paton *et al.*, 1990).

In recent years considerable advances have been made in our understanding of the molecular genetics of the SAPs. In particular, it has been shown that SAP-1 and SAP-2 are formed from the same precursor protein (Fürst *et al.*, 1988; O'Brien *et al.*, 1988). Two other putative activators, saposin A (Morimoto *et al.*, 1988) and component C (Fürst *et al.*, 1988) or saposin D (Morimoto *et al.*, 1988), are formed from the same SAP precursor [termed prosaposin by Morimoto *et al.* (1988)] and flank (first and fourth domains respectively) the SAP-1 and SAP-2 region (second and third domains respectively) on the precursor (O'Brien *et al.*, 1988). Saposin A has been shown to mimic the effects of SAP-2 on β-glucocerebrosidase and β-galactocerebrosidase (D-galactosyl-*N*-acylsphingosine galactohydrolase; EC 3.2.1.46) *in vitro* (Morimoto *et al.*, 1989), and saposin D was reported to stimulate sphingomyelinase *in vitro* (Morimoto *et al.*, 1988). Biosynthetic studies indicate that the precursor protein is co-translationally glycosylated to give a 65–68 kDa species, which is further glycosylated to 70–73 kDa, before it is processed to lower-molecular mass forms (Fujibayashi & Wenger, 1986*a*,*b*).

Abbreviations used: FCS, fetal calf serum; MEM, minimal essential medium; MLD, metachromatic leucodystrophy; SAP, sphingolipid-activator protein; SGP-1, sulphated glycoprotein 1; 4-MU, 4-methylumbelliferyl.

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More recently, molecular biology studies have determined the structure of the SAP precursor gene (Holtschmidt *et al.*, 1991*a*). The cDNA for SAP-3 has also been isolated (Schröder *et al.*, 1989) and is genetically distinct from prosaposin (Fürst *et al.*, 1990).

Collard *et al.* (1988) first noted the considerable sequence similarity between prosaposin and a 70 kDa rat secretory protein, the Sertoli sulphated glycoprotein 1 (SGP-1), and this raised the possibility that they were equivalent proteins from different species. Using antibodies to SAP-1 or SAP-2, it has been shown that the concentrations of intact, partially processed and fully processed forms of prosaposin vary between different tissues, both in humans and rats (O'Brien *et al.*, 1988; Sano *et al.*, 1989), raising the question of how tissue-specific processing and targeting of the gene product occurs and what function the different forms have *in vivo*. An hypothesis by Potier *et al.* (1990) that lysosomal neuraminidase (EC 3.2.1.18) is a 60 kDa processed form of prosaposin would, if substantiated, suggest that the processing of prosaposin may be even more complex.

The physiological significance of SAPs has been confirmed largely through the discovery of patients with sphingolipid-storage disorders, whose conditions result from a deficiency of a SAP rather than a sphingolipid hydrolase enzyme. Patients have now been described with deficiencies in the G_{M2}-ganglioside activator (SAP-3) (Conzelmann & Sandhoff, 1978) and the G_{M1}-ganglioside, globotriaosylceramide and cerebroside sulphate activator (SAP-1) (Shapiro *et al.*, 1979; Stevens *et al.*, 1981). More recently, Christomanou *et al.* (1986, 1989) have described two patients who had a deficiency in an A_1 activator, now known to be identical with SAP-2 (Fürst *et al.*, 1988; O'Brien *et al.*, 1988).

We have also described a patient (Harzer *et al.*, 1989) who was deficient in immunologically cross-reacting material to SAP-2, but who was far more severely affected than the patients described by Christomanou *et al.* (1986, 1989). The patient's fetal sibling was similarly affected. In view of the severity of the disease and the recent evidence that SAP-1, SAP-2 and two other putative SAPs are synthesized on a common precursor (Fürst *et al.*, 1988; O'Brien *et al.*, 1988), we hypothesized that our patient and his fetal sibling may have a defect in the SAP precursor which affected other products of the SAP precursor gene in addition to the SAP-2 locus. This has been confirmed in Dr. Sandhoff's laboratory (Schnabel *et al.*, 1992), where an A to T transversion has been identified in the initiation codon of the gene, the affected patient and fetal sibling being homozygous for this mutation.

We have previously shown that our patient and his fetal sibling had decreased β-glucocerebrosidase, β-galactocerebrosidase and ceramidase (*N*-acylsphingosine deacylase; EC 3.5.1.23) activities (assayed in the presence of detergents), but that the activities were not as low as in patients with Gaucher disease, Krabbe disease and Farber disease respectively (Harzer *et al.*, 1989). We attributed the decreased activities of at least the first two of these enzymes to an observed deficiency in immunologically cross-reacting material to SAP-2 (Harzer *et al.*, 1989). Interestingly, sphingomyelinase activity, also measured in the presence of detergents, was normal. Results from glucosyl[^{3}H]ceramide- and phosphocholine [^{3}H]ceramide-loading experiments were consistent with a defect in ceramide catabolism. Liver concentrations of ceramide, glucosylceramide and dihexosylceramide were elevated in the patient and affected fetus, but sphingomyelin concentrations were normal.

In this paper we present further biochemical data that indicate that SAP-1 function is also defective in this disorder. However, we found no evidence that the lysosomal neuraminidase enzyme was deficient.

MATERIALS AND METHODS

Materials

Globotriaosylceramide was purified from the formalin-fixed myocard of a Fabry patient. The cerebroside sulphate was from bovine brain and was purchased from Serva, D-6900 Heidelberg, Germany, and the lactosylceramide (ceramide dihexoside), also from bovine brain, was obtained from Sigma, D-8024, Deisenhofen, Germany. The tritium-labelled globotriaosylceramide, cerebroside sulphate and lactosylceramide were prepared commercially by catalytic hydrogenation with tritium across the double bonds in the fatty acid and sphingosine groups of the ceramide moiety (Amersham Buchler, Braunschweig, Germany, code TR.3). The labelled substrates were all purified by preparative t.l.c. before use. 4-Methylumbelliferyl (4-MU)-α-D-*N*-acetylneuraminide and 2-amino-1-methylpropan-1-ol were also obtained from Sigma.

Cell lines and tissue samples

Skin fibroblasts for the enzyme assays and substrate-loading studies were cultured in minimal essential medium (MEM) containing 20% (v/v) fetal calf serum (FCS), except for the lactosylceramide-loading studies and neuraminidase assays where 15% FCS was used. Dr. M. Elleder kindly provided the fibroblast cell line from the Fabry case, which was from an adult male. All the other fibroblastic cell lines were obtained from our routine diagnostic laboratory in Tübingen, the infantile control lines all being from patients with non-lipidotic diseases. Clinical, biochemical and molecular genetic findings for the SAP-1-deficient patient have already been published (Schlote *et al.*, 1991; Holtschmidt *et al.*, 1991*b*).

The fetal tissues were from our prenatal diagnostic laboratory in Tübingen and had been stored deep-frozen for 1–3 years. The fetuses were from pregnancies which the parents elected to terminate after a positive prenatal diagnosis or, in the case of the control, because of a suspected, but unconfirmed, X-chromosomal non-lipidotic disease. Autopsies were performed in our German laboratory. The index patients in the families had SAP deficiency (present patient), late-infantile metachromatic leucodystrophy (MLD) or Sandhoff disease (G_{M2} gangliosidosis, variant O).

Analysis of fetal kidney lipids

Kidney tissue from 20-week-old fetuses was extracted with 50 vol. (per wet weight) of chloroform/methanol (2:1, v/v). The lipid extracts were analysed by a two-dimensional chromatographic system. The first solvent (bottom to top) was chloroform/methanol/water (14:6:1, by vol.) and the second (left to right) was chloroform/methanol/acetic acid/water (170:35:4:5, by vol.). Lipids were detected by spraying with anisaldehyde/conc. H_2SO_4/acetic acid (3:10:490, by vol.) and heating at 130 °C for 20 min. After stain stabilization, densitometry was performed with transmission light at 550 nm. For more precise identification and quantification, the lipid spots from similar chromatograms were detected by iodine vapour and the essential fractions were scraped from the two-dimensional chromatograms. These silica fractions were then extracted and compared with calibrated standards using one-dimensional t.l.c., followed by staining and densitometry as above. Our experience suggests that, overall, the errors in such determinations can be relatively high (up to 80% for values below 0.5 μg of lipid/mg dry weight, up to 40% for values around 2 μg/mg and up to 20% for higher lipid concentrations) largely because of the small amounts of tissue, and, in some instances, of standard, available for analysis. However, these errors have been decreased in the reported analyses by taking the following precautions. First, all

the samples were handled in the same way to minimize any differences in the errors between samples. In addition, for each lipid the samples from all individuals were run on a single t.l.c. plate and compared with the same standard to minimize differences arising from staining and densitometry. It should also be noted that any errors in quantifying the absolute amount of a lipid, because of errors arising from the standard, would not affect the relative concentrations of a particular lipid in different tissue samples.

Neuraminidase activity

4-MU-α-D-*N*-acetylneuraminide (1 mg) was dissolved in 1 ml of water and purified, by phase partitioning twice with 1 ml of ethyl acetate, immediately before use. The substrate (100 nmol dissolved in 1.6 ml of 0.05 M-sodium acetate buffer, pH 4.0) was applied to a 75 cm^2 flask of cultured fibroblasts (early confluent stage with 0.6–1.0 mg of cell protein) which had been washed once with iso-osmotic NaCl and once, for 10 s, with water. After a 10, 20 and 30 min incubation at 37 °C, 200, 175 and 153 μl (one-eighth of the remaining volume in each case) respectively of reaction mixture was withdrawn for the determination of released 4-MU, after adjustment of the pH to 10.3 by the addition of 0.5 ml of 0.3 M-2-amino-1-methylpropan-1-ol. Blank assays were performed in culture flasks without any cells. Protein was determined by the Lowry procedure (Lowry *et al.*, 1951) by using homogenates of the cell layer that remained attached to each flask at the end of the assay. We have independently shown that neuraminidase remains associated with this cell layer. Specific activities were calculated from the amounts of 4-MU that were released from the substrate after 20 and 30 min, the two values being averaged. There was a steady increase in the amount of substrate that was released after 10, 20 and 30 min, although slight non-linearity was tolerated.

Chromatography and identification of radiolabelled products

For the following enzyme assays and sphingolipid-loading studies, the radioactive products were analysed by radio-t.l.c. Samples of the lipid extracts were chromatographed on silica-gel t.l.c. plates (either glass- or plastic-backed, as indicated) with chloroform/methanol/acetic acid/water (170:35:4:5, by vol.). The radioactive peaks were located, cut-out and the amount of radioactivity in each peak was determined by liquid-scintillation counting. Identification of the radioactive products was based on their co-migration with standard lipids in different solvent systems. In some instances additional evidence came from the colour reaction obtained with the anisaldehyde t.l.c. reaction (see earlier) or from comparisons with the pattern found for cells from patients with known enzyme deficiencies.

Cerebroside sulphatase activity

Cerebroside sulphatase activity was measured *in vitro* with 1 nmol (1 kBq) of [*ceramide*-^{3}H]cerebroside sulphate, 1 mg of crude sodium taurocholate, 8 μmol of sodium acetate buffer, pH 5.0, and approx. 30 μg of fibroblast homogenate protein (Lowry *et al.*, 1951) in a final volume of 0.17 ml. The assay tubes were incubated at 25 °C for 15 h. After being dried, the lipids were extracted and analysed on plastic-backed silica-gel plates. The data were corrected for a radioactive contaminant in the substrate (about 5% of the added radioactivity) which co-chromatographed with the ceramide/ceramide-degradation-products fraction.

Globotriaosylceramide galactosidase (α-D-galactoside galactohydrolase; EC 3.2.1.22) activity

Globotriaosylceramide galactosidase activity was measured *in vitro* with 1 μg (100 kBq) of [*ceramide*-^{3}H]globotriaosylceramide, 1 mg of crude sodium taurocholate, 15 μmol of sodium acetate buffer, pH 5.0, and approx. 60 μg of fibroblast homogenate protein (Lowry *et al.*, 1951) in a final volume of 0.15 ml. The assay tubes were incubated at 37 °C for 15 h and 20 μl of each assay suspension was analysed by radio-t.l.c. on plastic-backed plates.

Cerebroside sulphate-loading studies

[*ceramide*-^{3}H]Cerebroside sulphate (600 nmol, 1 kBq/nmol) in 0.1 ml of ethanol was added to freshly confluent skin fibroblast cultures (0.2–0.5 mg of protein) in 25 cm^3 culture flasks containing 5 ml of MEM with 20% FCS. After culture at 37 °C for 3 days, the medium was replaced with fresh medium, without the added sulphatide, and culture was continued for an additional day. The cell layer was washed twice with iso-osmotic NaCl and the lipids were extracted *in situ* for 2 h with chloroform/methanol (1:9, v/v). The extracts contained 5–11% of the radioactivity added to the cell cultures. The lipids were analysed by radio-t.l.c. on glass-backed plates. The data were corrected for small amounts of radioactive contamination in the substrate which co-chromatographed at the solvent front (4–5% of added radioactivity) and with the free ceramide peak (1.5%). The glass-backed plates gave better resolution in this region than the plastic-backed plates used for the '*in vitro*' enzyme assays.

Globotriaosylceramide-loading studies

[*ceramide*-^{3}H]Globotriaosylceramide (7 μg, 100 kBq/μg) in 0.1 ml of ethanol was added to freshly confluent skin fibroblast cultures (about the second to fifth passage and about 0.2–0.4 mg of protein) in 25 cm^2 culture flasks containing 5 ml of MEM with 20% FCS. After culture at 37 °C for 4 days, the medium was replaced with fresh medium, without the added globotriaosylceramide, and culture was continued for an additional day. The cell layer was washed twice with iso-osmotic NaCl and the lipids were extracted *in situ* with 0.7 ml of methanol for 30 min. The extract was mixed with 0.3 ml of chloroform and the mixture was used to re-extract the cell layer for 30 s. The final extracts, which contained 5–12% of the radioactivity added to the cell cultures, were analysed on glass-backed plates.

Lactosylceramide-loading studies

[*ceramide*-^{3}H]Lactosylceramide (4 nmol, 40 kBq/nmol) in 60 μl of ethanol was added to freshly confluent skin fibroblasts cultures (0.3–0.5 mg of protein) in 25 cm^2 flasks containing 5 ml of MEM with 15% FCS. After culture at 37 °C for 3 days, the cell layer was washed three times with phosphate-buffered saline (g/litre: NaCl, 8.0; KCl, 0.2; Na_2HPO_4, 1.15; KH_2PO_4, 0.2) containing EDTA and then extracted *in situ* with 0.7 ml of methanol for 4 h. Chloroform (0.3 ml) was then added to the methanol in the flasks. After 20 s the extract was removed and dried, then redissolved in chloroform/methanol (2:1, v/v). The lipid extracts contained 7–10% of the radioactivity added to the cell cultures and portions (usually one-half) were applied to plastic-backed silica-gel plates.

RESULTS

Turnover of cerebroside sulphate

Loading studies of fibroblasts with cerebroside sulphate indicated a clear defect in turnover of this substrate in cells from the patient and his fetal sibling, with a deficiency in turnover similar to that seen in cells from patients with late infantile and juvenile MLD (Table 1). The result was clearly different from that seen with a juvenile Krabbe patient, where turnover of sulphatide was normal but the product of the sulphatase reaction, galactosyl-

Table 1. Cerebroside sulphate turnover

Cerebroside sulphate turnover was measured in cultured fibroblasts using a 3 day pulse followed by a 1 day chase as described in the Materials and methods section. The percentage of radioactivity found in the different metabolites was then determined.

	Percentage radioactivity			
Fibroblasts	Sulphatide and polar lipids	Galactosyl-ceramide	Free ceramide	Ceramide-degradation products
Patient	98.8, 97.9	0.6, 0.5	0.6, 0.5	0.0, 1.1
Juvenile Krabbe	87.2	9.6	3.2	0.0
Late infantile MLD	97.5	1.6	0.9	0.0
Juvenile MLD	98.2	0.8	0.7	0.3
Controls ($n = 4$)*	89.4–90.6	1.7–2.9	4.4–7.1	0.6–2.2
Affected fetus	97.9	0.4	0.7	1.0
Fetal control	87.9	3.3	8.8	0.0

* The reproducibility of this assay is indicated by the results of seven additional experiments on a single control cell line, where the percentage radioactivity in the sulphatide substrate (and polar lipids) was 89.9 ± 2.1 (mean ± S.D.).

Table 2. Globotriaosylceramide turnover

Globotriaosylceramide turnover was measured in cultured fibroblasts using a 4 day pulse followed by a 1 day chase as described in the Materials and methods section. The percentage of radioactivity found in the different metabolites was then determined.

	Percentage radioactivity				
Fibroblasts	Globo-triaosyl-ceramide*	Lactosyl-ceramide	Glucosyl-ceramide	Free ceramide	Ceramide-degradation products
Patient	82.0	7.7	3.4	2.7	4.2
Affected fetus	87.0	7.0	2.5	1.5	2.0
Juvenile MLD	60.0	9.4	5.8	3.8	21.0
Fabry (adult)	94.0	2.7	2.0	0.7	0.6
Controls ($n = 3$)†	55.3–71.0	7.7–11.0	5.7–11.0	2.3–3.7	12.2–22.9

* The globotriaosylceramide peak also contained small quantities of biosynthetic phospholipids (less than 10% of the radioactivity), which chromatographed close to or with the substrate. The amount of biosynthetic products was greatest in the controls, so the tabulated results slightly underestimate the difference between turnover in normal controls and that in the SAP-deficient and Fabry cells.

† The reproducibility of this assay is indicated by the results of seven additional experiments on a single cell line, where the percentage radioactivity in the globotriaosylceramide substrate was 62.5 ± 5.4 (mean ± S.D.).

ceramide, accumulated relative to controls (Table 1). Other investigators (Tanaka & Suzuki, 1978; Kudoh & Wenger, 1982; Kobayashi *et al.*, 1985*a*,*b*; Ida *et al.*, 1990) have shown that, although there is considerable catabolism of galactosylceramide in fibroblasts from patients with Krabbe disease, the turnover of this substrate is less than in controls.

Turnover of globototriaosylceramide

Since a SAP-1 deficiency was suspected in our patient, globotriaosylceramide turnover was also investigated. In fibroblasts from both the patient and his fetal sibling the turnover of globotriaosylceramide was lower than in control cell lines, but was not as severely affected as in a patient with Fabry disease, whereas cells from a patient with juvenile MLD showed normal breakdown (Table 2). The globotriaosylceramide-loading studies also indicated that the turnover of lactosylceramide was impeded in our patient and his fetal sibling, since a relatively large percentage of the metabolized radioactivity appeared in this product (43% for the patient, 54% for the affected fetus) compared with controls (23–29%).

Turnover of lactosylceramide

Lactosylceramide-loading studies (Table 3) confirmed that turnover of this lipid was decreased in fibroblasts from the affected patient and fetus. Radioactivity from the small amount of lactosylceramide that was metabolized was largely found in the ceramide/fatty acid fraction and, unlike control incubations, virtually none appeared in biosynthetic products (sphingomyelin, phosphatidylcholine, phosphatidylethanolamine and, presumably, gangliosides at the origin). Patients with deficiencies in β-galactosidase (EC 3.2.1.23) (G_{M1} gangliosidosis and galactosialidosis), β-galactocerebrosidase (Krabbe disease) or an isolated SAP-1 deficiency showed normal turnover of lactosylceramide and normal incorporation of radioactivity into biosynthetic products. In controls the incorporation of radioactivity into biosynthetic products was greater than seen for the other substrates used.

Cerebroside sulphatase

Under the assay conditions used (bile salts were present) the activity of cerebroside sulphatase was normal in homogenates of fibroblasts of the affected fetus, with, in two determinations, 28.6% and 30.5% of the added substrate (equivalent to 0.64 and 0.68 nmol of substrate hydrolysed/h per mg of protein respectively) appearing in the hydrolysis products, galactosylceramide and ceramide. In five controls, 7.4–38.5% (mean 29.6%) of the substrate was hydrolysed, whereas in a cell homogenate from a patient with late infantile MLD only 1.5% of the substrate was hydrolysed.

Globotriaosylceramide galactosidase

Globotriaosylceramide galactosidase activity (assayed in the presence of bile salts) was normal in the patient's fibroblast homogenate, both the control and patient samples hydrolysing 2.7% of the added substrate to lactosylceramide, glucosylceramide and ceramide. By contrast, there was no detectable globotriaosylceramide galactosidase activity in cells from a Fabry case, where hydrolysis of the substrate was the same as for a blank incubation (both 0.7%). Unfortunately, we were unable to repeat these assays owing to limited supplies of the substrate. However, we have independently shown that the assay procedure is diagnostic for Fabry disease when leucocyte homogenates are used for the tissue source. In addition, the present results on our patient's fibroblasts are consistent with the previously reported normal α-galactosidase activity (using 4-MU-α-D-galactoside as the substrate and either leucocyte or fibroblast homogenates) for this patient (Harzer *et al.*, 1989).

4-MU-α-D-*N*-acetylneuraminide neuraminidase

In view of the suggestion that lysosomal neuraminidase may be a processed form of prosaposin, neuraminidase activity was determined in fibroblasts using an assay procedure *in situ*. In three experiments using cells from the patient, the activity of this enzyme was clearly normal (43, 72 and 89 nmol of neuraminide hydrolysed/h per mg of protein) when compared with controls (47.6 ± 24.3 units, $n = 14$). Cells from a patient with an isolated SAP-1 deficiency also had normal activity of this enzyme.

Table 3. Lactosylceramide turnover

Lactosylceramide turnover was measured in cultured fibroblasts using a 3 day pulse as described in the Materials and methods section. The percentage radioactivity found in the different metabolites was then determined.

	Percentage radioactivity							
Fibroblasts	Origin*	Sphingomyelin	Phosphatidyl-choline	Lactosyl-ceramide	Phosphatidyl-ethanolamine	Glucosyl-ceramide	Unknown	Ceramide and its degradation products
Blank								
Expt. 1	0.8	0.6	1.6	94.2	1.5	0.4	0.3	0.6
Expt 2	0.8	0.1	1.8	95.3	1.3	0.2	0.1	0.4
Patient								
Expt. 1	1.2	1.1	1.3	84.4	1.0	2.5	0.3	8.2
Expt. 2	1.5	2.0	1.6	84.0	0.8	2.7	2.8	4.6
Expt. 3	1.1	1.1	1.1	82.8	3.0	2.4	0.5	8.0
Affected fetus	1.0	1.0	1.0	89.1	0.6	2.6	1.7	3.0
SAP-1 deficiency								
Expt. 1	5.3	21.5	11.6	35.5	5.7	4.5	3.4	12.5
Expt. 2	3.0	11.8	7.0	55.8	3.1	2.0	1.1	16.2
G_{M1} gangliosidosis								
Case 1	5.2	15.6	6.2	50.9	3.3	3.4	2.3	13.1
Case 2	4.7	18.7	12.4	42.2	6.1	3.8	1.0	11.1
Galactosialidosis	5.4	20.1	12.6	28.7	3.5	5.0	1.2	23.5
Infantile Krabbe								
Case 1	2.9	12.0	10.8	43.4	4.5	2.5	1.4	22.5
Case 2	4.6	21.5	15.8	31.8	6.2	3.1	1.1	15.9
Controls ($n = 8$)†								
Mean	4.8	17.0	11.6	37.7	4.8	3.9	1.6	18.6
Highest	6.5	21.5	19.6	52.1	7.6	5.2	3.4	23.5
Lowest	2.9	9.6	6.2	23.3	2.3	2.5	0.7	12.5

* Possibly containing gangliosides.

† The S.D. of the percentage radioactivity in lactosylceramide for the controls was 9.8. The lactosylceramide values for the patient and affected fetus are more than 4 S.D.s removed from the mean (37.7).

Table 4. Fetal kidney lipids

Lipid extracts of kidney tissue from 20-week-old fetuses were analysed as described in the Materials and methods section. The results are expressed as μg/mg dry weight of tissue

	Affected fetus	Normal control	Late infantile MLD	Sandhoff disease
Total lipids*	245	140	230	180
Ceramide				
Monohexosides	2.2	0.2	0.3	0.4
Dihexosides	2.0	0.5	0.3	0.5
Trihexosides	1.0	0.3	0.7	0.8
Tetrahexosides (including globoside)	1.4	0.7	1.2	2.0
Sulphatides	2.1	0.5	2.4	0.6
G_{M3} ganglioside	1.2	0.8	0.9	0.8
G_{M2} ganglioside	n.d.†	n.d.	n.d.	Trace
Sphingomyelin	11.0	5.0	10.5	9.0

* The differences in the total lipid content of the kidney samples were essentially due to differences in the levels in non-sphingolipid lipids.

† n.d., not detected.

However, cells from two cases each of infantile sialidosis and galactosialidosis all showed defective neuraminidase activities, with only 0–2 and 4–6 units of activity respectively for each disease state. The fact that our assay procedure was diagnostic for the latter patients, and that it was carried out at pH 4, indicates that we were measuring the activity of the lysosomal enzyme.

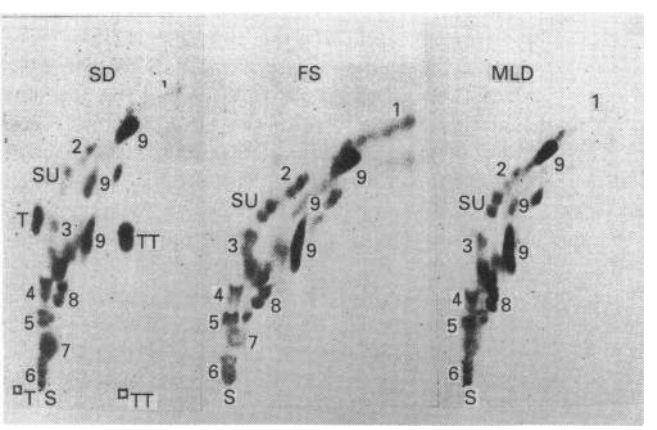

Fig. 1. Two-dimensional t.l.c. of fetal kidney lipids

Total lipids from the equivalent of 2 mg dry weight of kidney tissue from 20-week-old fetuses were applied to the starting point (S) of individual t.l.c. plates as indicated: SD, Sandhoff disease fetus; FS, present SAP-deficient fetus; MLD, metachromatic leucodystrophy fetus (sibling of patient with late infantile MLD). On the SD chromatogram, two additional starting points (squares T and TT) are shown to which 5 (left) and 10 (right) μg of reference globotriaosylceramide were applied resulting in the reference spots T and TT on this chromatogram. On all chromatograms, the most hydrophobic fractions found at the upper, right-hand edges of the original plate were excluded from the photograph. The fraction numbers correspond to the following lipids: 1, ceramide monohexosides; 2, ceramide dihexosides (lactosylceramide); SU, sulphatides; 3, ceramide trihexosides; 4, ceramide tetrahexosides (including globoside); 5, G_{M3} ganglioside; 6, major gangliosides; 7, glucose (including, only in SD, a small G_{M2} ganglioside spot analysed on a different chromatogram); 8, sphingomyelin; 9, phospholipids.

Fetal kidney sphingolipids

Clear increases in monohexosylceramide, dihexosylceramide and sulphatide were seen in the kidney of the affected fetus (Fig. 1, Table 4). Indeed, the concentration of sulphatide was similar to that found in kidney tissue from a fetus with MLD. The concentration of sphingomyelin was a little higher for the affected fetus than for the normal control, but was similar to those found in the two pathological controls (Table 4). These results are in agreement with our previous findings on liver tissue from the patient and affected fetus, where concentrations of ceramide, glucosylceramide and lactosylceramide, but not sphingomyelin, were elevated (Harzer *et al.*, 1989). Although the quantitative estimations reported here (Table 4) have not been repeated in their present form, the trends observed (in particular the increases in ceramide mono- and di-hexosides and sulphatides in the affected fetus's kidney) are also apparent from a visual assessment of the anisaldehyde-stained two-dimensional chromatograms presented in Fig. 1, and from the relative peak sizes of the relevant spots after densitometric analysis of similar two-dimensional chromatograms (results not shown). The limited supply of remaining tissue from the affected fetus has been used for a more comprehensive quantitative lipid study (V. Bradová, K. Harzer, F. Šmíd, B. C. Paton, B. Ulrich-Bott & W. Roggendorf, unpublished work). As part of that study the observed differences reported here have been confirmed. As noted in Table 4, the amount of total lipid/mg dry weight varied between the samples. However, clear elevations in the concentrations of ceramide mono- and di-hexosides and sulphatide in the kidney of the affected fetus, and of sulphatide for the MLD sample, are still apparent even when the data are expressed relative to the total lipid content rather than tissue dry weight.

DISCUSSION

Molecular biology studies of prosaposin cDNA from members of the present family have indicated that the affected siblings are homozygous for a defect in the initiation codon (Schnabel *et al.*, 1992). It is unlikely therefore that any prosaposin gene products are produced in this disorder, since the next in-frame AUG codon (Nakano *et al.*, 1989) is within the saposin A domain. In addition, no biosynthesis of prosaposin products has been detected with anti-(SAP-1) and anti-(SAP-2) sera [Schnabel *et al.* (1992) and our own preliminary findings with a different anti-(SAP-2) serum].

Previously, we have provided biochemical and immunological evidence that SAP-2 is deficient in this disorder (Harzer *et al.*, 1989; Paton *et al.*, 1990). Biochemical evidence of an additional SAP-1 deficiency in our patient and his fetal sibling comes from the following observations. First, both sulphatide and globotriaosylceramide turnover were decreased in fibroblasts from the patient and his fetal sibling, but cerebroside sulphatase and globotriaosylceramide α-galactosidase activities were normal when measured *in vitro* in the presence of bile salts. Second, a kidney sample from the affected fetus showed a clear increase in sulphatide. The deficiency in SAP-1 has been confirmed immunologically in Dr. Sandhoff's laboratory (Schnabel *et al.*, 1992).

Five other patients with SAP-1 deficiencies (two from one family) have been reported in the literature as variants of MLD (Shapiro *et al.*, 1979; Hahn *et al.*, 1982; Wenger *et al.*, 1989; Schlote *et al.*, 1991). They also all showed a deficiency in sulphatide turnover in fibroblasts associated with a normal or near-normal activity of arylsulphatase A or cerebroside sulphatase in leucocyte and/or fibroblast extracts. Considerable phenotypic differences between the cases were noted (Wenger *et al.*, 1989). Increases in globotriaosylceramide, indicating a problem with the catabolism of this lipid, have also been reported for some of the patients (Li *et al.*, 1985; Wenger *et al.*, 1989).

SAP-1 has also been shown to stimulate the hydrolysis of G_{M1} ganglioside *in vitro* (Li *et al.*, 1985; Banks *et al.*, 1987; Vogel *et al.*, 1987). However, G_{M1} accumulation has not been an apparent feature of the previously described cases of SAP-1 deficiency (Hahn *et al.*, 1982; Li *et al.*, 1985; Wenger *et al.*, 1989; Schlote *et al.*, 1991), with even the most severely affected case (Wenger *et al.*, 1989) having little or no accumulation of G_{M1} ganglioside in the brain. Recent studies have indicated that the turnover of G_{M1} ganglioside to G_{M2} ganglioside was close to normal in fibroblasts from our patient (Schmid *et al.*, 1992), making it unlikely that SAP-1 is essential for G_{M1} ganglioside catabolism *in vivo*.

The clinical history of our patient (Harzer *et al.*, 1989) indicated a more severe disorder than reported for the other cases of either SAP-2 deficiency (Christomanou *et al.*, 1986, 1989) or SAP-1 deficiency (Shapiro *et al.*, 1979; Hahn *et al.*, 1982; Schlote *et al.*, 1991; Wenger *et al.*, 1989). This presumably reflects the combined deficiency of these two activators, and possibly the other prosaposin gene products, in this disorder. Whereas the increase in sulphatide in the kidney of our affected fetus can be explained by a deficiency in SAP-1 and the increase in monohexosylceramide by a deficiency in SAP-2, the increased concentration of dihexosylceramide probably reflects the combined activator deficiency. Increases in dihexosylceramides, in some instances in both the lactosyl and digalactosyl forms, have sometimes been reported in SAP-1 deficiency, but they have not been consistently observed (see Shapiro *et al.*, 1979; Li *et al.*, 1985; Wenger *et al.*, 1989; Schlote *et al.*, 1991). Digalactosylceramide with an α-linked terminal galactose residue accumulates in Fabry disease (α-galactosidase deficiency) (Handa *et al.*, 1971) and this might also occur in SAP-1 deficiency if this activator is also required to solubilize this substrate. However, Vogel *et al.* (1991) have suggested that SAP-1 is more likely to be involved with the solubilization of more highly glycosylated sphingolipids. Recent studies have indicated that the dihexosylceramide fraction in the kidney of our affected fetus contains elevated concentrations of both lactosylceramide and digalactosylceramide (V. Bradová, K. Harzer, F. Šmíd, B. C. Paton, B. Ulrich-Bott & W. Roggendorf, unpublished work). Our lactosylceramide-loading studies using fibroblast cultures showed a normal turnover for patients with Krabbe disease (β-galactocerebrosidase deficiency) and G_{M1} gangliosidosis (β-galactosidase deficiency), in agreement with Kobayashi *et al.* (1985*b*), as well as for cells from patients with galactosialidosis (also deficient in β-galactosidase) or an isolated SAP-1 deficiency. It would appear therefore that the turnover of this glycolipid is only severely compromised when the activities of both of the indicated enzymes (towards lactosylceramide) are affected. This is presumably the case for our patient and affected fetus, where lactosylceramide turnover was defective. It is likely that saposin A and/or SAP-2 are required for activation of galactocerebrosidase. However, the activator needed for hydrolysis of lactosylceramide by β-galactosidase is less certain. Vogel *et al.* (1991) have reported that SAP-1 did not stimulate this activity *in vitro*, but the situation may be different *in vivo*.

An interesting feature of the lactosylceramide-loading studies in the present disorder was that, even though some of the substrate was metabolized, very little of the radioactivity from the metabolized substrate appeared in biosynthetic products. In cells from the controls and the other disease states studied, considerable amounts of radioactivity appeared in sphingomyelin, phosphatidylcholine and, to a lesser extent, phosphatidylethanolamine and possibly gangliosides. In cells from our patient and affected fetus, radioactivity from the lactosylceramide appeared mainly at the solvent front with smaller amounts in glucosylceramide. Both ceramide and fatty acids migrate at the

solvent front with the solvent system used. However, we have previously shown that ceramide turnover is severely compromised in the present disorder (Harzer *et al.*, 1989). Studies by Trinchera *et al.* (1990) on rat liver have indicated that, in that tissue, glycosphingolipids must be metabolized to sphingosine before recycling of the long-chain base to sphingomyelin can occur, whereas recycling for ganglioside synthesis can take place at the level of glucosylceramide. Breakdown of ceramide would also have to occur for labelled fatty acids to be incorporated into phospholipids. We believe the decreased incorporation of radioactivity from metabolized lactosylceramide into biosynthetic products may reflect the defect in ceramide catabolism in this disorder; however, we cannot exclude the possibility that prosaposin, or its products, has some other function.

Morimoto *et al.* (1989) proposed that saposin A has similar properties to SAP-2, in that both activate β-galactocerebrosidase and β-glucocerebrosidase *in vitro*. If this applies *in vivo*, one might expect that each of these activators could compensate for the other. However, Fabbro & Grabowski (1991) have shown that saposin A and SAP-2 have a synergistic effect on β-glucocerebrosidase under their assay conditions. This might explain why the two individuals with apparently isolated SAP-2 deficiencies [Christomanou *et al.* (1986, 1989); Schnabel *et al.* (1991) have shown that one of these patients has a point mutation which only affects the SAP-2 locus] did have clinical features of Gaucher disease (β-glucocerebrosidase deficiency). However, in spite of their SAP-2 deficiencies, no features of Krabbe disease (β-galactocerebrosidase deficiency) were reported for these patients. In fact, a normal β-galactocerebrosidase activity, measured in the presence of taurocholate, has recently been found for a spleen homogenate from one of these individuals (K. Harzer, unpublished work). By comparison, cells from our patient and fetus did show decreased β-galactocerebrosidase activity, as well as β-glucocerebrosidase activity, when assayed either in the presence of taurocholate (Harzer *et al.*, 1989) or without any detergent present (B. C. Paton, A. Poulos & K. Harzer, unpublished work), perhaps suggesting that β-galactocerebrosidase is more dependent on saposin A than SAP-2 *in vivo*.

Controversy (O'Brien & Kishimoto, 1991) still surrounds the effects *in vitro* of saposins on sphingomyelin degradation, as both SAP-2 (Wenger *et al.*, 1982; Poulos *et al.*, 1984) and saposin D (Morimoto *et al.*, 1988) have been reported to be stimulatory. Christomanou & Kleinschmidt (1985) have suggested that the former may act by solubilizing the substrate, whereas saposin D had its effect in the presence of detergent (Morimoto *et al.*, 1988). In spite of the various findings *in vitro*, our evidence suggests that prosaposin gene products are probably not involved in sphingomyelin catabolism *in vivo*. First, there was no clear-cut accumulation of sphingomyelin in either liver tissue (Harzer *et al.*, 1989) or kidney in the present disorder. It is possible, though, that the decreased biosynthesis of sphingomyelin from sphingolipid-degradation products in this disorder compensates for a defect in sphingomyelin degradation. (The extent to which sphingomyelin synthesis is dependent on recycling of sphingolipid-degradation products rather than synthesis *de novo* is presently unknown.) Second, when sphingomyelin-loading studies were performed on cultured cells from the patient and fetus (Harzer *et al.*, 1989), no accumulation of labelled sphingomyelin was seen, but it is possible that the conditions were not appropriate for detecting a defect in turnover. Last, as we previously reported, sphingomyelinase (EC 3.1.4.12) activity was normal when assayed in the presence of bile salts (Harzer *et al.*, 1989). Since it is possible that the bile salts may have masked a detergent-like activator role, we have also assayed sphingomyelinase in fibroblasts in the absence of detergents using a liposomal substrate (Paton & Poulos, 1988) and again found normal activities for cells from the patient and fetus (B. C. Paton, A. Poulos & K. Harzer, unpublished work). Although there is no clear evidence that sphingomyelin catabolism *in vivo* is dependent on prosaposin gene products, at this stage we cannot totally exclude this possibility.

We previously reported (Harzer *et al.*, 1989) that our patient also had a partial deficiency in ceramidase activity, presumably because of a missing SAP. Neither ceramide concentrations nor the activity of ceramidase have been reported for any of the other patients with SAP-1 or SAP-2 deficiencies; however, nor were any features of Farber disease noted. It still remains to be determined which of the four activator domains on the SAP precursor corresponds to the putative ceramidase activator.

Evidence now suggests that a single neuraminidase enzyme is responsible for the lysosomal degradation of gangliosides and oligosaccharides (Mancini *et al.*, 1986; Zeigler *et al.*, 1989; Leiser *et al.*, 1989; Hiraiwa *et al.*, 1987) and our data indicate that the activity of this enzyme is normal in our patient [workers in Dr. Sandhoff's laboratory (Schnabel *et al.*, 1992) have also found a normal lysosomal neuraminidase activity using a different assay procedure]. This observation and the findings of Hiraiwa *et al.* (1991) clearly argue against the hypothesis of Potier *et al.* (1990) that the prosaposin gene codes for lysosomal neuraminidase. Although the lysosomal neuraminidase activity was normal in our patient's cells, G_{M1} ganglioside-loading studies have indicated that there is an impairment in the turnover of G_{M3} ganglioside in cells from this patient and also in cells from a patient with SAP-1 deficiency (Schmid *et al.*, 1992). Zeigler *et al.* (1989) hypothesized that an activator protein may be required for lysosomal ganglioside sialidase activity *in vivo*, since detergents were required to measure any activity *in vitro*. It now seems likely that SAP-1 is the necessary solubilizing agent.

Our findings have clearly demonstrated that studies on patients with a defective SAP(s) can teach us much about the physiological role of that SAP(s). As a caveat, it should be noted that certain activities observed *in vitro* may not play a role *in vivo*. In part, this may reflect the detergent-like properties of the saposins. SAP-1 has been shown to solubilize a number of sphingolipid substrates, and the sequence similarities to the other saposins indicate that they also have potential detergent-like properties. It is possible that such properties may be elicited *in vitro* but not *in vivo*. Also, substrate-loading studies may give misleading results depending on the level of residual enzyme activity and/or the amount of substrate added.

In prosaposin deficiency the effects of the defect are profound, with the catabolism of several sphingolipids adversely affected. If the unprocessed gene product has a physiological role as well, perhaps in sphingolipid transport or cell regulation, its function must also be deficient in this disorder. O'Brien *et al.* (1988) reported that there were higher concentrations of the high-molecular mass forms of prosaposin in human brain and testis than in kidney, spleen or liver, so any defect related to the function of the precursor protein is more likely to be manifested in the former tissues. The precursor forms of prosaposin may also have a function in extracellular fluids (Hineno *et al.*, 1991). Whether or not the precursor forms have a distinct physiological role, our studies have clearly emphasized the importance of prosaposin gene products for normal sphingolipid metabolism.

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