Purification and Some Properties of the Glycolipid Transfer Protein from Pig Brain*

Akira Abe and Terukatsu Sasaki†

From the Department of Biochemistry, Sapporo Medical College, Sapporo 060, Japan

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A glycolipid-specific lipid transfer protein has been purified to apparent homogeneity from pig brain postmitochondrial supernatant. The purified protein was obtained after about 6,000-fold purification at a yield of 19%. Evidence for the homogeneity of the purified protein includes the following: (i) a single band in acidic gel electrophoresis, in sodium dodecyl sulfate-gel electrophoresis, (ii) a single band in analytical gel isoelectric focusing, (iii) exact correspondence between the glycolipid transfer activity and stained protein absorbance in the acidic gel electrophoresis, and (iv) coincidence between the transfer activity and protein absorption at 280 nm in gel filtration through Ultragel AcA 54. The protein has an isoelectric point of about 8.3 and a molecular weight of 22,000, as measured by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. A molecular weight of 15,000 was calculated from AcA 54 gel filtration. The amino acid composition has been determined. The protein binds [3H]galactosylceramide but not [3H]phosphatidylcholine. Under the conditions used, 1 mol of the transfer protein bound about 0.13 mol of [3H]galactosylceramide. The glycolipid transfer protein-[3H]galactosylceramide complex was isolated by a Sephadex G-75 chromatography. An incubation of the complex with liposomes resulted in the transfer of [3H]galactosylceramide from the complex to the acceptor liposomes. The result indicates that the complex functions as an intermediate in the glycolipid transfer reaction. The protein facilitates the transfer of [3H]galactosylceramide from donor liposomes to acceptor liposomes lacking in glycolipid as well as to acceptor liposomes containing galactosylceramide.

Proteins that facilitate the transfer of phospholipids between membranes in vitro have been found in animal cells, plant cells, yeast, and a Gram-negative bacterium (for reviews, see Refs. 1-3). The transfer of phospholipids between intracellular membranes is presumably mediated by the activity of phospholipid transfer proteins. Phospholipid transfer proteins different in their phospholipid specificities have been purified from bovine liver (4, 5), heart (6), and brain (7), rat liver (8-10) and hepatoma (11), yeast (12), and a bacterium (13).

The mechanism of intracellular translocation of glycosphingolipids in animal cells remains to be elucidated. It is possible that glycosphingolipids are transferred by a mechanism different from that found in phospholipids. Still, it is interesting to find proteins which facilitate the transfer of glycosphingolipids between membranes because such proteins may possibly be involved in the biosynthesis or degradation of glycosphingolipids. Metz and Radin (14) found in bovine spleen a protein which accelerates the transfer of glucosylceramide between erythrocytes and liposomes. The protein was partially purified (15). Blu and Zilversmit (16) showed that a nonspecific lipid transfer protein purified from bovine liver (5), which is identical to sterol carrier protein II (17, 18), accelerates the transfer of globoside and GM1 ganglioside from liposomes to erythrocyte ghosts.

A sensitive and specific determination of the glycolipid transfer activity is possible by a modified procedure of the phospholipid transfer assay which we previously described (19). By the use of this assay, we studied the glycolipid transfer activity present in the postmicrosomal supernatant of rat brain and liver (20, 21). We obtained a small amount of purified glycolipid transfer protein from pig brain (22). The purified protein facilitates the transfer of various glycosphingolipids and glycerolipids between membranes (23). However, the transfer of phosphatidylcholine, phosphatidylinositol, phosphatidylethanolamine, cholesterol, or cholesteryl oleate was not accelerated by the protein (23).

In this paper, we wish to present an improved purification procedure of pig brain glycolipid transfer protein. The molecular weight and the isoelectric point of the protein were estimated. Amino acid composition of the protein was determined. It was found that the protein binds [3H]galactosylceramide. The protein-galactosylceramide complex functions as an intermediate in the glycolipid transfer reaction.

EXPERIMENTAL PROCEDURES

Materials—Galactosylceramide containing nonhydroxy fatty acids was purchased from Sigma. The glycolipid was tritium-labeled (525 μCi/μmol) by the galactose oxidase/sodium borohydride method according to the procedure of Radin (24). [3H]Phosphatidylcholine (200 μCi/μmol) was prepared from cells of a human lymphoblastoid cell line, CCRF-CEM, grown in RPMI 1640 medium containing [9,10-3H]palmitic acid, which had been bound to fatty acid-free bovine serum albumin. Phosphatidylcholine was prepared from rat liver by the method described previously (19). [14C]Cholesteryl oleate (62.5 μCi/μmol) was prepared by a modification of the method of Metz and Radin (14).

The abbreviations and trivial names used are: GM1, IIα-N-acetyllactosaminoglucosylceramide; ConA, concanavalin A; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; GM2, IIβ,N-acetyllactosaminoglucosylceramide; Tened, N,N',N,N'-tetramethylethylenediamine. Portions of this paper (including part of "Experimental Procedures" and Figs. 1-4) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 80M-0280, cite the authors, and include a check or money order for $3.60 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.
Young et al. (25) on 15% acrylamide gels at pH 4.3, except (i) pre-electrophoresis of separating gel was conducted in 0.06 M KOH-acetic acid, pH 4.3, at 4 mA/tube for 2 h, (ii) stacking gel was prepared from a buffered monomer solution to which glycerol and 2-mercaptoethanol were added at a final concentration of 5% and 0.6 mM, respectively, (iii) stacking gel was polymerized by the addition of ammonium persulfate, and (iv) the electrophoresis was carried out for 2 h with 2 µA/tube.

When the glycolipid transfer activity was recovered from a polyacrylamide gel, two gels were loaded with the purified glycolipid transfer protein (6.2 µg of protein/gel) in Buffer A (10 mM sodium phosphate, pH 6.0, 1 mM dithiothreitol, and 0.02% NaN₃) (70 µl) at 25 °C in cylindrical 5% acrylamide gels (5 mm in diameter and 8 cm in length) with 10 mM sodium phosphate, pH 7.4, 1 mM dithiothreitol, and 0.02% NaN₃ in the anode and cathode compartments, respectively. The ratio of the anode and the other gel was sliced into 2-mm segments. Each gel slice was fragmented by a glass bar and soaked overnight in 0.5 ml of Buffer B (0.15 M NaCl, 10 mM sodium phosphate, pH 7.4, 1 mM dithiothreitol, and 0.02% NaN₃) at 4 °C. The samples were then centrifuged to remove the gel particles, and the galactosylceramide transfer activity was measured for each sample using 100 pCi/pmol, [6-3H]galactosylceramide (1.4 × 10⁶ dpm/nmol) at 50 °C for 20 h. The samples were mixed with 5 ml of toluene scintillation medium containing 33% Triton X-100 (28) and radioactivity was determined.

In experiments of [3H]phosphatidylcholine binding to the glycolipid transfer protein, liposomes were prepared by sonication from a lipid mixture containing phosphatidylcholine, dicetyl phosphate, and cholesterol in a molar ratio of 86:5:45. The liposomes were incubated with the glycolipid transfer protein or with the glycolipid transfer protein-[3H]galactosylceramide complex as described in the legends to Figs. 10 and 12. Each incubated mixture was subjected to gel electrophoresis, and the gels were processed as described above.

### Table 1

<table>
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<th>Step</th>
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<th>Activity*</th>
<th>Recovery</th>
<th>Specific activity</th>
<th>Purification factor</th>
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<td>82,375</td>
<td>545.3³</td>
<td>100</td>
<td>0.00620</td>
<td>1</td>
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<tr>
<td>(NH₄)₂SO₄ precipitation</td>
<td>670</td>
<td>26,670</td>
<td>786.8</td>
<td>144</td>
<td>0.0275</td>
<td>4.2</td>
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<td>4,242</td>
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<td>62</td>
<td>0.080</td>
<td>12.1</td>
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<td>367.6</td>
<td>67</td>
<td>0.945</td>
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<tr>
<td>Sephadex G-75</td>
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<td>64</td>
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<td>37</td>
<td>3.11</td>
<td>470</td>
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<tr>
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<td>155.4</td>
<td>28</td>
<td>34.3</td>
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<tr>
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<td>2.6</td>
<td>105.6</td>
<td>19</td>
<td>40.6</td>
<td>6,133</td>
</tr>
</tbody>
</table>

*Galactosylceramide transfer activity.

In order to obtain figures in this row, the postmitochondrial supernatant was centrifuged at 100,000 × g for 60 min and the protein concentration and the specific activity of the membrane-free supernatant were determined.

*This figure is exceptionally small probably due to a partial inactivation of the activity during the storage of a sample for the assay. In most preparative runs, the recovery of the transfer activity in Step 2 was 70-90% of the activity in Step 1.
RESULTS

Purification of Glycolipid Transfer Protein from Pig Brain—Table I summarizes the results of the purification procedure for pig brain glycolipid transfer protein. A seven-step procedure was employed and the transfer activity was assayed by the galactosylceramide transfer throughout the purification.

The postmitochondrial supernatant was fractionated with ammonium sulfate. The protein precipitated by 35–70% ammonium sulfate was applied to a phosphocellulose column, and the column was eluted by a stepwise gradient. A Cm-cellulose column was utilized in Step 4. This step produced a purification factor of about 12-fold. The fifth step employed a Sephadex G-75 gel filtration column, which produced an additional 3.3-fold purification. In the sixth step phenyl-Sepharose CL-4B column was utilized. Addition of 50% ethylene glycol to the elution buffer was required to elute the transfer activity from the column. This step produced an additional 11-fold purification. The final step was a chromatography on a phosphocellulose column, from which purified glycolipid transfer protein was eluted at about 98 mm NaCl. The resulting purified protein was about 6000-fold purified as compared with the membrane-free supernatant. The yield from 44 brains (3950 g) was 2.6 mg with a specific activity of 40.6 nmol/min/mg of protein under the conditions of the [3H]galactosylceramide transfer assay. The overall recovery of the activity amounted to 15%.

Purity and Estimation of Molecular Weight and Isoelectric Point—When each fraction in the second phosphocellulose chromatography (Step 7) was analyzed by SDS-polyacrylamide gel electrophoresis using the Laemmli gel system (26), a single band was observed in all the fractions (fractions 84–93 in Fig. 4) under the peak of the glycolipid transfer activity. The purified protein moved as a single band on polyacrylamide gel electrophoresis at pH 4.3 (Fig. 5). Fig. 5 also shows the recovery of the glycolipid transfer activity from a gel after the gel electrophoresis in acidic buffer. A precise correspondence was observed between the absorbance of the single protein band and the transfer activity. An analytical gel isoelectric focusing experiment also showed a single band with a pH equal to about 8.3 (Fig. 6).

The purified protein was subjected to molecular sieve chromatography on a column of Ultrogel AcA 54. The results of this procedure showed coincident profiles of protein and the glycolipid transfer activity (Fig. 7). When the same column was calibrated with several proteins of known molecular weight, the elution behavior of the glycolipid transfer protein
Glycolipid Transfer Protein

Fig. 8. SDS-polyacrylamide gel electrophoresis of the glycolipid transfer protein. Electrophoresis on 12.5% acrylamide gels in the presence of 0.1% SDS was performed by the method of Weber and Osborn (27). The ratio of acrylamide and bisacrylamide was 37.5 in the monomer solution used to prepare the gel. Electrophoresis was performed at 3.33 mA/tube for 0.7 h and then at 6.7 mA/tube for 3.5 h. Purified glycolipid transfer protein (13.4 μg) was applied to the gel shown in b. Molecular weight standards and the glycolipid transfer protein were applied to the gel shown in a. Arrow indicates the transfer protein. Molecular weight standards are bovine serum albumin (68,000), ovalbumin (43,000), chymotrypsinogen A (25,000), myoglobin (17,500), and cytochrome c (12,500).

indicates a molecular weight of 15,000 (Fig. 7). Furthermore, electrophoresis on 12.5% acrylamide gels in the presence of 0.1% SDS according to the method of Weber and Osborn (27) revealed one single band (Fig. 8). Calculation of the molecular weight by this technique gives a value of 22,000 (Fig. 8).

Amino Acid Analysis—The amino acid composition (Table II) indicates that the glycolipid transfer protein is rich in glutamate (including glutamine), leucine, alanine, lysine, and aspartate (including asparagine). No hexosamine was detected by the amino acid analysis. The amino acid composition differs from the compositions of phosphatidylcholine-transfer proteins from bovine liver (4, 30) and from rat liver (10) and of nonspecific lipid transfer proteins from bovine liver (5), and from rat liver (9, 17, 18, 31). The percentage of amino acids with nonpolar side chains, which include proline, alanine, valine, methionine, isoleucine, leucine, and phenylalanine, is 46.3% in the glycolipid transfer protein. In this calculation, it is assumed that the transfer protein does not contain half-cystine. Tryptophan is not included in the calculation because the number of tryptophan residue in the protein is not yet determined. The percentage obtained on the glycolipid transfer protein is larger than the values calculated for other lipid transfer proteins, which have been counted as proteins high in the proportion of the amino acids with nonpolar side chains.

\[^{[H]}\text{Galactosylceramide Binding to the Glycolipid Transfer Protein}\]—Binding of \[^{[H]}\text{galactosylceramide}\] to the purified glycolipid transfer protein was examined by gel electrophoresis at pH 4.3. A complex between the glycolipid transfer protein and \[^{[H]}\text{galactosylceramide}\] was found when an incubation mixture containing the transfer protein and liposomes was subjected to the electrophoresis (Fig. 9A); the liposomes were prepared from a lipid mixture containing phosphatidylcholine, dicetyl phosphate, cholesterol, and \[^{[H]}\text{galactosylceramide}\] in molar ratio of 86:5:45:5. About 30 pmol of \[^{[H]}\text{galactosylceramide}\] was located at the position of the transfer protein, whose quantity was about 230 pmol if the molecular weight of the protein is assumed to be 20,000. Therefore, it was estimated that about 13% of the transfer protein contained bound \[^{[H]}\text{galactosylceramide}\] under the conditions used in this experiment. A tailing of \[^{[H]}\text{galactosylceramide}\] in the gel from the protein band towards anode probably implies dissociation of the \(^3\text{H}\) lipid from the transfer protein during the electrophoresis. Addition of \(^N\text{ethylmaleimide}\) to the incubation mixture at 4.2 mM resulted in a 35% reduction of the bound \[^{[H]}\text{galactosylceramide}\] (Fig. 9A). The rate of galactosylceramide transfer facilitated by the transfer protein was also reduced to about one-half by the addition of \(^N\text{ethylmaleimide}\) to the assay mixture at 4.2 mM (data not shown). The inhibition of the transfer activity by \(^N\text{ethylmaleimide}\) had previously been reported (21, 25).

When liposomes prepared from a lipid mixture containing \[^{[H]}\text{phosphatidylcholine}\], dicetyl phosphate, and cholesterol were used in the incubation with the glycolipid transfer protein, no complex between \[^{[H]}\text{phosphatidylcholine}\] and the transfer protein was found (Fig. 9B); the appearance of \(^3\text{H}\) radioactivity near the tracking dye was not dependent on the addition of the transfer protein to the incubation mixture (Fig. 9B), and this \(^3\text{H}\) peak probably represents \[^{[H]}\text{phosphatidylcholine}\] monomer. In the experiment shown in Fig. 9B, the specific radioactivity of the liposomal \[^{[H]}\text{phosphatidylcholine}\] was adjusted to the same value as that of the liposomal \[^{[H]}\text{galactosylceramide}\] used in

\begin{table}[h]
\centering
\caption{Amino acid composition of purified glycolipid transfer protein}
\small
\begin{tabular}{|l|c|}
\hline
Amino acid & mol % \\
\hline
Aspartic acid & 8.79 \\
Threonine & 5.12 \\
Serine & 2.86 \\
Glutamic acid & 11.67 \\
Proline & 6.09 \\
Glycine & 4.54 \\
Alanine & 9.50 \\
Half-cystine plus cysteine & 1.31 \\
Valine & 6.21 \\
Cysteine & 2.22 \\
Isoleucine & 4.95 \\
Leucine & 11.50 \\
Tyrosine & 3.90 \\
Phenylalanine & 4.81 \\
Histidine & 1.90 \\
Lysine & 9.38 \\
Arginine & 2.96 \\
Tryptophan & 4.4 \\
\hline
\end{tabular}
\label{table2}
\end{table}

\(^a\) Amide content has not been determined.
\(^b\) Tryptophan was detected by fluorescence spectroscopy. An excitation at 280 nm gave an emission maximum of 342 nm. The UV absorption spectrum of the protein exhibits a shoulder at 290 nm, suggesting the presence of tryptophan.

The data is the average value of two analyses of one protein preparation.


the experiments shown in Fig. 9A. The results shown in Fig. 9 are in good agreement with the lipid specificity of the transfer reaction accelerated by the glycolipid transfer protein (22, 23).

The experiments described under "Experimental Procedures." Liposomes containing either [3H]galactosylceramide or [3H]phosphatidylcholine were incubated at 25°C for 1 h in the presence or in the absence of the glycolipid transfer protein. Portions of the incubation mixture, containing 4.6 μg of protein when the protein had been added to the incubation mixture, were subjected to electrophoresis at pH 4.3 on 15% acrylamide gels. The procedures for staining, slicing, and analyzing for radioactivity are described under "Experimental Procedures." A, [3H]galactosylceramide binding to the glycolipid transfer protein. B, experiments on [3H]phosphatidylcholine binding to the glycolipid transfer protein. The experiments determined under "Experimental Procedures". Liposomes containing either [3H]galactosylceramide or [3H]phosphatidylcholine were incubated at 25°C for 1 h in the presence or in the absence of the glycolipid transfer protein. Portions of the incubation mixture, containing 4.6 μg of protein when the protein had been added to the incubation mixture, were subjected to electrophoresis at pH 4.3 on 15% acrylamide gels. The procedures for staining, slicing, and analyzing for radioactivity are described under "Experimental Procedures." A, [3H]galactosylceramide binding to the glycolipid transfer protein. B, experiments on [3H]phosphatidylcholine binding to the glycolipid transfer protein. The experiments determined under "Experimental Procedures".

The experiments shown in Fig. 9A. The results shown in Fig. 9 are in good agreement with the lipid specificity of the transfer reaction accelerated by the glycolipid transfer protein (22, 23).

Isolation of the Glycolipid Transfer Protein-[3H]Galactosylceramide Complex—The complex between [3H]galactosylceramide and the glycolipid transfer protein was isolated by a Sephadex G-75 chromatography of the incubated mixture of the protein and liposomes containing [3H]galactosylceramide (Fig. 10). The liposomes were eluted at the void volume (fractions 18–22 in Fig. 10) and did not contain protein (Fig. 11). A small amount of radioactivity corresponding to 0.46 nmol of [3H]galactosylceramide was eluted at the position of the protein, which was located both by the absorption at 280 nm and by gel electrophoresis (Figs. 10 and 11). The optical density indicates that about 4 nmol of the glycolipid transfer protein is present in this peak if we assume the molecular weight of this protein to be 20,000. This estimation was made based on the data that the optical density at 280 nm of a solution (100 μg of protein/ml) of the glycolipid transfer protein is 0.128 cm. Therefore, about 12% of the glycolipid transfer protein contained bound [3H]galactosylceramide. It was found that the appearance of the small radioactive peak at around fraction 34 was totally dependent on the presence of the transfer protein in the applied material. The glycolipid transfer activity was found in the fractions around number 34 but not in the fractions at the void volume (Fig. 10). The material with absorption at 280 nm at around fraction 57 in Fig. 10 represents oxidized dithiothreitol present in the applied incubation mixture. In the Sephadex G-75 chromatography shown in Fig. 10, about 7 nmol of the glycolipid transfer protein was applied to the column but only 4 nmol of the protein was recovered from the column. SDS-gel electrophoresis shown in Fig. 11 indicates that all the protein eluted from the column is present in the fractions around number 34 where 3H radioactivity, material with absorption at 280 nm, and the glycolipid transfer activity were coeluted (Fig. 10). Low recovery of the transfer protein from the column is due to the adsorption of the protein to the column.

Participation of the Glycolipid Transfer Protein-[3H]Galactosylceramide Complex as Intermediate in Glycolipid Transfer Reaction—An incubation of the glycolipid transfer protein-[3H]galactosylceramide complex with liposomes resulted in
the transfer of \[^{3}H\]galactosylceramide from the complex to the acceptor liposomes (Fig. 12). When the complex was incubated with liposomes lacking in glycolipids, about 46% of the \[^{3}H\]galactosylceramide originally present as a complex with the transfer protein was transferred to the liposomes, which had been prepared from phosphatidylcholine, cholesterol, and dicetyl phosphate (Fig. 12A). About 54% of the \[^{3}H\]galactosylceramide remained bound to the transfer protein after incubation of the complex with the liposomes for 1 h at 20 °C (Fig. 12A). When the glycolipid transfer protein-\[^{3}H\]galactosylceramide complex was incubated with liposomes containing galactosylceramide, almost all the \[^{3}H\]galactosylceramide originally present as the complex was transferred to the liposomes during the incubation (Fig. 12B). In this experiment, the incubation mixture contained liposomal galactosylceramide in about 300-fold excess of the \[^{3}H\]galactosylceramide added as the complex. Therefore, the result in Fig. 12B suggests an extensive mixing of the protein-bound \[^{3}H\]galactosylceramide with the liposomal galactosylceramide. The results in Fig. 12 indicate that the glycolipid transfer protein-glycolipid complex functions as the intermediate in the glycolipid transfer reaction. The use of different acceptor liposomes with or without galactosylceramide resulted in a marked difference in the amount of the \[^{3}H\]galactosylceramide eventually transferred from the complex to the acceptor liposomes at an equilibrium point.

Glycolipid Transfer Protein-facilitated Transfer of Galactosylceramide from Donor Liposomes to Acceptor Liposomes either Containing or Lacking in Galactosylceramide—The glycolipid transfer protein facilitated the transfer of \[^{3}H\]galactosylceramide from donor liposomes to acceptor liposomes lacking in glycolipids as well as to acceptor liposomes containing galactosylceramide at the same molar ratio as in the donor liposomes. The extents of \[^{3}H\]galactosylceramide transfer from the donor to acceptor liposomes approached the same equilibrium point for both glycolipid-containing and glycolipid-deficient acceptor liposomes. The results indicate that the transfer reaction to both acceptor liposomes proceeds in the same way up to the point where \[^{3}H\]galactosylceramide evenly distributes on the surfaces of the donor and acceptor liposomes.

DISCUSSION

In a purification of glycolipid transfer protein, we used pig brain as a starting material because it had been found that the specific activity of the glycolipid transfer reaction facilitated by the membrane-free supernatant of rat brain was about 10 times higher than the specific activity of the reaction facilitated by rat liver supernatant (21). Metz and Radin (15) reported partial purification and properties of a cerbroside transfer protein from bovine spleen which is similar to but not identical with the glycolipid transfer protein from pig
phosphatidylcholine or cholesterol (15, 22, 23). Metz and protein from bovine spleen at a yield of 2%. Their purest colipid transfer as described under "Experimental Procedures." In membranes, but are not active in facilitating the transfer of Radin (15) obtained partially purified cerebroside transfer analyzed by SDS-gel electrophoresis (15). They found that complex (a portion of fractions choline)

Buffer actosylceramide complex (a portion of fractions galactosylceramide-containing liposomes (715 nmol as phosphatidylcholine) were incubated at 20 °C for 1 h in 2.0 ml of Buffer B. In the glycolipid transfer protein-[H]galactosylceramide complex (a portion of fractions 33-36 in Fig. 10, 19,000 dpm) and galactosylceramide-containing liposomes (715 nmol as phosphatidylcholine) were incubated at 20 °C for 1 h in 2.1 ml of Buffer B. The incubated mixtures were subjected to Sephadex G-75 chromatography as described under "Experimental Procedures." In A, the glycolipid transfer activity was found in fraction 33 but not in fraction 19.

brain. Similarities between the pig brain protein and the bovine spleen protein include the following properties: (i) the molecular weights are similar, for the pig brain protein, 22,000 (SDS-gel electrophoresis) and 15,000 (gel filtration), and for the bovine spleen protein, 20,300 (gel filtration); (ii) the isoelectric points are similar, for the pig brain protein, about 8.3, and for the bovine spleen protein, about 8.8 and 9.1; and (iii) both are active in facilitating the transfer of galactosylceramide, glucosylceramide, and lactosylceramide between membranes, but are not active in facilitating the transfer of phosphatidylcholine or cholesterol (15, 22, 23). Metz and Radin (15) obtained partially purified cerebroside transfer protein from bovine spleen at a yield of 2%. Their purest preparation still contained several protein components when analyzed by SDS-gel electrophoresis (15). They found that the activity of cerebroside transfer protein was unstable during the last two steps of their purification procedure. We had no problem with the stability of the activity of pig brain transfer protein during the purification procedure described in this paper. Moreover, the purified transfer protein retained its original activity for at least 4 months when stored at 0 °C in Buffer A containing 0.1 M NaCl at a protein concentration of 0.1 mg/ml. The purity of the glycolipid transfer protein prepared by the procedure described in this paper has been assessed by gel electrophoresis in acidic buffer, by gel filtration on Ultrogel AcA 54, by gel isoelectric focusing, and by SDS-gel electrophoresis (Figs. 5–8). All the results indicate that the protein has been purified to homogeneity.

The molecular weight of pig brain transfer protein estimated by SDS-gel electrophoresis was significantly larger than the molecular weight estimated by molecular sieve chromatography (Figs. 7 and 8). At present, we don't know the reason for the discrepancy of the estimated molecular weights of the protein. One possibility is the presence of covalently bound carbohydrate in the protein. However, no hexosamine was found by the amino acid analysis. The glycolipid transfer protein contains amino acids with nonpolar side chains at a high proportion (Table II), which suggests the presence of a hydrophobic binding site for glycolipids in the protein. The presence of a highly hydrophobic region in the transfer protein may account for the effectiveness of phenyl-Sepharose chromatography in the purification of the transfer protein.

Conzelmann et al. (32) reported that an activator protein of lysosomal β-hexosaminidase A has an activity to facilitate the transfer of G_{M2} and other glycosphingolipids from donor to acceptor liposomes. The glycolipid transfer protein we purified is most likely different from the activator protein. The pig brain protein has a pI of 8.3 whereas the activator protein has a pI of 4.8. The activator protein accelerates the transfer of G_{M2} much faster than that of asialo-G_{M2}, whereas the rate of lactosylceramide and globotriaosylceramide transfer facilitated by the pig brain protein is faster than the rate of G_{M2} transfer (23). It seems important to determine the intracellular localization of the glycolipid transfer protein, i.e., whether the glycolipid transfer protein is a cytoplasmic protein, a lysosomal protein, or a protein in the Golgi complex.

The glycolipid transfer protein does not facilitate the transfer of phosphatidylcholine, phosphatidylinositol, cholesterol, or dimannosyldiacylglycerol between membranes (22, 23). Therefore, galactosylceramide is the only one constituent of the liposomes used in this paper which can be transferred by the glycolipid transfer protein. Nevertheless, the transfer protein facilitates the transfer of [H]galactosylceramide from donor liposomes to acceptor liposomes lacking in glycolipid. The results are consistent with the capability of the glycolipid transfer protein to facilitate a net mass transfer of glycolipid molecules from donor to acceptor membranes. The net mass transfer of galactosylceramide facilitated by the glycolipid transfer protein was shown by the use of monomolecular lipid film spread at the air-water interface (33).

A complex formation of the glycolipid transfer protein with pyrene-labeled galactosylceramide was indicated by fluorescence measurements of a mixture of the protein and liposomes containing the fluorescent glycolipid analogue (29). The results shown in Figs. 9 and 10 directly proved that the glycolipid transfer protein binds [H]galactosylceramide. The lack of [H]phosphatidylcholine binding to the transfer protein (Fig. 9) is in good agreement with the specificity of the transfer reaction accelerated by the glycolipid transfer protein (23). Under the conditions used in Figs. 9 and 10, 1 mol of the transfer protein bound 0.12–0.13 mol of [H]galactosylcer-
amidine. Although it may be possible to find $[^1]H$galactosylceramide-containing lipid film which will load a larger portion of the transfer protein with $[^1]H$galactosylceramide (33), we take it significant to find a large portion of the transfer protein presumably in a lipid-free state. The results presented in Fig. 12 indicate that the transfer protein-glycolipid complex functions as the intermediate in the glycolipid transfer reaction. Most likely, the intermediate is the transfer protein containing 1 mol of bound glycolipid/mol of the protein as is the case with phosphatidylcholine-specific transfer protein purified from bovine liver (4, 34). Then, in order to perform a net mass transfer of a glycolipid molecule from the donor to acceptor liposomes, the transfer protein carrying a glycolipid monomer must unload the bound glycolipid onto an acceptor liposome. Thus the glycolipid transfer protein without a bound lipid probably represents a state of the protein after net mass transfer of a glycolipid molecule to the acceptor membrane.

Wong et al. (38) showed an association of partially purified glycolipid transfer protein from bovine brain with phosphatidylcholine/$[^1]H$glucosylceramide vesicles. We did not find any such binding of the purified glycolipid transfer protein to liposomes (Figs. 10 and 11). The significance of the results reported by Wong et al. (38) in considering the transfer mechanism by the glycolipid transfer protein is questionable in the light of the fact that the purity of the glycolipid transfer protein used in their experiments was too low to study the specific interaction of the transfer protein with glycosylceramide. They found that 30–40% of the protein present in the partially purified glycolipid transfer protein coeluted with the vesicle. The value is too large because the purification procedure and data reported by Wong et al. (38) suggest to us that the purity of their preparation is at most 10%. It is not possible to find the glycolipid transfer protein—$[^1]H$glycolipid complex by the use of a transfer protein preparation with low purity, which was the case in the experiments reported by Wong et al. (38). The glycolipid transfer protein is a basic protein and tends to form nonspecific complexes. It is important to study the complex formation between the glycolipid transfer protein and lipids by the use of a pure protein.

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REFERENCES

Step 4: G-75 Column Chromatography — Ammonium sulfate (60.3 g/100 ml) was added to 95% saturation in order to precipitate all protein. The solution was allowed to stand overnight and then centrifuged at 30,000 x g for 10 min. The precipitate was dissolved in 40 ml of buffer A. After dialysis against 4:1 liter of the same buffer, the solution was centrifuged at 72,400 x g for 30 min to remove the small amount of insoluble materials. The supernatant was applied to the Phenyl-Sepharose column. The column was washed with 1.6 liters of the buffer and eluded with a 3.6-liter linear gradient of sodium chloride 0 to 0.5 M in the same buffer at a flow rate of 30 ml per h. The major peak of glycolipid transfer activity was eluted at about 80 ml NaCl, but some transfer activity appeared before the main peak (Fig. 1).

Fig. 1. Elution pattern of glycolipid-transfer protein from a G-75 column. Protein (35.64 mg in 151 ml of Buffer A) from the Sephadex G-75 (column) step was applied to the G-75 column and eluted as described above. Seventeen-milliliter fractions were collected. The shape of the gradient (X—X) was established from conductivity measurements. Fifteen-milliliter fractions were assayed for galactosylceramide transfer activity (A—A). Protein was measured by absorbance at 280 nm (O--O). Horizontal bar indicates fractions pooled for the next step.

Step 5: Sephadex G-75 Chromatography — Fractions of the major peak were pooled and BSA was added at 0.1 mg/ml. Ammonium sulfate (45 g/100 ml) was added to 95% saturation. After allowing it to stand overnight, the precipitate was removed by centrifugation at 25,300 x g for 10 min. The precipitate was washed with 1.6 liters of the buffer and eluted with a 3.6-liter linear gradient of sodium chloride 0 to 0.5 M in the same buffer at a flow rate of 30 ml per h. The major peak of glycolipid transfer activity was eluted as a broad peak at an elution volume of 280 ml, and the column void volume was 140 ml (Fig. 2).

Fig. 2. Elution pattern of glycolipid-transfer protein from a Sephadex G-75 column. Protein (35.64 mg in 127 ml of Buffer A) from the Sephadex G-75 column was applied to the G-75 column and eluted as described above. Fifteen-milliliter fractions were collected. Fifteen-milliliter fractions were assayed for galactosylceramide transfer activity (A—A). Protein was measured by absorbance at 280 nm (O--O). Horizontal bar indicates fractions pooled for the next step.

Step 6: Phenyl-Sepharose Chromatography — The combined active fractions were dialyzed against a 3.5 M linear gradient of phenyl-Sepharose. The eluate was chromatographed on a column (1.4 x 80 cm) of phenyl-Sepharose 6B (column) with Buffer A. The column was washed with 180 ml of Buffer A and eluted with a 220 ml linear gradient of 0.5 M to 3.5 M imidazole in the same buffer at a flow rate of 20 ml per h. Most of the transfer activity and protein were eluted in a single peak at about 90 ml NaCl (Fig. 3). Fractions 84-121 also contained the same band as the major component. A minor band of faster-mobility in the 155 ml fraction was absent from fractions 84-121 and fractions 122-140, which contained the same band as the major component. Fractions 84-121 were assayed for a constant specific activity of galactosylceramide transfer. The purified fractions, indicated by a bar, were pooled and stored at 0°C. The transfer protein retained its original activity at least four months.

Fig. 3. Elution pattern of glycolipid-transfer protein from a Phenyl-Sepharose column. Protein (64.6 mg in 100 ml of Buffer A) from the Sephadex G-75 chromatography step was applied to the Phenyl-Sepharose column. The column was eluted as described above. Arrow and A indicate the peaks at which elution buffers were changed to 0.1 M Tris-ME (pH 6.8) and 1 M imidazole (pH 6.8). Protein was measured by absorbance at 280 nm (O--O). Absorbance at 280 nm (O--O). Arrow and A indicate fractions pooled for the next step.

Step 7: Phenyl-Sepharose Chromatography — The combined active fractions were dialyzed against a 3.5 M linear gradient of phenyl-Sepharose. The dialyzed solution was applied to the Phenyl-Sepharose column (column) with Buffer A. The column was washed with 180 ml of Buffer A and eluted with a 220 ml linear gradient of 0.5 M to 3.5 M imidazole in the same buffer at a flow rate of 20 ml per h. Most of the transfer activity and protein were eluted in a single peak at about 90 ml NaCl (Fig. 4). Fractions 84-121 also contained the same band as the major component. A minor band of faster-mobility in the 155 ml fraction was absent from fractions 84-121 and fractions 122-140, which contained the same band as the major component. Fractions 84-121 were assayed for a constant specific activity of galactosylceramide transfer. The purified fractions, indicated by a bar, were pooled and stored at 0°C. The transfer protein retained its original activity at least four months.

Fig. 4. Elution pattern of glycolipid-transfer protein from a phenyl-Sepharose column. Protein (64.6 mg in 100 ml of Buffer A) from the Phenyl-Sepharose chromatography step was applied to the Phenyl-Sepharose column. The column was eluted as described above. Arrow and A indicate the peaks at which elution buffers were changed to 0.1 M Tris-ME (pH 6.8) and 1 M imidazole (pH 6.8). Protein was measured by absorbance at 280 nm (O--O). Absorbance at 280 nm (O--O). Arrow and A indicate fractions pooled for the next step.