Concentrations of an Activator Protein for Sphingolipid Hydrolysis in Liver and Brain Samples from Patients with Lysosomal Storage Diseases

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ABSTRACT The hydrolysis of sphingolipids by lysosomal enzymes requires the presence of additional proteins, which have been called activator proteins. The number of activator proteins, their specificity, exact mechanism of action, and response to a storage process all remain to be determined. In this study, antibodies to an activator protein known to bind sphingolipids and activate the enzymatic hydrolysis of G_{M1} ganglioside and sulfatide were used to estimate the concentration of this activator protein in small samples of liver and brain from patients with lysosomal storage diseases. By using rocket immunoelectrophoresis, the concentration of cross-reacting material (CRM) was determined. Control livers had an average of 0.95±0.18 (mean±1 SD) μg CRM/mg protein in the extracts, and control brains had an average of 0.25±0.14 μg CRM/mg protein. Extremely high levels of CRM were found in extracts of livers from patients with type 1 G_{M1} gangliosidosis (15.1 and 16.9), and type A Niemann-Pick disease (10.7). Extracts of brain samples revealed a large amount of CRM in type 1 G_{M1} gangliosidosis (14.8), Tay-Sachs disease (5.3 and 8.7), and Sandhoff disease (13.5). Significantly elevated CRM was also measured in brain samples from patients with type 2 G_{M1} gangliosidosis, type A Niemann-Pick disease, metachromatic leukodystrophy, and Krabbe disease. The highest levels are found in those genetic diseases where the lipids stored, primarily or secondarily to the genetic defect, bind to this activator protein. This activator protein may have an important function in regulating intralysosomal lipid catabolism, and changes in its concentration in certain genetic diseases may be the cause of clinical, biochemical, and pathological heterogeneity found in the patients.

INTRODUCTION

Sphingolipids are degraded in the lysosomes by the sequential action of specific acid hydrolases. A mutation in one of these enzymes can result in a lack of sufficient activity to prevent accumulation of one or more substrates, and this can lead to severe clinical deterioration in a patient. The activity of these lipid hydrolases can be measured in vitro using either a synthetic substrate or a suitably labeled natural substrate usually assayed in the presence of a specific detergent. Recently, it has become evident that mammalian tissues contain proteins that act either as natural activators by binding the lipid substrates and carrying them to the specific hydrolase or as a cofactor by binding to the hydrolase to prepare it for the substrate. These include the activators of sulfatide sulfatase (1, 2), glucosylceramide β-glucosidase (3), G_{M1} ganglioside β-galactosidase (4, 5), G_{M2} ganglioside β-hexosaminidase A (6, 7), sphingomyelinase (8, 9), and galactosylceramide β-galactosidase (10). The exact number of activator proteins is not known, and some activator proteins with different specificities appear to be identical. For example, recent studies in this laboratory (10) demonstrated that the activator protein for glucosylceramide β-glucosidase (3) may be identical to the activator of galactosylceramide β-galactosidase. Recently, the activator protein originally reported to activate G_{M1} ganglioside β-galactosidase (5) was found to be missing in cultured cells from a patient who was unable to properly metabolize sulfatide (11). This provides evidence that the activator of G_{M1} ganglioside may be identical to the activator of sulfatide sulfatase originally described by Mehl and Jatzkewitz (1). This

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activator's exact mechanism of action and response to a defect in lysosomal function is not known.

The role that activator proteins play in the etiology of certain genetic lipidoses has only recently been elucidated. Conzelmann and Sandhoff (7) and Hechtman et al. (12) have described patients who store G_{M2} ganglioside because of a deficiency of the activator protein toward G_{M2} ganglioside β-hexosaminidase A. Li et al. (13) and our laboratory (14) reported that other patients who store G_{M2} ganglioside had normal (or elevated) levels of this activator protein and apparently normal β-hexosaminidase A levels (using synthetic substrates for assay). The defect in these patients appears to be in the activator protein or lipid-binding site on their β-hexosaminidase A. Christomanou (8) reported a defect in a sphingomyelinase activator protein in some patients with juvenile Niemann-Pick disease, but this has not been confirmed. Shapiro et al. (15) reported on patients with a juvenile form of metachromatic leukodystrophy who had only partially deficient arylsulfatase A activity. Recently, our laboratory (11) demonstrated correction of in situ sulfatide metabolism in the skin fibroblasts from one of these patients by the addition of the activator protein originally demonstrated to stimulate G_{M1} ganglioside β-galactosidase (5). In addition, by using monospecific antiserum to this activator protein, the patient's cultured cells were found to contain no cross-reacting material (CRM)[1] (11).

It is known that the activator protein that stimulates the enzymatic hydrolysis of G_{M1} ganglioside and sulfatide acts by binding the lipid substrates and presenting them to their specific lysosomal hydrolase (16).[2] However, it can also bind other sphingolipids but not stimulate their hydrolysis when presented with the specific lysosomal hydrolase.[2] Because of this finding, it was important to examine the levels of this activator protein in organ samples from a variety of lysosomal storage diseases. As shown previously (17), a liver sample from a patient who died with type 1 G_{M1} gangliosidosis had about 35 times more of this activator protein than a sample from a control. However, large tissue samples are not always available from patients, and the purification procedure is laborious. By using rocket immunoelectrophoresis, the concentration was estimated easily in small samples. The results demonstrate that tissues with storage of certain sphingolipids known to bind this activator protein have elevated concentrations of this activator protein. This elevation may influence the ability of other lysosomal enzymes to act on their substrates and this could result in clinical heterogeneity among patients with the same enzymatic defect.

METHODS

Purification of the activator protein. The activator protein, originally demonstrated to stimulate the enzymic hydrolysis of G_{M1} ganglioside by β-galactosidase, was purified from liver samples from a control and a patient who died with type 1 G_{M1} gangliosidosis by using the method of Li and Li (5). The purification was described in our previous communication (17). Although the liver from the patient had about 35 times more of the activator protein than the control used, both had identical properties with regard to lipid binding, ability to stimulate only specific enzymatic reactions, and both gave only one diffuse band with Coomassie Blue staining on polyacrylamide gel electrophoresis in the presence and absence of sodium dodecyl sulfate (11, 17).[2] From 200-g samples of liver, 0.65 mg and 24 mg of activator protein were isolated from the control and the patient, respectively.

Preparation of immune serum to this activator protein. Each of two young female rabbits was injected with 10 μg of purified activator protein from G_{M1} gangliosidosis liver emulsified in Freund's incomplete adjuvent into each shoulder region as recently described (15). After 2½ wk another 20 μg per rabbit was injected into the haunches in the same manner. 4 wk after the initial injection, precipitin lines were detected by Ouchterlony double immunodiffusion in 1.2% agarose gels. The rabbits were boosted by injecting 20 μg activator protein followed by collection of blood from an ear artery 1 wk later. Sera were prepared and frozen in aliquots after addition of 1/100 part of 1 M ε-aminocaproic acid. No precipitin lines were detected with the preimmune rabbit sera. The crude IgG fraction was prepared by the addition of an equal volume of saturated ammonium sulfate to the serum and collection of the precipitate.

The specificity of the antiserum toward this protein was determined by several criteria. Ouchterlony double immunodiffusion using the crude antiserum gave only one precipitin line with crude extracts of liver and brain. Two precipitin lines were obtained when the crude IgG fraction was used with pure activator protein and crude tissue extracts. This could reflect some denaturation of the antibodies by the treatment with ammonium sulfate as reviewed by Crowle (18). Crossed immunoelectrophoresis (19, 20) of crude extracts of liver and brain and purified activator proteins from control and type 1 G_{M1} gangliosidosis liver resulted in only one intense loop and one very faint loop of precipitation that ran parallel and directly under the intense loop. When the purified activator protein was preincubated with [^{14}C]sulfatide or [^{14}C]sphingomyelin before crossed immunoelectrophoresis, subsequently stained and exposed to x-ray film, the intense loop contained almost all of the radioactivity. Crude extracts of liver, brain, and cultured skin fibroblasts as well as purified activator protein were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described by Laemmli (21). The proteins were transferred to nitrocellulose using the electrophoretic transfer method of Burnette (22). The nitrocellulose was incubated with this antiserum, washed well, and subsequently reacted with horseradish peroxidase-goat anti-rabbit IgG. After reaction with 4-chloro-1-naphthol, multiple bands with estimated molecular weights between 8,500 and 10,000 were visualized. This agrees with our previous report of a subunit size of ~10,000 (17). Cells from the patient shown to have no

[1] *Abbreviation used in this paper:* CRM, cross-reacting material.

[2] Inui, K., and D. A. Wenger, manuscript in preparation.

CRM (11) had no visible bands by this technique. The multiple bands probably reflect different size species of the same gene product or carbohydrate chain differences. Previously, we had shown that this activator protein had two isoelectric points (17). Rocket immunoelectrophoresis using this antiserum gave one loop of precipitation with purified activator protein and crude tissue extracts. Some slight diffusion in the precipitin line with crude extracts probably reflects a broad range for precipitation of complexes as described by Laurell (19).

Tissue samples. Pieces of liver and brain obtained at autopsy from patients with lysosomal storage diseases and controls were frozen at −60°C until they were thawed for analysis. The liver samples from seven controls ranged in age from 2 d to 11 yr and the brain samples from four controls ranged from 4 mo to 13 yr. This covered the age range of the patients with lysosomal storage disease, except for the adult who died with type 1 Gaucher disease. The patients, with their age at death, are listed in Table I. They were diagnosed by enzyme assay and/or lipid analysis, and were categorized by their clinical presentation. The small samples (average 200 mg) were homogenized with ground glass homogenizers in 4 vol of distilled water for liver and 2 vol for brain. The homogenates were centrifuged at 20,000 *g* for 1 h at 4°C and the supernatant fractions were isolated. Over 85% of the protein cross-reacting with the antibodies to the activator protein was found in the supernatant fraction, as opposed to the very small amount remaining in the resuspended or detergent-extracted pellet. This was true of samples from controls and patients with lysosomal storage diseases. The protein concentration was determined according to the method of Lowry et al. (23).

Immunological techniques. Samples of the supernatant fractions were subjected to rocket immunoelectrophoresis as described (19, 20). Between 5 and 28 μg of protein in 1 μl from the extracts of liver and 5 to 15 μg of protein in 1 μl from the extracts of brain from patients were placed in wells in antiserum-containing gels along with known amounts of pure activator. Because of the low concentration of CRM in samples from control brain, these supernatant fractions were lyophilized and redissolved in a smaller volume of distilled water. Between 30 and 40 μg of protein in 1 μl was placed in the wells. After electrophoresis at 90 V for 2½ h, the washing and staining procedures of Emmett and Crowle were followed (20). The height of the loops from the tissue extracts were compared with the height of the loops from known amounts of pure activator protein. The amount of activator protein in tissues was expressed as micrograms CRM per milligram protein in the tissue extract supernatant.

RESULTS

In Table I, the patients used in this study are presented. They include patients with a primary defect in sphingolipid metabolism and some patients with evidence of sphingolipid storage secondary to another primary genetic defect. In Table I, we also list the lipids identified as being found in increased levels in samples analyzed for activator protein. In some instances, the liver and brain samples were obtained from the same patient and in other instances only liver or brain was available. Time from death to autopsy and length of storage at −60°C did not appear to appreciably change the results obtained.

TABLE I
CRM in Extracts of Liver and Brain from Patients Used in this Study*

Initials of patients	Age at death	Liver	Brain
Disease name (major lipids stored in organs analyzed)			
Type 1 G_{M1} gangliosidosis (23050)‡ (G_{M1} ganglioside and G_{A1} in liver and brain)			
D.V.	13 mo	15.1	14.8
J.K.	18 mo	16.9	ND§
Type 2 G_{M1} gangliosidosis (23060) (G_{M1} ganglioside and G_{A1} in liver and brain, less than in 23050)			
M.T.	6½ yr	2.2	2.9
C.E.	3 yr	5.1	ND
M.J.	6 yr	5.1	3.0
Type A Niemann-Pick disease (25720) (Sphingomyelin and cholesterol in liver and sphingomyelin and G_{M2} and G_{M3} gangliosides in brain)			
A.S.	26 mo	10.7	3.8
S. fetus	20 wk	3.0	ND
Type B Niemann-Pick disease (Sphingomyelin in liver)			
H. fetus	19 wk	1.8	ND
G_{M2} gangliosidosis (B variant, Tay-Sachs disease, 27280) (G_{M2} ganglioside in liver and brain)			
X.Y.	36 mo	3.7	5.3
L.L.	4½ yr	ND	8.7
G_{M2} gangliosidosis (0 variant, Sandhoff disease, 26880) (G_{M2} ganglioside and G_{A2} in liver and brain)			
Y.Z.	30 mo	2.6	13.5
G_{M2} gangliosidosis (A^MB variant) (G_{M2} ganglioside in liver and brain, less than in 27280 and 26880)			
J.M.	8½ yr	0.59	0.86
Metachromatic leukodystrophy (25010) (Sulfatide in brain)			
S.H.	8 yr	3.4	2.3
J.P.	?	ND	5.2
Type 1 Gaucher disease (23080) (Glucosylceramide in liver)			
K.S.	51 yr	1.2	0.82
Type 2 Gaucher disease (23090) (Glucosylceramide in liver and brain)			
K.C.	32 mo	1.3	0.67
R.P.	13 mo	2.9	1.8
M.W.	20 mo	2.1	ND

TABLE I (*Continued*)

Disease name (major lipids stored in organs analyzed) Initials of patients	Age at death	Liver	Brain
I-Cell disease (Mucolipidosis II, 25250) (Lactosylceramide in liver)			
K.Z.	6½ yr	2.2	ND
Hurler syndrome (25280) (Lactosylceramide in liver and brain, G_{M2} and G_{M3} ganglioside in brain)			
M.R.	9½ yr	1.9	0.62
Hunter syndrome (30990) (Lactosylceramide and G_{M3} ganglioside in liver)			
M.S.	16 yr	2.2	ND
Krabbe disease (24520) (Galactosylceramide in certain regions of the brain)			
C.Y.	12 mo	1.3	ND
D.H.	17 mo	1.1	ND
M.G.	13 mo	2.2	2.4
M.P.	15 mo	ND	3.3
Controls Mean (*n*) ±SD		0.95 (7) ±0.18	0.25 (4) ±0.14

* Micrograms CRM per milligram protein in the extract.
‡ Disease number as given in McKusick, V. A. 1978. Mendelian Inheritance in Man. The Johns Hopkins University Press.
§ Not determined.

All extracts were initially subjected to Ouchterlony double immunodiffusion to see if any precipitin lines exhibited nonidentity with each other or the pure activator protein. Examples of double immunodiffusion are shown in Fig. 1. In all cases the precipitin lines fused, indicating antigenic identity between liver and brain samples from patients and controls and between pure activator proteins.

Using rocket immunoelectrophoresis, the concentration of the activator protein in the liver and brain extracts was estimated. The typical patterns obtained are demonstrated on Figs. 2 and 3. The peak heights obtained from the extracts were compared with the peak heights of known concentrations of pure activator protein. The results obtained for each sample are given in Table I. Samples from control human livers had a mean concentration of 0.95±0.18 μg CRM/mg protein in the supernatant. Control human brain had less CRM than liver with an average of 0.25±0.14 μg CRM/mg protein in the supernatant. It was easier to see the loops of precipitation in the control brains when the sample was concentrated 5 to 10 times. Very high levels of

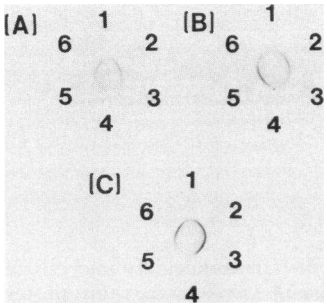

FIGURE 1 Ouchterlony double immunodiffusion of extracts of brain and liver from controls and patients and purified activator proteins from control and type 1 G_{M1} gangliosidosis livers. The center wells contained 10 μl of crude IgG fraction prepared from the immune serum. Six 10-μl samples were placed around each central well. The initials refer to patients listed in Table I and the number in parenthesis refers to micrograms protein in the 10-μl sample. (*A*) *1*, control liver (115); *2*, type 1 G_{M1} gangliosidosis, D.V. liver (18); *3*, type 2 G_{M1} gangliosidosis, C.E. liver (25); *4*, control brain (66); *5*, type 2 G_{M1} gangliosidosis, M.J. brain (38); *6*, type 1 G_{M1} gangliosidosis, D.V. brain (16). (*B*) *1*, same as 1 above; *2*, metachromatic leukodystrophy, S.H. liver (55); *3*, Tay-Sachs disease, X.Y. liver (36); *4*, same as 4 above; *5*, Tay-Sachs disease, L.L. brain (25); *6*, metachromatic leukodystrophy, S.H. brain (41). (*C*) *1*, same as 1 above; *2*, purified activator protein from control liver (0.38); *3*, purified activator protein from type 1 G_{M1} gangliosidosis liver (0.38); *4*, same as 4 above; *5*, type A Niemann-Pick disease, A.S. brain (16); *6*, type A Niemann-Pick disease, A.S. liver (16).

CRM were found in the extracts from liver samples from the patients with type 1 G_{M1} gangliosidosis and type A Niemann-Pick disease. Elevated concentrations were also measured in the liver samples from patients with type 2 G_{M1} gangliosidosis, metachromatic leukodystrophy, Tay-Sachs disease, Sandhoff disease, type 2

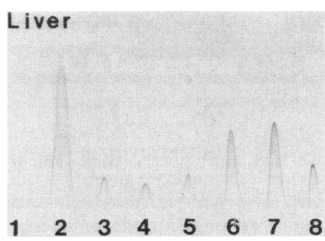

FIGURE 2 Rocket immunoelectrophoresis of extracts from liver samples and purified activator protein. All samples were applied in 1-μl aliquots and treated as described in Methods. The initials refer to patients in Table I and the numbers in parenthesis refer to the μg protein in the 1-μl sample. *1*, Control human liver (11.5); *2*, type 1 G_{M1} gangliosidosis, D.V. (9.0); *3*, type 2 G_{M1} gangliosidosis, C.E. (5.0); *4*, metachromatic leukodystrophy, S.H. (5.5); *5*, Tay-Sachs disease, X.Y. (7.1); *6*, type A Niemann-Pick disease, A.S. (7.5); *7*, purified activator protein (0.076); *8*, purified activator protein (0.038).

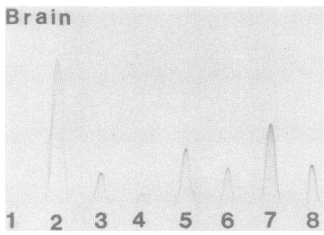

FIGURE 3 Rocket immunoelectrophoresis of extracts from brain samples and purified activator protein. Sample size and codes are the same as Fig. 2. *1*, Control human brain (6.6); *2*, type 1 G_{M1} gangliosidosis, D.V. (8.0); *3*, type 2 G_{M1} gangliosidosis, M.J. (7.6); *4*, metachromatic leukodystrophy, S.H. (4.1); *5*, Tay-Sachs diseases, L.L. (5.0); *6*, type A Niemann-Pick disease, A.S. (8.1); *7*, purified activator protein (0.076); *8*, purified activator protein (0.038).

Gaucher disease (2 out of 3), I-Cell disease, Hurler syndrome, Hunter syndrome, and Krabbe disease (1 out of 3). Near normal levels were measured in liver samples from patients with G_{M2} gangliosidosis (A^MB variant), type 1 Gaucher disease, type 2 Gaucher disease (1 out of 3), and Krabbe disease (2 out of 3). Liver samples from a fetus predicted and confirmed to have type A Niemann-Pick disease had a concentration more than three times above the mean for control liver and 1.6 times above the concentration measured in the liver from a fetus predicted and confirmed to have type B Niemann-Pick disease.

The results for the extracts from brain samples show an interesting pattern with the highest levels found in patients with type 1 G_{M1} gangliosidosis, Tay-Sachs disease, and Sandhoff disease. Significantly elevated concentrations were also found in extracts from patients with metachromatic leukodystrophy, type A Niemann-Pick disease, type 2 G_{M1} gangliosidosis, Krabbe disease, and type 2 Gaucher disease (1 out of 2). Near normal concentrations were found in extracts from G_{M2} gangliosidosis (A^MB variant), type 1 Gaucher disease, type 2 Gaucher disease (1 out of 2), and Hurler syndrome.

DISCUSSION

Using immunochemical techniques we have examined small brain and liver samples from controls and from patients with lysosomal storage disease for protein cross-reacting with antibodies to a sphingolipid activator protein. This activator protein was reported by Li and co-workers (4, 5) to activate the hydrolysis of G_{M1} ganglioside, trihexosylceramide, and G_{M2} ganglioside in the presence of the specific lysosomal hydrolase. Recent studies in our laboratory (11) provide evidence that this activator protein also activates sulfatide hydrolysis by arylsulfatase A, and that at least one patient has a genetic disease caused by the lack of this activator protein. This indicates that this activator protein is identical to the one originally described by Mehl and Jatzkewitz (1). Other studies in this laboratory have demonstrated that this activator protein binds certain sphingolipids, namely, G_{M1} ganglioside, G_{M2} ganglioside, G_{M3} ganglioside, sulfatide, sphingomyelin, and G_{A1}.[2] Although it has the ability to bind specific sphingolipids, it only activates the enzymatic hydrolysis of a few of these substrates in the presence of the specific hydrolase. Hydrolysis took place when G_{M1} β-galactosidase was added to G_{A1} or G_{M1} bound to this activator protein. The same occurred when purified arylsulfatase A was added to the sulfatide-activator complex. On the other hand, no reaction occurred when purified β-hexosaminidase A was added to a mixture of G_{M2} ganglioside and this activator protein. G_{M2} ganglioside can be hydrolyzed by β-hexosaminidase A when a specific activator protein is present (6, 7).

Antibodies to the activator protein for G_{M1} ganglioside and sulfatide hydrolysis were raised in rabbits and they were determined to be monospecific by several criteria. There appears to be more than one form of this protein as evidenced by the finding of two isoelectric points (17) and multiple bands for the subunits on sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by immunoblotting. All bands are missing in the cells from the patient with a variant form of metachromatic leukodystrophy, indicating that they are probably coded for by a common gene and that our rabbit antibodies react with all of the molecular species of this protein. These antibodies gave only one loop of precipitation on rocket immunoelectrophoresis of all samples and therefore they could be used to estimate the concentration of CRM in small tissue samples.

The finding of an extremely high concentration of activator protein in the livers of patients with type 1 G_{M1} gangliosidosis agrees with our previous study, which required large tissue samples and time-consuming purification (17) (Table I). This high level of activator protein correlates with storage of G_{M1} ganglioside and G_{A1}, which accumulate because of the defect in a specific β-galactosidase. The liver samples from patients with a less severe form of the same disease (called type 2) have significantly lower levels of this activator protein. The high level of activator protein in type A Niemann-Pick disease liver reflects either the greatly increased concentration of sphingomyelin (10 times normal), which we have shown binds to this activator protein, or the proliferation of lysosomes due to the sphingomyelinase deficiency. However, livers from patients with Gaucher disease are also greatly enlarged owing to the lysosomal storage of glucosylceramide but the level of activator protein is not nearly

as high. Glucosylceramide does not bind to this activator protein.[2] Small but significant increases in activator protein were found in liver samples from patients with Tay-Sachs disease, Sandhoff disease, metachromatic leukodystrophy, I-Cell disease, and possibly Hurler and Hunter syndromes. This reflects the storage of gangliosides and sulfated and nonsulfated glycolipids primary or secondary to the enzymatic defect (Table I).

As expected, the extracts of brains from patients with type 1 G_{M1} gangliosidosis, Tay-Sachs disease, and Sandhoff disease had the highest levels of CRM. These diseases have the highest concentrations of G_{M1} and G_{M2} gangliosides, which are known to bind to this activator protein. It is interesting that the brain extract from the patient with G_{M2} gangliosidosis (A^MB variant) had near normal levels of this activator protein but elevated levels of the specific activator required for reaction between G_{M2} ganglioside and β-hexosaminidase A (14). The level of CRM in brain from the type A Niemann-Pick patient was elevated 15 times the normal level, which may reflect the increase in G_{M2} and G_{M3} gangliosides rather than sphingomyelin storage. Increased levels of this activator protein were found in the brains of the patients with metachromatic leukodystrophy. This activator protein binds sulfatide and stimulates its enzymatic hydrolysis by sulfatide sulfatase (16). Although galactosylceramide and glucosylceramide do not bind to this activator protein to any great degree, significantly elevated levels of CRM were found in brain samples from two Krabbe disease patients and three Gaucher disease patients. Both of these diseases result in specific pathological storage vesicles that are of lysosomal origin. This increase in lysosome number and size should require elevated levels of an activator protein necessary for the degradation of other brain sphingolipids.

The finding of increased concentrations of a protein known to bind certain lipids and to activate specific lysosomal hydrolases in extracts of brain and liver from patients with defined lysosomal storage diseases raises a number of questions regarding its role in the chemical-pathological changes observed in these patients. Is it elevated in response to a call for increased synthesis of the enzyme and activator required for hydrolysis? If the enzyme is defective in a given disease is the activator protein stored along with the undegraded substrate? Will excess activator protein bind other lipids, thereby preventing their efficient degradation by lysosomal enzymes? Could an excess of one activator protein compete with another activator protein for a given lipid? As more is learned about this and other activator proteins these and other questions will be answered.

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