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Uptake and degradation of virus-associated fluorescent sulfatide by skin fibroblasts

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A fluorescent derivative of cerebroside sulfate, pyrene-dodecanoyl sphingosylgalactosylsulfate (P12-CS) was incorporated into the envelope of vesicular stomatitis virus (VSV). When the P12-CS-containing virus was incubated for 24 h with skin fibroblasts, up to 40% of the sulfatide was located in the cells – a value at least 10 fold greater than that observed using sulfatide suspensions without virus. In a similar experiment, in which the 24 h 'pulse' was followed by a 48 h 'chase', about 15–20% of the virus-associated fluorescence was recovered in the skin fibroblasts. Of the latter, about one-third was present as desulfated degradation products of P12-CS. The high uptake and degradation of the virus-associated sulfatide by intact skin fibroblasts suggested that enveloped viruses could be used for introducing other lipids into cells. This could be utilized for studying lipid catabolism and diagnosing lipid storage disorders in intact living cells.

Cerebroside sulfate; Fluorescent sulfatide; Pyrene sulfitide; Receptor-mediated endocytosis; Sulfatide uptake; Intracellular sulfatide metabolism; Vesicular stomatitis virus

1. INTRODUCTION

Investigations of this laboratory, aimed at administering fatty acids and lipids into cultured cells and following their intracellular metabolism, utilized fluorescent fatty acids and lipids for this purpose [1–13].

Cerebroside sulfate (sulfatide) accumulates in patients with MLD, a lipidosis characterized by a deficiency of the lysosomal hydrolase arylsulfatase-A [14]. Because of the presence of a 'pseudodeficiency' of this enzyme which provides false negative results in in vitro assays, Kihara et al. administered radioactively labelled sulfatides into skin fibroblasts and analyzed the degradation of the sulfatide in the intact cells [15]. While cells derived from individuals with MLD pseudodeficiency degraded this compound at rates similar to those of normal cells, those from patients with MLD did not do so [16]. We synthesized a fluorescent derivative of cerebroside sulfate containing covalently linked P12-CS and in previous studies administered its dispersions or complexes with albumin into normal or MLD cells and followed its uptake and degradation [11,17]. Either of these modes of dispersion most probably resulted in initial incorporation of the sulfatide into the plasma membrane, from where they reached the lysosomes by as yet unclear and slow pathways. In this paper we describe the incorporation of pyrene-cerebroside sulfate into the envelope of VSV and application of receptor-mediated endocytic uptake of the virus for transporting the lipid into the lysosomal compartment of skin fibroblasts where it was degraded.

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Abbreviations: P12-CS, pyrene-dodecanoyl sphingosylgalactosyl-3-sulfate; VSV, vesicular stomatitis virus; BSA, bovine serum albumin; MLD, metachromatic leukodystrophy; E/M, excimer to monomer ratio, i.e. the ratio of fluorescence emission at 475 and 378 nm, respectively

2. EXPERIMENTAL

2.1. *Materials*

P12-CS was synthesized as described by Marchesini et al. [18]. Fatty acid-free bovine serum albumin was obtained from Sigma.

2.2. *Isolation of VSV*

Ten day fertilized hen eggs were infected with VSV, incubated for 4 days at 37°C and the allantoic fluid was loaded on a discontinuous gradient of sucrose (2 ml of 22% layered over 0.5 of 50%) in 100 mM NaCl and 10 mM Tris buffer, pH 7.5. After centrifuging 1 h at 25000 rpm in a SW-27 rotor, the 22% sucrose layer was collected, the virus again sedimented, dispersed in the above Tris-buffered NaCl solution, divided into several portions and stored at −70°C. Growing the virus on fertilized eggs rather than cultured cells resulted in a lesser infectivity [19] and prevented lysis of the skin fibroblasts.

2.3. *Dispersion of P12-CS*

(i) 30 nmol of P12-CS were dried in a glass tube, 150 μl saline (0.9% NaCl) were added and the tube was heated for 2 min at 90°C. After cooling, 150 μl saline was added and the tube was kept overnight at room temperature. (ii) 30 nmol P12-CS were dried in a glass tube and dissolved in 14 μl of dimethylsulfoxide. A solution of BSA (containing 15, 30 or 60 nmol to provide 0.5, 1.0, or 2.0 mol ratios of BSA to P12-CS, respectively) was added. After 15 min at 37°C the volume was brought to 300 μl with saline and the tube was kept overnight at room temperature.

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2.4. *Introduction of P12-CS into VSV membrane*

P12-CS (30 nmol in saline or complex with BSA) was incubated with a virus suspension (200 μg protein) in a final volume of 300 μl overnight at room temperature. The dispersion was transferred to ultra-clear centrifuge tubes of 250 μl (Beckman) and centrifuged in a Beckman Airfuge for 20 min at 100000 × g. The supernatant was collected and the sediment was dispersed in 100 μl of saline. An aliquot was diluted with 2 ml of saline and its fluorescence recorded using an excitation at 343 nm and emission at 378 nm (monomeric) or 475 nm (excimeric) in a Perkin-Elmer LS5 spectrofluorometer. Another aliquot was added to 2 ml of chloroform-methanol 1:1 (by vol.) and the fluorescence emission at 378 nm recorded. Control experiments using P12-CS dispersions or BSA-complexes, without virus, were processed by precisely the same procedure.

2.5. *Introduction of P12-CS into cells*

Skin fibroblasts were grown in 3 cm Petri dishes using minimal essential medium containing L-glutamine (200 mM), non-essential amino acids (1 mM) and supplemented with heat-denatured 10% fetal calf serum. The medium was removed and the supernatant or sediment (see above), were dispersed in 2 ml of serum-free medium. After 24 h at 37°C ('pulse') the medium was replaced with 2 ml medium containing 10% fetal calf serum and incubation continued for 48 hours more ('chase'). The cells were then dislodged by mild trypsinization and the trypsin neutralized with serum-containing medium. The cells were sedimented, washed 3 times with saline, dispersed in 2 ml of saline and the fluorescence emission was determined at 378 (monomeric) and 475 (excimeric) nm. The cells were resedimented and the lipids extracted for 5 min at 60°C with 2 ml of chloroform/methanol, 1:1 (v/v). After centrifuging, the fluorescence of the lipids in the organic solvent was read at an excitation and emission of 343 and 378 nm.

3. RESULTS AND DISCUSSION

Table 1 shows the degree of association of P12-CS with the sedimented virus, incubated overnight with P12-CS in saline or complexed to albumin. For comparison, data are also presented on P12-CS dispersions treated similarly but without virus. As shown in table 1, the virus carried with it 80% of the P12-CS dispersed in the medium and about 50% of BSA-precomplexed sulfatide; the corresponding values in the sediments of controls devoid of virus, were only 15% and 5%, respectively. Incubation of the aqueous dispersion of the sulfatide with virus resulted in an overall recovery of about 90%, while the corresponding value for the dispersion without virus was only 25%, suggesting an adsorption of the dispersed P12-CS to the walls of the tubes which was not removed by the aqueous medium but was nearly completely desorbed by the virus.

A comparison of the 'excimer to monomer' ratios (E/M, the ratio of the fluorescence emission at 475 to that at 378 nm), an indicator of the degree of aggregation of the pyrene-sulfatide in intact cells [20,21], suggests that the virus which had been incubated with a P12-CS dispersion might have adsorbed rather than incorporated the lipid into the viral envelope. Incubating a P12-CS–albumin complex having an excimer to monomer ratio of 0.45 with the virus, resulted in an increased E/M value of 1.2, suggesting a close packing of the P12-CS molecules in the envelope of the virus. The 47% recovery, in the virus, of the BSA-complexed P12-CS is equivalent to 14 nmol per 200 μg virus or 70 nmol per mg, a value probably high enough to make plausible the excimer to monomer ratio of 1.2.

When virus-associated P12-CS was incubated for 24 h with skin fibroblasts in medium without serum ('pulse'), about 40% of the P12-CS was recovered in the cells. Three experiments provided an average of 5 nmol per dish or more than 50 nmol per mg protein. The corresponding value for cells incubated with a suspension or BSA-complex of P12-CS was about 10 times smaller (data not shown). After washing the cells and replacing with fresh, serum-containing medium (chase) about 20% of the virus-associated fluorescence (i.e. 1.8 nmol) was recovered in the cells (table 2). The decrease during the 48 h 'chase' of the cell fluorescence from 5.75 to 1.8 nmol suggests lysosomal degradation

Table 1

Association of P12-CS with VSV

	Minus virus		Plus virus	
	% recovery	E/M	% recovery	E/M
P12-CS dispersion				
Sediment	15	15	80	2.4
Supernatant	10	6	6	0.6
P12-CS:BSA 2:1 (mol ratio)				
Sediment	5	0.45	47	1.2
Supernatant	93	0.8	30	0.5

P12-CS (30 nmnol), dispersed in 0.9% NaCl, or precomplexed with BSA, was incubated overnight in a volume of 0.3 ml without or with virus and the tubes were centrifuged at 100000 × g. The respective supernatants were collected, the sediments dispersed and their fluorescence recorded in a spectrofluorometer. The lipids were extracted with chloroform-methanol mixtures and the fluorescence again recorded. For details see section 2. E/M, 'excimer to monomer' ratio [20,21]

Table 2

Uptake of VSV-associated P12-CS by skin fibroblasts

Exp. no.	Virus-associated P12-CS		Cell-associated fluorescence	
	nmol	% of total	nmol	% of virus-associated
1	16.5	55	2.64	16
2	13.2	44	1.45	11
3	7.4	25	2.35	32
4	13.75	46	1.50	11
5	13.2	44	1.32	10
6	9.6	32	1.34	14
7	6.0	20	1.98	33
Average	11.4	38	1.80	18

P12-CS (30 nmol) was complexed with BSA at a mol ratio of 2:1 and incubated in a volume of 0.3 ml with virus overnight; the virus was sedimented, redispersed and a portion was taken for determination of virus-associated fluorescence. The remainder was incubated with normal skin fibroblasts for 72 h (24 h 'pulse' and 48 h 'chase'), the cells were collected and their associated fluorescence was recorded. For details see section 2

of the cell-associated P12-CS and elimination of about two-thirds of the fluorescent split products.

When the chloroform-methanol extracts of the cells were filtered through DEAE-Sephadex, undegraded sulfatide was adsorbed and the effluent contained the metabolic degradation products of CS in the lipid extract. In a typical experiment 12 nmol of virus-associated P12-CS was incubated with fibroblasts. After the 24 h 'pulse' and 48 h 'chase' periods, 1.3 nmol of fluorescent lipids were present in the cell extract; of these, about one-third were eluted as non-sulfated degradation products (data not shown).

The very high uptake, by skin fibroblasts of P12-CS, incorporated into the envelope of VSV-virus (about 50 nmol per mg protein in 24 h) suggests a receptor mediated endocytosic uptake [22] and transport of the sulfatide, via endocytic vesicles [23–25] into the cell lysosomes [24], where they were efficiently degraded. Experiments are now planned to shorten the 'pulse' and 'chase' periods and compare relative uptake and degradation by cells derived from normal individuals and patients with metachromatic leukodystrophy, which is characterized by absence of arylsulfatase A.

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