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A FLUORIMETRIC DETERMINATION OF THE ACTIVITY OF GLYCOLIPID TRANSFER PROTEIN AND SOME PROPERTIES OF THE PROTEIN PURIFIED FROM PIG BRAIN

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The fluorimetric method of Correa-Freire et al. (Correa-Freire, M.C., Barenholz, Y. and Thompson, T.E. (1982) Biochemistry 21, 1244–1248) to measure glucosylceramide transfer between phospholipid bilayers has been applied to the determination of the activity of glycolipid transfer protein purified from pig brain. The transfer of pyrene-labeled galactosylceramide (PyrGalCer) from donor to acceptor vesicles was measured by a decrease in the intensity ratio of eximer (E) to excited monomer (M). A sensitive determination of the glycolipid transfer activity is possible by the fluorimetric method without separation of the donor and acceptor vesicles. The newly developed fluorimetric assay of glycolipid transfer protein was used to study the effects of *N*-ethylmaleimide, $HgCl_2$ and sugars on the transfer activity. The treatment with *N*-ethylmaleimide inactivated the activity to about 40%. The activity was almost completely inactivated by the treatment with $HgCl_2$. Monosaccharides and methyl-α-D-glucoside had no inhibitory effect on the transfer activity. A marked and immediate drop of the *E*/*M* ratio was observed by the addition of glycolipid transfer protein to vesicles containing PyrGalCer at a protein-to-PyrGalCer molar ratio of 1.56:1. The result suggests a complex formation of glycolipid transfer protein with PyrGalCer.

Introduction

A glycolipid-specific lipid transfer protein was purified to apparent homogeneity from pig brain [1]. The protein facilitates the transfer of various glycosphingolipids and glyceroglycolipids between membranes in vitro. In the purification of the protein, we determined the glycolipid transfer activity by the measurement of translocation of [6-^{3}H]galactosylceramide (GalCer) from donor liposomes to acceptor liposomes [2]. In this assay and also in commonly used assay of phospholipid transfer proteins [3–9], separation of donor membranes from acceptor membranes is required for the determination of the transfer of radioisotope-labeled lipids.

In the last couple of years, fluorimetric methods were introduced in the measurement of phospholipid transfer activity [10–12]. These methods allow a sensitive determination of phospholipid transfer activity without separation of donor and acceptor vesicles. Correa-Freire et al. [13] determined the rate of spontaneous transfer of glucosylceramide between phospholipid bilayers by a fluorimetric method. In the present paper, this fluorimetric method was applied to the determination of galactosylceramide transfer reaction facilitated by glycolipid transfer protein. The assay is based on the principle described by Correa-Freire

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Abbreviations: GalCer, galactosylceramide; PyrGalCer, 1-*O*-(β-D-galactopyranosyl)-*N*-(10-1-pyrene-9-enedecanoyl)-*D*-*erythro*-sphingosine; *E*/*M* ratio, eximer/excited monomer ratio.

et al. [13] and briefly summarized below. We have used 1-*O*-(β-D-galactopyranosyl) – *N*-(10-1-pyrene-9-enedecanoyl)-*D-erythro*-sphingosine (PyrGalCer) as a galactosylceramide analogue incorporated into phosphatidylcholine donor vesicles. Transfer of PyrGalCer from donor vesicles to unlabeled acceptor vesicles results in the decrease in the local concentration of PyrGalCer. PyrGalCer can form an excited complex (eximer, E) between a PyrGalCer in the ground state and an excited monomer (M). The excited monomer M has a characteristic emission peak at 396 nm, while the eximer emits at 475 nm. The presence of the eximer depends on the local concentration of PyrGalCer. Therefore, transfer of PyrGalCer from donor to acceptor vesicles is reflected in a decrease of the observed E/M intensity ratio.

Materials and Methods

Materials. PyrGalCer and GalCer containing nonhydroxy fatty acids were purchased from Sigma Chemical Company (St. Louis). PyrGalCer was kept in chloroform/methanol (2 : 1, v/v) at −20°C in the dark. The concentration of PyrGalCer was estimated by the galactose determination [14]. Phosphatidylcholine from rat liver and dimannosyldiacylglycerol from *Micrococcus lysodeikticus* were prepared by the method previously described [9].

Preparation of lipid vesicles. Single-bilayer phospholipid vesicles prepared by the method of Batzri and Korn [15] were used in all the experiments presented in this paper. The vesicles were prepared by injecting an ethanolic solution of lipids into buffer A (0.15 M NaCl/10 mM sodium phosphate (pH 7.4)/1 mM EDTA/0.02% NaN_3) as described below. The vesicle preparation was dialyzed against buffer A at room temperature in order to remove ethanol. In the preparation of donor vesicles, a mixture (180 nmol) of PyrGalCer and rat-liver phosphatidylcholine, at a molar ratio of 6–7 : 94–93, dissolved in 40 μl ethanol was injected into 3 ml buffer A under stirring. Acceptor vesicles were prepared by injecting 2.1 μmol rat-liver phosphatidylcholine in 70 μl ethanol into 3 ml buffer A under stirring. In the preparation of concanavalin A-reactive acceptor vesicles, a mixture (2.35 μmol) of rat-liver phosphatidylcholine and dimannosyldiacylglycerol, at a molar ratio of 96 : 4, dissolved in 80 μl ethanol was injected into 3 ml buffer A under stirring.

Glycolipid transfer protein-facilitated PyrGalCer transfer from donor vesicles to concanavalin A-reactive acceptor vesicles. A mixture of donor vesicles (36 nmol lipids in 600 μl), concanavalin A-reactive acceptor vesicles (235 nmol lipids in 300 μl) and glycolipid transfer protein (2.18 μprotein in 20 μl) was incubated at 27°C for 60 min. The incubation was stopped by adding 25 μl of 0.1 M *N*-ethylmaleimide, and 200 μl of 14 mM $HgCl_2$ in this sequence. The concanvalin A-reactive vesicles were aggregated by adding 400 μl concanavalin A (20 mg/ml) and, after several hours at room temperature, separated from the donor vesicles by centrifugation [9]. The precipitates were washed twice with buffer A. Lipids in the precipitates were extracted by the method of Bligh and Dyer [16] and separated by thin-layer chromatography on a precoated silica-gel plate (Merck) with chloroform/methanol/water (65 : 25 : 4, v/v) as a developing solvent. The fluorescent lipids were located under ultraviolet illumination.

Fluorimetric assay of the activity of glycolipid transfer protein. The assay is based on the principle described by Correa-Freire et al. [13]. The transfer of PyrGalCer from the donor to acceptor vesicles was measured by a decrease of the E/M intensity ratio. The donor vesicles (250 μl) containing PyrGalCer and the acceptor vesicles (250 μl) made of phosphatidylcholine were mixed with 2.5 ml buffer A in a cuvette. This mixture gives a vesicle suspension with 63.3 nmol lipids/ml at an estimated donor-to-acceptor vesicle ratio of 1 : 11.7. The mixture was kept at 27°C for 30 min. Then the transfer reaction was started at 27°C by the addition of glycolipid transfer protein. The PyrGalCer emission spectra were recorded at suitable time intervals in the scanning range 370–600 nm. The excitation wavelength was kept constant at 345 nm. Excitation and emission slits were 10 nm. The cuvettes received the excitation light only during fluorescence measurements. The E (at 475 nm)/M (at 396 nm) intensity ratio was calculated from the recorded emission spectra. Fluorescence measurements were carried out at 27°C using a Shimadzu RF520 spectrofluorometer equipped with a thermostated cuvette holder.

The following equation presented by Roseman and Thompson [17] relates the observed values of E/M ratio to the concentration of PyrGalCer remaining in the donor vesicles during the transfer experiments:

$$\frac{E}{M} = \left[\left[C_D^2(C_{D_0} - C_D) + RC_D^2C_h + (C_{D_0} - C_D)^2(D_D + C_h)\right] \times \left[C_hC_D(C_{D_0} - C_D) + RC_h^2C_D + RC_h(C_{D_0} - C_D)(C_D + C_h)\right]^{-1}\right] \cdot \frac{E_{max}}{M_{max}}$$

Here C_{D_0} is the initial concentration of PyrGalCer in the donor vesicles, C_D is the PyrGalCer concentration in the vesicles at any time t, and R is the ratio of acceptor to donor vesicles. E_{max} is the eximer intensity that would be observed as the number of phospholipid molecules approaches zero, M_{max} is the monomer intensity observed as the number of phospholipid molecules approaches infinity, and C_h is the half-value concentration. The constants C_h and E_{max}/M_{max} were determined as described by Roseman and Thompson [17] and Correa-Freire et al. [13]. $C_h = 0.0640$ and $E_{max}/M_{max} = 0.6353$ for our system. C_D values were calculated from E/M ratio by using the equation shown above on a Sharp pocket computer PC-1245.

The transfer reaction was analyzed as a first-order process. The rate constant k was obtained from the following equation:

$$\ln\frac{C_{D_0} - C_{D_{eq}}}{C_D - C_{D_{eq}}} = kt$$

Here $C_{D_{eq}}$ is the PyrGalCer concentration in the donor vesicles at equilibrium. It is assumed that the flip-flop process (the transbilayer migration) of PyrGalCer in the single-bilayer vesicles is very slow. Therefore, we assign the value $C_{D_0}[0.65/(R + 1) + 0.35]$ for $C_{D_{eq}}$ on the assumption that the ratio of the area of the external to the internal surface of the vesicle wall is 1.857 [18]. The initial velocity of PyrGalCer transfer from the donor to acceptor vesicles is given by $0.65(R/(R + 1)) \cdot k[C_{D_0}]$.

Preparation of glycolipid transfer protein. The protein was purified from pig brain by a modification of the method described previously (Ref. 1, and Abe, A., Yamada, K. and Sasaki, T., unpublished data). The purified glycolipid transfer protein gave a single band with an estimated molecular weight of 22000 on gel electrophoresis in the presence of sodium dodecyl sulfate according to the method of Weber and Osborn [19]. Protein was determined by the method of Bensadoun and Weinstein [20].

Results and Discussion

Glycolipid transfer protein facilitates transfer of PyrGalCer, as measured by the separation of donor and acceptor vesicles

Donor vesicles containing 7 mol% PyrGalCer were incubated at 27°C for 60 min with concanavalin A-reactive acceptor vesicles in the absence or presence of glycolipid transfer protein. After the incubation, the two populations of vesicles were separated by aggregating the acceptor vesicles with concanavalin A [9]. Thin-layer chromatography of the lipids extracted from the acceptor vesicles indicates that the transfer of PyrGalCer to the acceptor vesicles occurred only in the presence of the transfer protein. The results indicate that the transfer protein is capable of transfering the GalCer analogue with *N*-pyrene-9-enedecanoyl moiety. This less strict specificity of glycolipid transfer protein on the hydrophobic portion of GalCer made it possible to apply the method of Correa-Freire et al. [13] to the determination of the activity of the transfer protein.

Fluorescence spectra of PyrGalCer in phosphatidylcholine vesicles and the dependence of E/M intensity ratio on the PyrGalCer concentration in the vesicles

In order to determine a PyrGalCer concentration range suitable for the transfer assay, the E/M ratio was measured as a function of the PyrGalCer concentration in single-bilayer vesicles of rat-liver phosphatidylcholine. The E/M ratio was linearly dependent on the PyrGalCer concentration up to 8 mol% (inset in Fig. 1). All the experiments in this paper were performed with the use of donor vesicles containing 6–7 mol% PyrGalCer.

The fluorescence spectra of PyrGalCer in rat-liver phosphatidylcholine vesicles are shown in Fig. 1. No noticeable change was found in the shape of the spectrum after addition of acceptor vesicles at a 12-fold excess of the donor vesicles (spectrum b in Fig. 1, E/M ratio = 0.6). Addition of glycolipid transfer protein (255 ng) and an incubation at 27°C for 60 min caused a marked increase in excited monomer (M, emission peak at 396 nm) and a large decrease in eximer (E, emission peak at 475 nm) (spectrum c in Fig. 1, E/M ratio = 0.2). The decrease in the E/M ratio indicates transfer of PyrGalCer from the donor to acceptor vesicles.

Fluorimetric assay of the activity of glycolipid transfer protein

Addition of 12-fold excess of unlabeled acceptor vesicles to the donor vesicles containing PyrGalCer induced a small decrease in the E/M ratio. This decrease ceased after about 15 min at 27°C, and then a new steady E/M ratio was established (Fig. 2A). Therefore, we routinely start the assay of transfer activity 30 min after the addition of the acceptor vesicles. Fig. 2A shows the time-course of changes of E/M ratio in the presence of various amounts of glycolipid transfer protein. The observed E/M ratio reflects the state of PyrGalCer molecules present in both donor vesicles and acceptor vesicles. Therefore, the change of PyrGalCer concentration in the donor vesicles was determined first by the method of Roseman and Thompson [17]. Then the rate constant of PyrGalCer transfer from the donor to acceptor vesicles was obtained from the slope of the lines shown in Fig. 2B which was obtained by

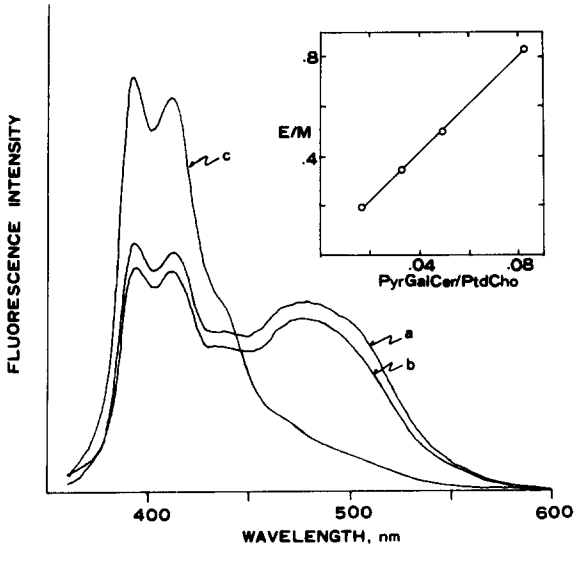

Fig. 1. Fluorescence spectra of PyrGalCer in rat-liver phosphatidylcholine vesicles and the dependence of E/M ratio on the PyrGalCer concentration in the vesicles. Emission spectra for the donor vesicles (12 nmol lipids), containing 7 mol% PyrGalCer, in 2.7 ml buffer A (a), a mixture of the donor vesicles and the acceptor vesicles (140 nmol phosphatidylcholine) in 2.9 ml buffer A (b), the donor and acceptor vesicles in 2.9 ml buffer A incubated at 27°C for 60 min in the presence of 255 ng protein of glycolipid transfer protein (c). The inset shows the dependence of the E/M intensity ratio on the PyrGalCer concentration in phosphatidylcholine (PtdCho) vesicles.

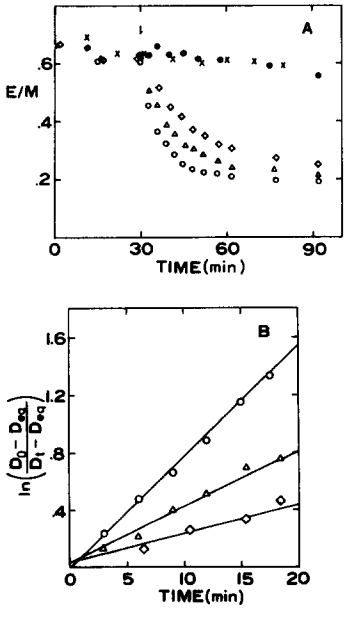

Fig. 2. Time-course of PyrGalCer transfer from the donor to acceptor vesicles, as measured by the decrease in the E/M ratio (A). The fluorimetric assay was performed as described in Materials and Methods in the absence (×) or presence of 80 ng (◇), 150 ng (△) and 300 ng (○) glycolipid transfer protein. In one assay (●), 300 μg of bovine serum albumin was added in place of the transfer protein. The proteins were added at the point indicated by an arrow. The concentration of PyrGalCer remaining in the donor vesicles during the transfer experiments, C_D, was calculated from E/M ratios as described in Materials and Methods. (B) was plotted by the use of this calculated values as described in Materials and Methods. Do, C_{D_0}; Dt, C_D; Deq, $C_{D_{eq}}$.

replotting the results in Fig. 2A. Under the conditions of the fluorimetric assay, it was found that the transfer rate of PyrGalCer was proportional to the amounts of glycolpid transfer protein at least from 80 to 300 ng protein per assay (Fig. 2B). The calculated initial velocity was 140 nmol of PyrGalCer transfer/min per mg protein. Bovine serum albumin added at 1000-fold excess of the transfer protein had no significant effect on the PyrGalCer transfer (Fig. 2A).

The results in Fig. 2 indicate that a convenient and sensitive determination of the activity of glycolipid transfer protein is possible by the use of the fluorimetric method. The use of fluorimetric methods in the assay of phospholipid transfer proteins has been described by Somerharju et al. [10] and Nichols and Pagano [12]. These two methods and the method described in this paper all use different properties of fluorescent lipid analogues in the determination of the transfer of the lipid analogues between vesicles. We found that the decrease of the E/M ratio is an accurate measure of the decrease in the PyrGalCer concentration in the plane of vesicular membrane.

Effects of N-ethylmaleimide, $HgCl_2$ and sugars on the activity of glycolipid transfer protein

Incubation of glycolipid transfer protein with 10 mM *N*-ethylmaleimide at 0°C for 30 min resulted in a decrease of the transfer activity to about 40% of the original activity (Fig. 3). This inactivation by *N*-ethylmaleimide had been found previously by the use of [^{3}H]GalCer transfer assay [1]. It has been our experience to find a decrease of the activity of glycolipid transfer protein after prolonged exposure of the protein to buffers without a SH-protecting reagent. Dithiothreitol had been added at 1 mM in all the buffers used in our procedures for the purification of glycolipid transfer protein from pig brain [1]. Addition of $HgCl_2$ to the assay mixture at 2.5 mM almost completely inactivated glycolipid transfer protein (Fig. 3). It was found that this inactivation by $HgCl_2$ is reversible. Addition of mercaptoethanol almost completely restored the activity of the transfer protein inactivated by $HgCl_2$-treatment. Additions of galactose, glucose, mannose and methyl-α-D-glucoside to the assay mixture at 5 mM had no effect on the activity of glycolipid transfer protein (data not shown). This result indicates that the binding of sugars to the transfer protein is not so strong. We interpret this observation to be related with the broad specificity of the

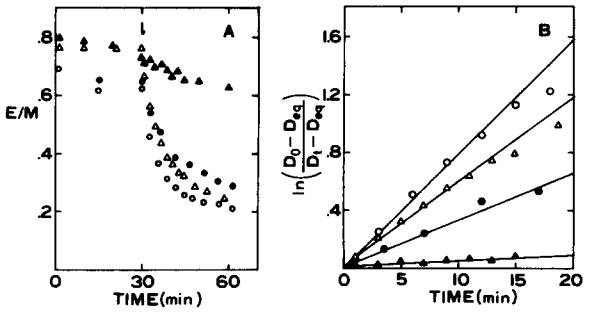

Fig. 3. Effects of *N*-ethylmaleimide and $HgCl_2$ on the activity of glycolipid transfer protein. The fluorimetric assay was performed as described in Materials and Methods in the presence of either 300 ng glycolipid transfer protein (○), 255 ng glycolipid transfer protein (△), 320 ng glycolipid transfer protein pretreated with 10 mM *N*-ethylmaleimide at 0°C for 30 min (●), or 255 ng glycolipid transfer protein and 2.5 mM $HgCl_2$ (▲). Results obtained from two paired experiments (○, ● and △, ▲) performed by the use of different vesicle preparations are shown. A, time-course of changes of E/M ratio; B, the data in A were replotted as described in the legend to Fig. 2B.

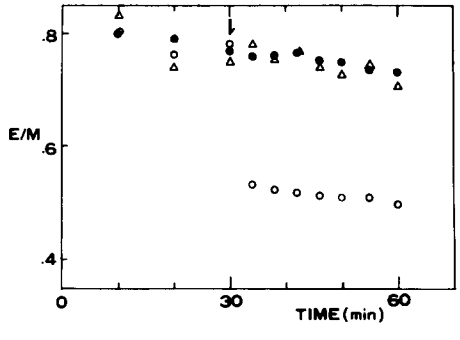

Fig. 4. Rapid decrease of the E/M ratio induced by the addition of glycolipid transfer protein to vesicles containing PyrGalCer: binding of PyrGalCer to the transfer protein. Either glycolipid transfer protein in buffer A (13.8 μg protein in 125 μl) (○), bovine serum albumin in buffer A (66 μg in 125 μl) (△), or buffer A (125 μl) (●) was added at the point indicated by an arrow to phosphatidylcholine vesicles (6.43 nmol lipids) containing 7 mol% PyrGalcer in 2.38 ml buffer A kept at 27°C. The mixtures were incubated at 27°C. Recording of emission spectra and calculation of the E/M ratio were done as described in Materials and Methods.

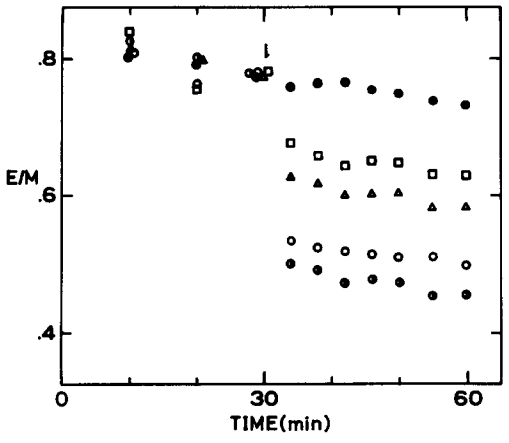

Fig. 5. Effect of various amounts of glycolipid transfer protein on the decrease of the E/M ratio, which is observed on addition of the transfer protein to vesicles containing PyrGalCer. The experiment was performed as described in the legend to Fig. 4, except that 3.3 μg (□), 6.6 μg (△), 13.8 μg (○) and 22 μg (◑) glycolipid transfer protein in 125 μl buffer A was added to the vesicles containing PyrGalCer at the point indicated by an arrow. To the control (●), 125 μl buffer A was added.

transfer protein toward the sugar moieties of glycolipids (Yamada, K. et al., unpublished data).

Binding of PyrGalCer to glycolipid transfer protein

A marked and immediate drop of the E/M ratio was observed by the addition of 13.8 μg (about 0.7 nmol) of glycolipid transfer protein to the vesicles containing 0.45 nmol PyrGalCer (Fig. 4). The decrease in E/M ratio has an increase in M and a decrease in E. Addition of 66 μg bovine serum albumin in place of the transfer protein had no effect on the ratio (Fig. 4). The decrease of the E/M ratio brought by the addition of the transfer protein was dependent on the amount of transfer protein (Fig. 5). The most simple interpretation of these results is that the transfer protein extracts PyrGalCer molecules from the vesicles and forms a complex with this molecules. A complex formation of glycolipid transfer protein with [^{3}H]GalCer had also been shown by the use of polyacrylamide gel electrophoresis at pH 4.3 (Abe, A., Yamada, K. and Sasaki, T., unpublished data). This complex is most probably an essential intermediate of the glycolipid transfer reaction facilitated by the protein.

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