Reconstitution and Further Characterization of the Cholesterol Transport Activity of the Small-Intestinal Brush Border Membrane†

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ABSTRACT: The sterol (free and esterified cholesterol) transport activity of the small-intestinal brush border membrane was solubilized with the short-chain detergent diheptanoylphosphatidylcholine and reconstituted to an artificial membrane system (proteoliposomes). The resulting proteoliposomes were identified as unilamellar membrane vesicles ranging in size between 50 and 200 nm with a broad maximum at 70−110 nm. That the sterol transport protein was indeed incorporated into the lipid bilayer was shown by density gradient centrifugation on a Ficoll gradient: the proteoliposomes yielded a single band with an apparent density of 1.035 g/mL. By subjecting solubilized brush border membrane vesicles (BBMV) to gel filtration on Sephadex G-200 prior to reconstitution, a 7-fold enrichment of the sterol transport activity was achieved relative to the original BBMV. The experimental evidence presented lends strong support to the notion that the sterol transport protein is an integral protein of the brush border membrane which is anchored in the lipid bilayer by at least one hydrophobic domain. The active center(s) is (are) exposed to the external side of the membrane. Anchoring of this protein to the lipid bilayer by a glycosylphosphatidylinositol moiety is unlikely. The reconstituted proteoliposomes behaved very similarly to the original BBMV in terms of facilitated sterol uptake. Using these proteoliposomes, a hitherto unknown activity of the brush border membrane was discovered. Long-chain triacylglycerols can be taken up by this membrane as such and need not be hydrolyzed prior to absorption.

The transport[1] of free and esterified cholesterol from either small unilamellar vesicles (SUV)[2] or mixed bile salt micelles as the donor to the small-intestinal brush border membrane (BBM) as the acceptor is protein-mediated (Thurnhofer & Hauser, 1990a; Compassi et al., 1995, 1997; Schulthess et al., 1996). This was shown for rabbit, pig, and human BBMV, for pig enterocytes, and for Caco-2 cells (Schulthess et al., 1996). Sterol transfer is a second-order reaction, and as its mechanism, collision-induced movement of lipid between donor and acceptor was postulated (Thurnhofer & Hauser, 1990a; Compassi et al., 1995). After digestion of BBMV with proteases, the rate of sterol absorption is significantly reduced, and the rate constants measured under these conditions are characteristic of passive exchange of cholesterol between two populations of SUV (McLean & Phillips, 1981; Thurnhofer & Hauser, 1990a; Compassi et al., 1995). Furthermore, the order of the reaction is changed to a first-order reaction (Thurnhofer et al., 1990a). Comparison of the rates of sterol absorption before and after proteolytic treatment of BBMV provides clear-cut evidence that a BBM protein exists which facilitates sterol transport (Thurnhofer & Hauser, 1990a; Compassi et al., 1995, 1997; Schulthess et al., 1996). Since BBMV are impermeable to proteases (Klip et al., 1979a,b), the putative sterol transport protein is very likely an integral BBM protein with its active center(s) being exposed on the external or luminal side of the membrane (Thurnhofer & Hauser, 1990a; Compassi et al., 1995).

Here we show that the sterol transporter of the small-intestinal BBM can be solubilized with the detergent diheptanoyl-PtdCho and partially purified by gel filtration of the resulting mixed detergent−lipid−protein micelles on Sephadex G-200. The partially purified protein is reconstituted to a fully functional, artificial membrane system (proteoliposomes). The successful reconstitution lends support to the notion that the protein responsible for facilitated sterol absorption is indeed an integral protein of the BBMV. Using the reconstituted system, we were able to show that triacylglycerols are taken up as such and that hydrolysis of these lipids to monoacylglycerol and fatty acid is not a prerequisite for the uptake of triacylglycerols by the BBM.

MATERIALS AND METHODS

Materials. Egg PtdCho was purchased from Lipid Products (South Nutfield, Surrey, U.K.); 1,2-diheptanoyl-PtdCho (purity ≥99%) from Avanti Polar Lipids (Alabaster, AL);

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[1] In this paper, cholesterol transport or uptake is defined as the process of cholesterol movement or transfer from the lipid donor particle to the outer half of the BBM or reconstituted system.

[2] Abbreviations: AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride; BBM, brush border membrane(s); BBMV, brush border membrane vesicle(s); cmc, critical micellar concentration; E-64, (*trans*-epoxysuccinyl-L-leucylamido)-4-guanidinobutane; EDTA, disodium salt of ethylenediaminetetraacetic acid; GPI, glycosylphosphatidylinositol; Hepes, N-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; PI-PLC, phosphatidylinositol-specific phospholipase C; PtdCho, phosphatidylcholine; SDS, sodium dodecyl sulfate; SUV, small unilamellar vesicle(s); TLC, thin-layer chromatography; Tris, tris(hydroxymethyl)aminomethane.

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cholesterol (≥99%) and sodium bromide (≥99.5%) from Fluka (Buchs, Switzerland); potassium isothiocyanate (≥99%) and potassium bromide (≥99.5%) from E. Merck (Darmstadt, Germany); Ficoll, SP-Sepharose Fast Flow, Q-Sepharose Fast Flow, Sephadex G-200, and Sepharose CL-4B from Pharmacia (Dübendorf, Switzerland); 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF), papain from papaya latex, lyophilized proteinase K from *Tritirachium album*, and phosphatidylinositol-specific phospholipase C (PI-PLC) from *Bacillus cereus* from Boehringer (Mannheim, Germany); 2,3-dihydroxy-1,4-dithiolbutane, ethylenediaminetetraacetic acid disodium salt (EDTA), *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid (HEPES), (*trans*-epoxysuccinyl-L-leucylamido)-4-guanidinobutane (E-64), cholesteryl hemisuccinate covalently linked to 4% beaded agarose, trioleoylglycerol (∼99%), and cholesteryl oleate (≥98%) from Sigma (Buchs, Switzerland); [1α,2α(N)-^{3}H]cholesterol (47.0 Ci/mmol) and [1α,2α(N)-^{3}H]cholesteryl oleyl ether (47.0 Ci/mmol) from Amersham (Amersham, U.K.); [*carboxyl*-^{14}C]trioleoylglycerol (80−120 mCi/mmol) from DuPont NEN (Regensdorf, Switzerland); bicinchoninic acid Protein Assay Reagent from Pierce (Lausanne, Switzerland). Diheptanoyl-PtdCho was pure by C,H,N microanalysis and by ^{1}H NMR. All lipids in this study were pure by TLC standards.

Preparation of Brush Border Membrane Vesicles (BBMV). Rabbits of different breed were killed in the rabbit slaughterhouse; the proximal small intestines of about 1.5 m length were excised, thoroughly rinsed with 0.15 M NaCl, frozen in liquid nitrogen, and stored at −80 °C prior to the preparation of BBMV. The frozen small intestines (120−140 g) were thawed and BBMV prepared by up-scaling the procedure of Hauser et al. (1980).

Preparation of Small Unilamellar Vesicles (SUV). SUV of egg PtdCho containing 1 mol % radiolabeled sterol (free or esterified cholesterol containing trace quantities of radiolabeled cholesterol or cholesteryl oleyl ether, respectively) or trioleoylglycerol were prepared by tip-sonication of the lipid dispersion in buffer A (0.01 M Hepes, pH 7.3, 0.15 M NaCl, 0.01 M EDTA) as described before (Brunner et al., 1978). The average size and size distribution of the resulting dispersion of SUV were checked by gel filtration on Sepharose CL-4B.

Reconstitution of the Cholesterol Transport Activity of Rabbit BBM to an Artificial Membrane System. Freshly thawed BBMV were diluted 25-fold with buffer A to a concentration of 0.7 mg of protein/mL. BBMV were pelleted at 130000*g* for 10 min at 4 °C in a Kontron Centrikon T-2050 ultracentrifuge using the TFT65.38 rotor. The supernatant was decanted and the pellet resuspended to 7 mg of protein/mL in buffer A containing 30 mM diheptanoyl-PtdCho, 1 mM AEBSF, and 10 μM E-64. The membrane dispersion was centrifuged (in the same ultracentrifuge using the TFT50.13 rotor) at 130000*g* for 10 min at 4 °C. Proteins not solubilized and recovered in the pellet were dissolved in sample buffer and analyzed by SDS−7.5% PAGE. The supernatant containing the solubilized BBMV was decanted and added to a film of egg PtdCho dried on the wall of a round-bottom flask. The egg PtdCho (0.8 mg of lipid/mg of solubilized protein) was dispersed by whirl mixing for about 1 min. The reconstitution of an artificial membrane system was accomplished by dropwise addition of buffer A to the solubilized BBMV over a period of 30 min. The final diheptanoyl-PtdCho concentration was 0.4 mM, which is well below the cmc of 1.5 mM of this detergent (Tausk et al., 1974). The reconstituted system was centrifuged at 142000*g* for 90 min at 4 °C using the TFT45.94 rotor, and the resulting pellet was resuspended in buffer A to a final concentration of 4.5−6.7 mg of protein/mL.

Partial Purification of the Solubilized Cholesterol Transport Activity. A dispersion of solubilized BBM proteins in buffer A (∼4.2 mL; 7 mg of protein/mL) was subjected to gel filtration on Sephadex G-200 at 4 °C. The column (1.2 cm diameter × 30.5 cm) was equilibrated with buffer A containing 2 mM diheptanoyl-PtdCho (Sigrist et al., 1975) and eluted with the same buffer at a flow rate of 4.4 mL/h. In each fraction, the optical density at 280 nm and the cholesterol transport activity were measured. Fractions containing the cholesterol transport activity and the fractions between elution volumes 30 mL and 120 mL containing sucrase−isomaltase were pooled, concentrated, and subjected to the reconstitution procedure described above.

Characterization of the Reconstituted System. An estimate of the average vesicle size and the size distribution of the reconstituted system was obtained by freeze−fracture electron microscopy (Hauser et al., 1983). To determine the apparent density of the resulting lipoproteins, the reconstituted system was subjected to density gradient centrifugation. The reconstituted sample (0.3 mL containing 6.7 mg of protein/mL) was layered on 1.7 mL of a 35% Ficoll solution in water and centrifuged in the TLS-55 rotor at 166000*g* for 24 h at 20 °C using a Beckman Optima TLX ultracentrifuge. The reconstituted vesicles formed a single narrow band at $d = 1.035$ g/mL, and the gradient was eluted by piercing the bottom of the centrifuge tube and collecting the effluent in milliliter fractions. The apparent density, d, of each fraction was obtained from refractive index measurements, n_D, according to: $d = 2.381 n_D - 2.175$ [this equation is valid for Ficoll solutions at 20 °C (Rickwood, 1980)]. All fractions were analyzed for phospholipid, protein, and cholesterol transport activity. Fractions containing the reconstituted system were pooled and diluted 28-fold with buffer A, and the reconstituted vesicles were pelleted by centrifugation at 120000*g* using the TFT50.13 rotor. The supernatant containing Ficoll was removed; the pellet was washed by redispersing it in buffer A and repeating the centrifugation. The washed pellet was dispersed in buffer A, and lipid uptake was measured as described below. As a control, the size and the size distribution were determined by freeze−fracture electron microscopy.

Determination of the Lipid Transport Activity in BBMV and in the Reconstituted System. The reconstituted cholesterol transport activity was measured at 25 °C in an exchange reaction using egg PtdCho SUV containing 1 mol % radiolabeled lipid ([^{3}H]cholesterol or cholesteryl oleate labeled with cholesteryl oleyl ether or [*carboxyl*-^{14}C]trioleoylglycerol) as the donor and BBMV or the reconstituted system as the acceptor. The kinetics of lipid uptake by BBMV and the reconstituted membrane system were determined as described previously (Thurnhofer et al., 1991; Compassi et al., 1995; Schulthess et al., 1996).

The specific sterol and trioleoylglycerol transport activities of the reconstituted system in picomoles of lipid per minute per milligram of protein were measured essentially as follows: prior to mixing, the donor and acceptor dispersions in buffer A were centrifuged in the Beckman airfuge at

115000*g* for 2 min at 4 °C using the A-100/18 rotor. The dispersion of the acceptor yielded a pellet under these conditions which was resuspended in the same volume of buffer A. At time zero, this dispersion of acceptor was mixed with an aliquot of the top 80% of the donor dispersion obtained after centrifugation of the donor dispersion in the airfuge. The final concentration of the donor in the incubation mixture was 0.2 mg of total lipid/mL, and that of the acceptor varied between 0.2 and 2.0 mg of protein/mL. After incubation of the mixture at 25 °C for 20 min, the lipid exchange reaction was stopped by dilution of the incubation mixture with 2 volumes of buffer A. Donor and acceptor were separated by centrifugation in the airfuge at 115000*g* for 2 min at 4 °C. The radioactivities in the supernatant containing donor vesicles and in the pellet containing the reconstituted membrane system (acceptor) were determined in a Beckman LS 7500 scintillation counter.

Papain Digestion of the Reconstituted System. The reconstituted proteoliposomes were dispersed at 2 mg of protein/mL in buffer A containing 0.04 mg of papain/mL, 5 mM 2,3-dihydroxy-1,4-dithiolbutane, and 5 mM EDTA. The resulting dispersion was flushed with nitrogen and incubated at 37 °C for 3 h. The digestion was stopped by the addition of E-64 to 20 μM, and the suspension was centrifuged at 120000*g* for 10 min at 4 °C. The pelleted proteoliposomes were redispersed in buffer A to about 1 mg of protein/mL.

Digestion with Phosphatidylinositol-Specific Phospholipase C (PI-PLC). BBMV and reconstituted proteoliposomes were digested with PI-PLC following essentially the same procedure (Hooper & Turner, 1992) except that frozen BBMV were thawed and washed once by centrifugation (at 100000*g* for 3 min at 4 °C) and resuspension in Tris buffer (2 mM Tris, pH 7.1, 50 mM D-mannitol, 1 mM EDTA). By this washing cycle, about 20% of the total BBM proteins were separated from the BBMV (Thurnhofer et al., 1991). The resulting suspension of BBMV (~1.5 mg of protein/mL) was homogenized 3 times in a Potter−Elvehjem homogenizer, and PI-PLC was added to this suspension or to a suspension of reconstituted proteoliposomes to 100 milliunits of PI-PLC/mL. The digestion was carried out at 37 °C, and at timed intervals, aliquots of the incubation mixture were centrifuged in the airfuge at 100000*g* for 2 min at 4 °C. The supernatant was carefully decanted, and the pellet was resuspended in the Tris buffer. Both supernatant and pellet were analyzed for protein and trehalose activity. In the pellet, the cholesterol uptake activity was determined as described above.

Digestion of Solubilized BBMV with PI-PLC. BBMV were washed once as described above, and the pellet of BBMV was dispersed in Hepes buffer, pH 7.3 (0.01 M Hepes, 25 mM NaCl, 10 mM EDTA, 30 mM diheptanoyl-PtdCho, 1 mM AEBSF, 10 μM E-64) to 7 mg of protein/mL. The dispersion was centrifuged at 130000*g* for 10 min at 4 °C (TFT50.13 rotor); the supernatant was collected and diluted 1:2 (by volume) with H_2O to 1.7 mg of protein and 10 mM diheptanoyl-PtdCho. PI-PLC was added (100 milliunits/mL), and the mixture was incubated for 3 h at 37 °C. The incubation mixture was then added to an egg PtdCho film (0.8 mg of egg PtdCho/mg protein) dried on the glass wall of a round-bottom flask, and the lipid suspension formed was diluted 25 times by the dropwise addition of Hepes buffer for 30 min. The final concentration of diheptanoyl-PtdCho was 0.4 mM, well below the cmc of this detergent. The suspension was centrifuged at 142000*g* for 90 min at 4 °C, the supernatant was removed, and the pellet of reconstituted proteoliposomes was resuspended in 2 mM Tris buffer, pH 7.1, 50 mM D-mannitol, 1 mM EDTA.

Table 1: Percentage of Total Protein and Cholesterol Transport Activity Solubilized from BBMV and Reconstituted to Proteoliposomes[a]

	protein (%)	activity (%)	specific activity (microunits/mg of protein)
BBMV	100	100	5.4 ± 1.3
solubilized BBMV	67 ± 4	nd	nd
reconstituted system	24 ± 4	64 ± 12	13.0 ± 3.7

[a] BBMV were solubilized with 30 mM diheptanoyl-PtdCho and reconstituted to an artificial membrane system (proteoliposomes) as described under Materials and Methods. nd = not determined; this means that we were unable to determine the cholesterol transport activity in the solubilized state of BBMV (for details, see text).

Miscellaneous. Phospholipid concentrations were determined according to Chen et al. (1956) and protein concentrations by the bicinchoninic acid method (Smith et al., 1985). SDS−PAGE was carried out in a Mini-Protean II dual slab cell from Bio-Rad according to the Bio-Rad instruction manual. Proteins were visualized by Coomassie blue staining. Protein standards from Bio-Rad were used as molecular weight markers. The trehalase activity of BBMV was determined as described by Kolinska and Semenza (1967); the sucrase−isomaltase activity was measured according to Banauch et al. (1975). The proteinase K digestion was carried out according to Thurnhofer and Hauser (1990b).

RESULTS

Reconstitution of the Cholesterol Transport Activity of the BBM to an Artificial Membrane System. In order to shed light on the nature of the cholesterol transport protein, BBMV were solubilized with diheptanoyl-PtdCho, and the solubilized BBM proteins were reconstituted to an artificial membrane system. As documented in Table 1, only about one-fourth of the total BBM protein or 35% (range 25−40%) of the solubilized proteins were recovered in the reconstituted membrane system. This amounts to ~65% of the cholesterol transport activity present in the original BBMV. The preferential reconstitution of the cholesterol transporter led to a 2.5-fold increase in the specific cholesterol transport activity (Table 1). Evidence for the selective solubilization and reconstitution of certain BBM proteins is provided in Figure 1. The SDS−7.5% PAGE pattern of BBMV (Figure 1, lane A) is dominated by the doublet above 116 kDa and the band at slightly less than 45 kDa assigned to sucrase−isomaltase and actin, respectively (Klip et al., 1979a,b; Gains & Hauser, 1981).The latter protein was partially solubilized but not reconstituted as evident from a comparison of lanes B and D (Figure 1). Although some actin was solubilized by diheptanoyl-PtdCho (Figure 1, lane B), no actin was detected in the reconstituted system (Figure 1, lane D). Most of the actin was insoluble in diheptanoyl-PtdCho and accumulated in the pellet (Figure 1, lane C). The band at 200 kDa assigned to myosin behaved similarly, and so did the bands at 95 and 110 kDa assigned to villin and a calmodulin binding protein (Mooseker, 1995), indicating that cytoskeletal BBM proteins are not participating in the solubilization−reconstitution process (cf. arrows in lane C of Figure 1). In contrast, the integral membrane protein

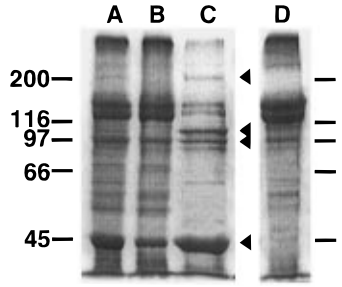

FIGURE 1: SDS−7.5% PAGE patterns of BBMV (lane A), BBMV solubilized with diheptanoyl-PtdCho (lane B), BBM proteins that were not solubilized with diheptanoyl-PtdCho and precipitated by centrifugation as described under Materials and Methods (lane C), and reconstituted proteoliposomes (lane D). The arrows in lane C mark cytoskeletal BBM proteins. Proteins were visualized by Coomassie blue staining. High-range molecular weight proteins were used as standards, and the positions of apparent molecular masses in kDa are marked on either side of the gel pattern.

sucrase−isomaltase, which is a dominant protein and a marker enzyme of the BBM (Kessler et al., 1978), was present mainly in the solubilized fraction and greatly enriched in the reconstituted system (Figure 1, lanes B and D).

Partial Purification of the Cholesterol Transporter. The solubilization−reconstitution process described above yielded an approximate 3-fold purification of the cholesterol transporter (Table 1). Further purification was accomplished by subjecting diheptanoyl-PtdCho-solubilized BBM protein to gel filtration on Sephadex G-200 prior to reconstitution. The gel filtration pattern is shown in Figure 2. About 70% of the protein and practically all of the cholesterol transport activity were eluted between the column void volume V_0 = 10 mL and the elution volume V_e = 19 mL. Fractions containing the cholesterol transport activity (represented by the bars in Figure 2) were visibly turbid; they were pooled and subjected to the reconstitution procedure, yielding proteoliposomes with a specific cholesterol transport activity of 36.4 microunits/mg of protein. This value was increased by a factor of about 7 relative to the specific cholesterol transport activity of the original BBMV (cf. Table 1). The SDS−10% PAGE pattern of these proteoliposomes shown in lane 1 of the inset of Figure 2 was similar to that of unpurified proteoliposomes (lane 5, inset of Figure 2) except that the sucrase−isomaltase content was reduced to 1/2−1/3. This was derived from a comparison of the densitometer scans of lanes 1, 4, and 5 (inset of Figure 2). As described by Sigrist et al. (1975), most of the sucrase−isomaltase was retained on the Sephadex column as evident from the retarded elution of this protein between 1 and 4 column volumes (cf. legend of Figure 2). These column fractions were pooled, concentrated, and subjected to the reconstitution procedure. The resulting proteoliposomes contained essentially pure sucrase−isomaltase as evident from SDS−10% PAGE (lane 4, inset of Figure 2) but no cholesterol transport activity.

Preliminary experiments subjecting diheptanoyl-PtdCho-solubilized BBM proteins to other standard protein purification procedures including gel filtration on Sepharose CL-4B, cation and anion exchange chromatography on SP-Sepharose and Q-Sepharose, respectively, and affinity chromatography on agarose beads with covalently bonded cholesterol showed that the purity and the specific activity of the cholesterol transporter were not significantly improved by these methods.

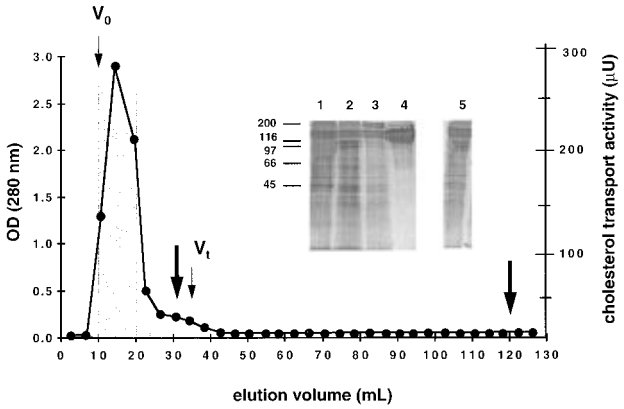

FIGURE 2: Gel filtration on Sephadex G-200 of BBMV solubilized with diheptanoyl-PtdCho. BBMV were solubilized with diheptanoyl-PtdCho as described under Materials and Methods, and ∼4.2 mL of the resulting mixed micelles dispersed in buffer A (∼7 mg of protein/mL) was applied to the Sephadex G-200 column (1.2 cm diameter × 30.5 cm; the void volume V_0 = 10 mL and the total column volume V_t = 35 mL are marked by small arrows). Fractions of about 4 mL eluted from the column with the same buffer were analyzed by optical density (OD) measurement at 280 nm and by measuring the cholesterol transport activity. Fractions were pooled, and the resulting pools were analyzed for protein and cholesterol transport activity and by SDS−PAGE (see below). Pool 1 (from elution volume V_e = 10 mL to V_e = 19 mL) contained 7.5 mg of protein; the cholesterol transport activity was 273 microunits, yielding a specific cholesterol transport activity of 36.4 microunits/mg of protein. Pool 2 (from V_e = 19 mL to V_e = 23 mL) contained 2.0 mg of protein, and the cholesterol transport activity was 2 microunits (specific cholesterol transport activity = 1 microunit/mg of protein). Pool 3 (from V_e = 23 mL to V_e = 31 mL) contained 0.47 mg of protein and no cholesterol activity. This is also true for pool 4 (from V_e = 31 mL to V_e = 120 mL marked by boldface arrows) containing 0.81 mg of protein. All fractions eluted between these elution volumes (pool 4) had an optical density above the background. Inset: SDS−10% PAGE patterns of reconstituted proteoliposomes produced from pools 1−4 (lane 1−4) and of control proteoliposomes produced as described under Materials and Methods (lane 5).

Characterization of the Reconstituted, Artificial Membrane System. In order to characterize the reconstituted artificial membrane system, it was subjected to density gradient centrifugation on a Ficoll gradient. Visual inspection of the density gradient at the end of the centrifugation showed a narrow turbid band at the top quarter of the centrifuge tube. This is entirely consistent with the analysis of the density gradient in terms of cholesterol transport activity and phospholipid and protein content. Almost all of the activity, 85% of the phospholipid, and 90% of the protein were recovered in the second fraction of the density gradient, corresponding to an apparent density d = 1.035 g/mL. Some lipids and proteins were present in the first (top) fraction of the gradient, corresponding to an apparent density d = 1.001 g/mL (data not shown). The lipid/protein weight ratios in the two top fractions were 6.7 (d = 1.001 g/mL) and 1.8 (d = 1.035 g/mL). In these two fractions, all of the cholesterol transport activity and the phospholipid and 93% of the total protein were recovered. No phospholipid and protein were detectable in fractions with densities higher than 1.035 g/mL. The density gradient centrifugation therefore provided evidence that the solubilized BBM proteins are reconstituted successfully to proteoliposomes, i.e., lipoprotein vesicles of an apparent density $d \approx$ 1.035 g/mL. This density is in the range of that of low-density serum lipoproteins (Chapman et al., 1986). Very similar results as described for the Ficoll

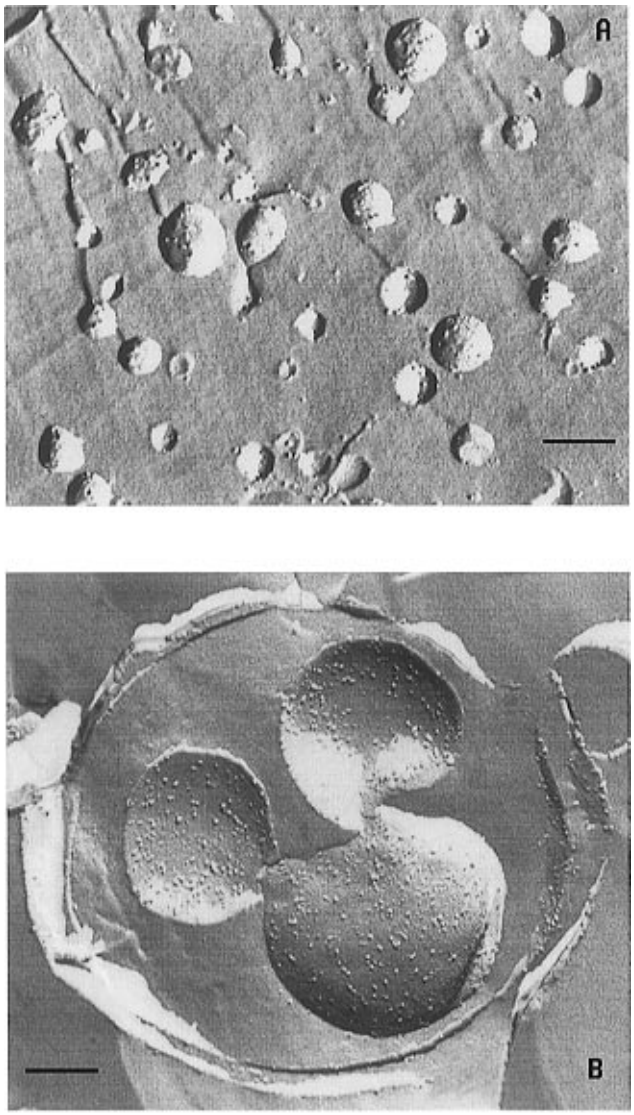

FIGURE 3: Electron micrographs of freeze-fractured samples of the proteoliposomes reconstituted as described under Materials and Methods. The concentration of the sample was 3.8 mg of protein/mL. Panels A and B are images of the same sample recorded from different regions of the electron microscopy grid. The bar represents 200 nm.

gradient were obtained when the reconstituted system was subjected to density gradient centrifugation on a continuous sodium bromide gradient (data not shown).

Electron micrographs of freeze-fractured samples of the reconstituted membrane system showed mainly spherical, unilamellar vesicles of a wide size distribution (Figure 3). The majority of vesicles ranged in size between 50 and 200 nm with a broad maximum at 70−100 nm (Figures 3A and 4). Occasionally some very large vesicles with diameters between 0.5 and 1.0 μm were observed (Figure 3B). With very few exceptions, the fracture faces of the vesicles were covered with proteinaceous spherical particles of an average size of 11 ± 2 nm (range of 8−15 nm). As evident from Figure 3A, B, the proteinaceous particles are randomly distributed over both the exoplasmic and the protoplasmic fracture faces of the reconstituted vesicles. BBM proteins, at least those visible by freeze−fracture electron microscopy, appear to be assembled into phospholipid bilayers symmetrically, i.e., with no apparent preference for the outer or inner half of the membrane. Some of the proteinaceous

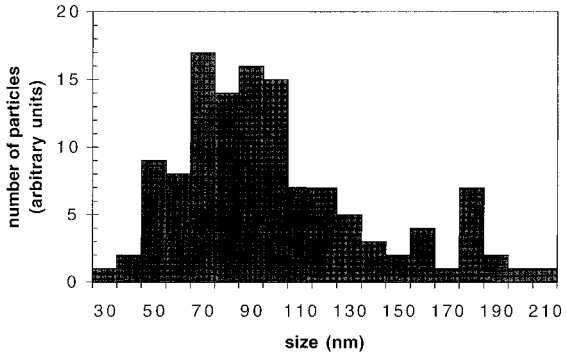

FIGURE 4: Bar histogram representing the size distribution of the reconstituted membrane vesicles as derived from electron micrographs of freeze-fractured samples (cf. Figure 2). The size of about 150 vesicles was measured.

particles appeared to be located outside of the reconstituted membrane vesicles. These are very likely lipid−detergent−protein complexes that were not incorporated into phospholipid bilayers during reconstitution. Electron micrographs of the reconstituted membrane system recovered from the second fraction of the Ficoll density gradient showed membrane vesicles indistinguishable from those presented in Figure 3. However, the number of proteinaceous particles located outside of the membrane vesicles appeared to be greatly reduced (data not shown).

Lipid Transport of the Reconstituted Artificial Membrane System. The reconstituted, artificial membrane system resembled BBMV in terms of facilitated sterol transport. The data presented in Figure 5 are qualitatively similar to results obtained with BBMV [cf. Figure 3 of Compassi et al. (1995)] despite the fact that the two acceptor membranes of BBMV and the reconstituted vesicles differed significantly in chemical composition. Figure 5 provides evidence that the uptake of both free and esterified cholesterol is protein-mediated. The kinetic curves of cholesterol uptake were monophasic and adequately fitted by a single exponential function (top panel, Figure 5). In contrast, the kinetics of cholesteryl oleate uptake were biphasic and fitted best by the sum of two exponentials (bottom panel, Figure 5). This behavior is entirely consistent with that of BBMV (Compassi et al., 1995). Pseudo-first-order rate constants k_1 (half-times $t_{1/2}$) and equilibrium values x_∞ derived from curve-fitting are summarized in Table 2. After papain treatment of the reconstituted membrane vesicles, cholesterol transport was markedly slowed down; the k_1 ($t_{1/2}$) values measured for free cholesterol under these conditions were 0.1 h^{-1} (7 h), characteristic of passive exchange of cholesterol between two populations of SUV (McLean & Phillips, 1981; Compassi et al., 1995). After proteolysis of the reconstituted system using either papain or proteinase K, the exchange of cholesteryl oleate was no longer detectable (bottom panel, Figure 5). The kinetic properties of proteolytically treated reconstituted proteoliposomes were identical to BBMV subjected to the same proteolytic treatment (Thurnhofer & Hauser, 1990a; Compassi et al., 1995).

Digestion of BBMV and Reconstituted Proteoliposomes with Phosphatidylinositol-Specific Phospholipase C (PI-PLC). In an attempt to elucidate the mode of anchoring of the cholesterol transporter to the BBM, BBMV were digested with PI-PLC. This phospholipase releases the carbohydrate moiety of the glycosylphosphatidylinositol (GPI) anchor by cleaving the glycerol phosphoester bond (Hooper & Turner,

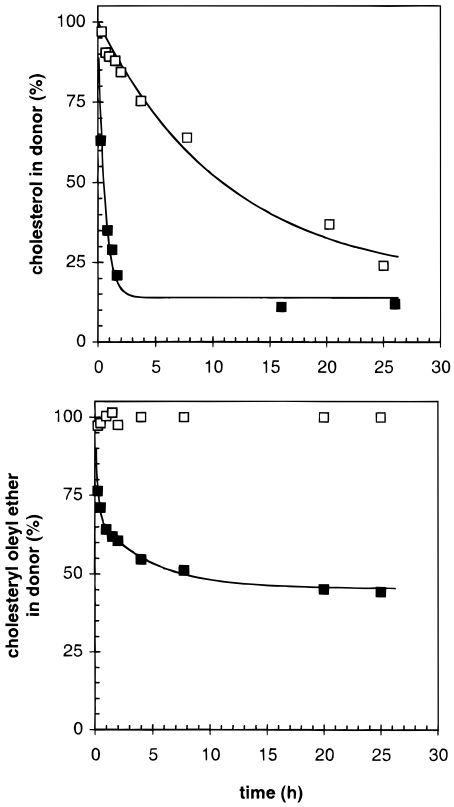

FIGURE 5: Kinetics of sterol exchange between SUV of egg PtdCho and reconstituted proteoliposomes. SUV of egg PtdCho containing either 1 mol % radiolabeled cholesterol (top panel) or 1 mol % cholesteryl oleate plus a trace amount of radiolabeled cholesteryl oleyl ether (bottom panel) as the donor were incubated with reconstituted proteoliposomes as the acceptor (solid symbols) as described under Materials and Methods. The kinetics of sterol exchange between the same donor vesicles and reconstituted proteoliposomes after papain treatment are included (open symbols). At time zero, donor and acceptor dispersions were mixed to yield a final concentration of the donor of 0.2 mg of total lipid/mL and a final acceptor concentration of 4.3 mg of protein/mL. The acceptor concentration after papain treatment was 1.2 mg of protein/mL. The experimental data points in the top panel were fitted by a single exponential function; the kinetic curve of cholesteryl oleate exchange in the bottom panel was fitted best by the sum of two exponentials. The equations used for curve fitting are given in the legend of Table 2.

1992). Incubation of BBMV with 100 milliunits/mL of PI-PLC at 37 °C for 24 h reduced the cholesterol transport activity associated with BBMV to about 75%. Within experimental error, this is consistent with the decrease in activity measured in the absence of PI-PLC. As a positive control, the release of trehalase from BBMV was determined, an ectoenzyme known to be GPI-anchored (Takesue et al., 1986). The PI-PLC treatment released on the average 81 ± 3% (Takesue et al., 1986) as compared to 15 ± 2% in the absence of PI-PLC. Furthermore, when reconstituted membrane vesicles were treated with PI-PLC in the same way, no loss in cholesterol transport activity relative to untreated proteoliposomes was observed. The average release of trehalase activity from these proteoliposomes by PI-PLC digestion was 40 ± 5%, compared to about 6% in the absence of PI-PLC. In another series of experiments, BBMV were solubilized with diheptanoyl-PtdCho, and the resulting mixed micelles were subjected to PI-PLC digestion at 37 °C for 24 h. After digestion with PI-PLC, the mixed micelles were reconstituted to proteoliposomes and tested for cholesterol uptake. The rate of cholesterol uptake in reconstituted proteoliposomes pretreated with PI-PLC was within experimental error indistinguishable from that measured in untreated control proteoliposomes (Figure 6). When diheptanoyl-PtdCho-solubilized BBMV were treated with PI-PLC, 90% of the trehalase activity was released into the supernatant, and only about 10% was reconstituted to proteoliposomes. In control BBMV, i.e., without the PI-PLC digestion, about 80% of the trehalase activity present in BBMV was solubilized and reconstituted to proteoliposomes. Taking all these results together, we conclude that GPI-anchoring of the cholesterol transporter in the small-intestinal BBM is unlikely.

Trioleoylglycerol Uptake by BBMV and the Reconstituted Membrane System. Measuring specific lipid transport activities in the reconstituted proteoliposomes, we discovered that trioleoylglycerol is taken up as such and need not be hydrolyzed to monoacylglycerol and fatty acid prior to uptake. After the trioleoylglycerol uptake experiment, total lipids were extracted from the reconstituted proteoliposomes, and TLC of the lipid extract revealed that hydrolysis of the trioleoylglycerol was negligible. This indicated that little if any lipolytic activity was left in the reconstituted system. The ability to take up trioleoylglycerol as such was confirmed with BBMV. However, in this case, about 33% of the triacylglycerol taken up was hydrolyzed to monoacylglycerol and fatty acid as evident from TLC of the total lipids extracted from BBMV after the lipid uptake experiment. After correcting for hydrolysis, the specific uptake of trioleoylglycerol by BBMV closely resembled that of the reconstituted system.

The relative specific activities of the uptake of free and esterified cholesterol and trioleoylglycerol were compared in BBMV and the reconstituted proteoliposomes. The data in Figure 7 provide further evidence that BBMV and the reconstituted system behave very similarly in terms of lipid uptake. However, the most remarkable result of Figure 7 is that the specific trioleoylglycerol uptake exceeds that of free and esterified cholesterol. This finding is quite unexpected; it is at variance with one of the dogmas of lipid absorption stating that long-chain triacylglycerols have to be hydrolyzed prior to their absorption.

DISCUSSION

Our laboratory presented evidence that sterol absorption in the small-intestinal BBM is protein-mediated (Thurnhofer & Hauser, 1990a; Compassi et al., 1995, 1997; Schulthess et al., 1996). In the first part of this paper, we addressed the question of the mode of association of the sterol transporter with the BBM. The fact that the sterol transport activity can be solubilized by detergents and reconstituted to an artificial membrane system (proteoliposomes) like other integral BBM proteins, e.g., sucrase−isomaltase and trehalase, lends strong support to the notion that the sterol transporter is an integral membrane protein. The sterol transport activity was liberated from the reconstituted proteoliposomes by protease treatment, resulting in proteoliposomes inactive in cholesterol uptake. This finding indicates that the active center(s) of the sterol transporter is (are) exposed on the external or luminal side of the BBM.

Proteins liberated from the BBM by washing with salt solutions or EDTA are operationally defined as extrinsic or

Table 2: Kinetic Parameters for the Uptake of Cholesterol and Cholesteryl Oleate by the Reconstituted Artificial Membranes[a]

				first phase		second phase	
reconstituted membrane system	lipid taken up	x_∞ (%)	kinetics	k_1 (h^{-1})	$t_{1/2}$ (h)	k_1' (h^{-1})	$t_{1/2}$ (h)
native	cholesterol	13.9	monophasic	1.47	0.41		
papain digested	cholesterol	19.1	monophasic	0.07	7.79		
native	cholesteryl oleyl ether	45.5	biphasic	2.52	0.15	0.22	3.15
papain digested	cholesteryl oleyl ether	~100		nd			
proteinase K digested	cholesteryl oleyl ether	~100		nd			

[a] Small unilamellar egg PtdCho vesicles were used as the donor and reconstituted proteoliposomes as the acceptor. The kinetic curves (Figure 5) were fitted by $x(t) = x_\infty + (x_0 - x_\infty)e^{-k_1[(a+b)/a]t}$ or $x(t) = x_\infty + (x_0 - x_{1\infty})e^{-k_1[(a_1+b_1)/a_1]t} + (x_{1\infty} - x_\infty)e^{-k_1't}$ depending on whether the experimental curves were monophasic or biphasic, respectively; x_0, x, $x_{1\infty}$, and x_∞ represent the percentages of radiolabel remaining in the donor at times 0, t, at an intermediate state, and at the final equilibrium, respectively, k_1 and k_1' are the pseudo-first-order rate constants of the first and second phase of lipid absorption, respectively, a and b are the effective lipid pools of acceptor and donor membrane, respectively, and a_1 and b_1 are the effective lipid pools of acceptor and donor, respectively, accessible during the first phase of lipid transfer. An estimate of the ratio $(a + b)/a$ was obtained from $(a + b)/a = 100/(100 - x_\infty)$. As a first approximation, the lipid pools a_1 and b_1 were assumed to be equivalent to a and b. Since the lipid pools involved in the second phase of lipid absorption are usually unknown, the lipid pool ratio is included in k_1'. The half-time for monophasic lipid uptake is $t_{1/2} = (\ln 2/k_1)[a/(a + b)]$; for biphasic lipid uptake, the half-times for the first and the second phase are $t_{1/2} = (\ln 2/k_1)[a_1/(a_1 + b_1)]$ and $t'_{1/2} = (\ln 2/k_1')$, respectively. nd = not detectable, implying that the k values were too small to be measurable, i.e., $k \rightarrow 0$ and $t_{1/2} \rightarrow \infty$.

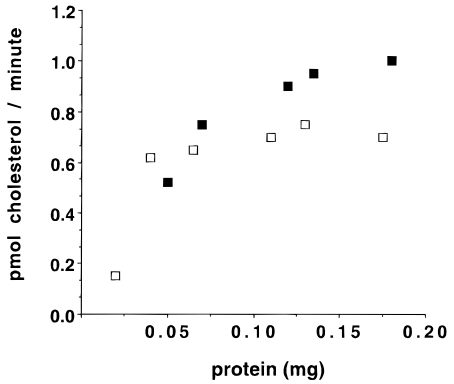

FIGURE 6: Rate of cholesterol uptake (pmol/min) by reconstituted proteoliposomes. BBMV were solubilized with 30 mM diheptanoyl-PtdCho; the resulting mixed micelles were subjected to digestion with PI-PLC (100 milliunits/mL) as described under Materials and Methods and reconstituted to proteoliposomes. In controls, BBMV were solubilized and reconstituted, omitting the digestion with PI-PLC. The rate of uptake of cholesterol by reconstituted proteoliposomes was measured using SUV of egg PtdCho containing 1 mol % [^{3}H]cholesterol as the donor at 0.2 mg of total lipid/mL and determined as a function of the protein concentration of proteoliposomes. Proteoliposomes pretreated with PI-PLC (■) and control proteoliposomes without pretreatment (□).

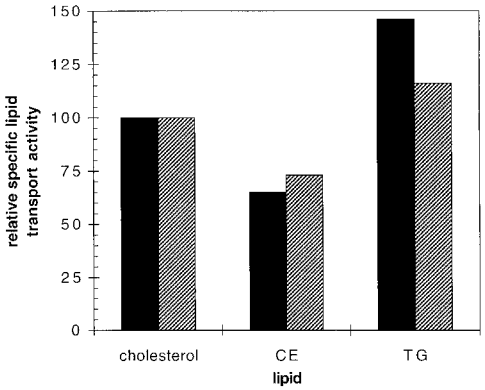

FIGURE 7: Comparison of specific lipid uptake activities in rabbit BBMV and in reconstituted proteoliposomes. Specific lipid uptake activities were measured as described under Materials and Methods and normalized to the cholesterol uptake activity. Black bars, BBMV; hatched bars, reconstituted proteoliposomes. CE = cholesteryl oleate, TG = trioleoylglycerol.

peripheral. Washing BBMV twice with 1 M NaCl, KBr, or KSCN released 23%, 23%, and 34% of total BBM protein, respectively, but did not reduce the sterol transport activity of the BBMV. As a control, BBMV were washed in the same way with buffer A, leading to a protein loss of 17% in good agreement with published data (Thurnhofer & Hauser, 1990b; Compassi et al., 1995; Lipka et al., 1995). Further, washing BBMV with chaotropic agents such as 1 M KBr or 1 M KSCN prior to solubilization and reconstitution did not reduce the cholesterol transport activity in the reconstituted membrane system. By noting that the buffer solutions used to prepare BBMV include EDTA, electrostatic interactions between the cholesterol transporter and the BBM involving divalent cations can also be ruled out. All these results are self-consistent and taken together provide good evidence for the cholesterol transporter being an integral BBM protein and not a peripheral one: it must possess at least one hydrophobic domain that anchors the protein to the phospholipid bilayer.

Density gradient centrifugation of the reconstituted membrane system confirmed that the sterol transporter is truly associated with the phospholipid bilayer. The resulting proteoliposomes have an apparent density d which is similar to that of low-density serum lipoproteins but significantly lower than the apparent density of BBMV. This is evident from a comparison of the lipid/protein weight ratio of reconstituted proteoliposomes and BBMV, the values of which were 2 and 0.6, respectively (Hauser et al., 1980).

About 60% of the original sterol transport activity is recovered in the reconstituted proteoliposomes. It is unknown whether the loss in activity is real incurred during the solubilization−reconstitution procedure or whether it is due to the random assembly of the sterol transporter into phospholipid bilayers. For sufficiently large proteoliposomes, such a random assembly would give rise to a random orientation of the transporter with about 50% of the active centers being exposed to the external medium and 50% being located within the internal cavity of the proteoliposomes. The latter protein molecules would be inaccessible to the donor and make no contribution to sterol uptake.

The sterol transporter of the BBM shares with hydrolases of the same membrane the requirement that the active site be close to the membrane surface. Hydrolases of the BBM such as alkaline phosphatase, aminopeptidase N, aminopeptidase A, dipeptidyl peptidase IV, sucrase−isomaltase, trehalase, and lactase−phlorizin hydrolase (Semenza, 1986; Danielsen, 1995) consist typically of a bulk, water-soluble, globular part located in the lumen of the small intestine and

a small hydrophobic anchor that fixes the enzyme in the bilayer. These enzymes are also known as ectoenzymes (Semenza, 1986; Hooper & Turner, 1988, 1989). Hydrolytic products such as amino acids and monosaccharides are released in the proximity of the BBM surface where appropriate carriers are located effecting the transport of these compounds across the membrane. In analogy, the active site of the sterol transporter is probably located close to the BBM surface in order to shuttle hydrophobic lipid molecules from donor particles (bile salt micelles or phospholipid vesicles) through the hydrophilic surface layer of the BBM to the outer monolayer of the BBM. Hydrophobic anchors of ectoenzymes fall essentially into two categories: (I) peptide anchors consisting of one α-helix at the N-terminus that span the phospholipid bilayer as for sucrase−isomaltase (Semenza, 1986; Hunziker et al., 1986), and (II) lipid anchors having one or more lipid molecules covalently bound to the protein. The most common anchor in the latter group is GPI as for trehalase (Takesue et al., 1986). To test whether the cholesterol transport activity is linked to the BBM by a GPI anchor, BBMV were incubated in the presence of PI-PLC known to release GPI-anchored proteins from membrane structures (Hooper & Turner, 1992). By this treatment, about 80% of the GPI-anchored trehalase was liberated, in contrast to only about 25% of the sterol transport activity. It is possible that PI-PLC is unable to access the putative GPI anchor of the sterol transporter in the tightly packed BBM. To tackle this question, mixed micelles obtained by solubilization of BBMV with diheptanoyl-PtdCho were subjected to PI-PLC digestion and then reconstituted to proteoliposomes. Even extensive digestion of solubilized BBMV with PI-PLC failed to release a significant proportion of the transport activity (Figure 6). This result makes GPI-anchoring of the sterol transporter unlikely.

The kinetic behavior of the sterol transporter present in the reconstituted membrane is comparable to that of the protein present in the original BBMV [cf. Table 2 and data published by Compassi et al. (1995)]. In both BBMV and reconstituted proteoliposomes, the kinetics of cholesterol transport were monophasic, and those of cholesteryl oleate were biphasic. Similar pseudo-first-order rate constants k_1 were derived for the initial fast phase of sterol uptake, and these rate constants depended linearly on the acceptor concentration (data not shown).The pseudo-first-order rate constant k_1' (half time $t_{1/2}$) of the second slow phase of cholesteryl oleate transport in the reconstituted membrane system is identical within the error of the measurement with that obtained with BBMV (Compassi et al., 1995). This rate constant was found to be independent of the acceptor concentration. The second slow phase of cholesteryl oleate transport was proposed to be due to the slow transverse or flip-flop motion of cholesteryl esters (Compassi et al., 1995).

BBMV are rich in endogenous proteases as well as proteases of pancreatic origin. Protein degradation was shown to be induced in the BBM by the addition of detergents which apparently activate these proteases (Gains & Hauser, 1981). Diheptanoyl-PtdCho, the detergent used in the present work, was previously shown to have good dispersing properties for both phospholipid bilayers (Tausk et al., 1974) and biological membranes (Kessi et al., 1994). Diheptanoyl-PtdCho acts as a mild, yet effective detergent. Its salient feature is the stabilization of the native conformation of proteins, and as a result, the activity of solubilized proteins is retained over a wide range of detergent concentrations (Kessi et al., 1994). This is due to the preferential interaction of diheptanoyl-PtdCho with the phospholipid bilayer, leaving intact or only minimally perturbing the interaction between membrane proteins and the phospholipid bilayer (Kessi et al., 1994). Thus, the natural environment of these proteins is preserved in the solubilized state even in excess diheptanoyl-PtdCho. This observation helps to explain the stability of BBM proteins in mixed diheptanoyl-PtdCho/BBM protein micelles relative to mixed micelles made with other detergents. Diheptanoyl-PtdCho selectively solubilized BBM proteins (cf. Figure 1 and Table 1), yielding proteoliposomes the specific cholesterol transport activity of which was increased by a factor of 2.5 (Table 1). The sterol transporter is shown here to be an integral membrane protein. The efficient solubilization of BBMV proteins leading to stable detergent−lipid−protein micelles is therefore a prerequisite to designing a successful purification scheme for such proteins. We note that the cholesterol transport activity cannot be monitored in the solubilized state, i.e., in the presence of excess diheptanoyl-PtdCho or any other detergent. Unfortunately, an activity test in the presence of detergents is still elusive (cf. second row of Table 1). In order to determine the cholesterol transport activity in fractions eluted from chromatographic columns, the activity has to be reconstituted to an artificial membrane system. Hence, monitoring the activity of column fractions is very tedious and represents a serious limitation to the purification of the sterol transporter via solubilization−reconstitution. In spite of these difficulties, an encouraging first purification step was achieved by subjecting diheptanoyl-PtdCho-solubilized BBM proteins to gel filtration on Sephadex G-200. Proteoliposomes were generated by this procedure that were depleted of sucrase−isomaltase. As a result, their sterol transport activity was increased by a factor of about 7 relative to the original BBMV.

We note here that the small-intestinal BBM has the capacity of facilitated trioleoylglycerol uptake (Figure 7). The conclusion that BBMV can take up long-chain triacylglycerols in a facilitated, protein-mediated way is supported by the following observation: (I) the rate of trioleoylglycerol uptake is faster than that of sterol uptake, and sterol uptake was previously shown to be protein-mediated (Thurnhofer & Hauser, 1990a; Compassi et al., 1995, 1997); (II) after proteolytic treatment of BBMV or of the reconstituted membrane system, the rate of trioleoylglycerol uptake was negligible. The finding of facilitated trioleoylglycerol uptake at the BBM is unexpected and to the best of our knowledge has not been reported before. This result challenges the generally accepted view that long-chain triacylglycerols are not absorbed per se but have to undergo hydrolysis to monoacylglycerol and fatty acid prior to absorption. In discussing the physiological significance of this result, it has to be borne in mind that the solubility of long-chain triacylglycerols is rather limited in lipid bilayers (Hamilton & Small, 1981; Hamilton, 1989; Hamilton et al., 1991; Li et al., 1993). Due to the severe limitation in solubility of long-chain triacylglycerols in lipid carriers transporting these lipids to the site of lipid absorption in the small intestine, the actual percentage of triacylglycerol absorption by the small-intestinal epithelial cells may be small. Nevertheless, from a mechanistic point of view, it is important to note that such an uptake activity exists in the small-intestinal

BBM. The question of whether the sterol and trioleoylglycerol transport activities of the BBM are due to one transport protein (system) or different ones cannot be answered from the data presented in this study. However, the data presented do clearly show that both sterol and triacylglycerol transport activities are due to integral membrane protein(s) and if there is more than one protein involved all the components required for this function are solubilized by diheptanoyl-PtdCho and reconstituted to an artificial membrane system. The question of the assignment of these activities is of great importance in elucidating the mechanism of neutral lipid uptake at the level of the BBM. It will therefore be addressed in a future study. A possible approach would be to search for an effective inhibitor of sterol uptake at the BBM and to determine if and to what extent this inhibitor will affect the protein-mediated uptake of trioleoylglycerol.

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