Intracellular events in the assembly of chylomicrons in rabbit enterocytes

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Abstract **The aim of this study was to determine the intracellular events in chylomicron assembly in adult villus enterocytes. We have used novel methods for separation of the intracellular components of the secretory compartment [rough and smooth endoplasmic reticulum (RER and SER, respectively) and Golgi], and their membrane and luminal components, from villus enterocytes isolated from rabbit small intestine. The steady state composition of the components of the secretory compartment and the intracellular pools of newly synthesized apolipoprotein B-48 (apoB-48) and triacylglycerol (TAG) was determined. The observations indicate that the SER is the main site of TAG synthesis and of chylomicron assembly. Newly synthesized apoB-48 and TAG accumulate in the SER membrane and are transferred into the lumen in a microsomal triglyceride transfer protein-dependent step. In enterocytes isolated from chow-fed rabbits, in which fat absorption is relatively slow, transfer of apoB-48 and TAG from the SER membrane into the lumen appears to be rate limiting. In enterocytes from fat-fed rabbits, TAG accumulates in the lumen of the SER, suggesting that movement out of the SER lumen becomes rate limiting, when chylomicron secretion is markedly stimulated. In these cells, the cytosolic TAG also increased to 450 μg/g enterocytes, compared with 12 μg/g enterocytes from chow-fed rabbits, indicating that transfer of TAG from the SER membrane into the secretory pathway can become saturated, so that newly synthesized TAG moves into the cytosol.**—Cartwright, I. J., D. Plonné, and J. A. Higgins. **Intracellular events in the assembly of chylomicrons in rabbit enterocytes.** ***J. Lipid Res.*** **2000.** 41: **1728–1739.**

Supplementary key words RER • SER • Golgi • iodixanol • cell fractionation • fat absorption • triacylglycerol • apolipoprotein B • MTP

Absorption of dietary lipids occurs in two main stages: digestion in the lumen of the small intestine and absorption across the mucosa [reviewed in refs. (1–3)]. The main products of digestion of dietary triacylglycerol (TAG), monoacylglycerides and free fatty acids, move across the brush border of the enterocytes, are re-esterified, and the TAG is assembled with cholesterol, cholesteryl ester, phospholipids, and apolipoproteins to form chylomicrons [reviewed in refs. (2 and 3)]. Apolipoprotein B-48 (apoB-48) is the major apolipoprotein and is essential for the assembly and secretion of chylomicrons (2, 4). Ultrastructural studies of fat absorption, including cytochemistry, have shown that chylomicron assembly follows the classic secretory pathway (5–7). ApoB is synthesized by bound ribosomes, and is associated with the rough endoplasmic reticulum (RER), lipid droplets are observed in the lumen of the smooth endoplasmic reticulum (SER), and the lipid and apoB-48 are assembled into chylomicrons that accumulate in the lumen of the Golgi before release at the lateral borders of the enterocytes. Apart from this outline. however, little is known about the molecular details of the intracellular events.

Chylomicrons resemble very low density lipoprotein (VLDL), the vehicle of transport of endogenous lipids from the liver. However, chylomicrons are larger, contain proportionally more TAG, and their main apolipoprotein is apoB-48, a truncated form of apoB-100, which is the main apolipoprotein of VLDL (8, 9). There has been considerable progress in elucidation of the molecular and intracellular details of VLDL assembly [reviewed in refs. (10–12)] and there has been a tendency to extrapolate finding from studies of hepatic cells to enterocytes (2). However, there have been few direct studies of chylomicron assembly. In part this is due to the lack of a suitable cell system for such studies. A number of investigators have used the Caco-2 cell line, which has been shown to secrete TAG-rich particles when incubated with bile salts and oleic acid (2, 13–18). However, Caco-2 is a colonic cell line that secretes particles of the density of VLDL/low density lipoprotein containing both apoB-100, which is characteristic of liver and fetal intestine, and apoB-48, which is characteristic of adult intes-

Abbreviations: ACAT, acylcholesterol acyltransferase; apoB, apolipoprotein B; DGAT, diacylglycerol acyltransferase; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethylsulfoxide; ER, endoplasmic reticulum; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; HPTLC, high performance thin-layer chromatography; MTP, microsomal triglyceride transfer protein; PDI, protein disulfide isomerase; RER, rough endoplasmic reticulum; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SER, smooth endoplasmic reticulum; TAG, triacylglycerol; VLDL, very low density lipoproteins.

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tine (2, 14, 18). Caco-2 cells are not therefore an ideal system for the study of chylomicron assembly and resemble fetal rather than adult enterocytes. It is also difficult to extrapolate findings from Caco-2, or indeed any cell line, to whole body physiological events. The small intestine is subjected to enormous fluctuations in the amount and composition of dietary lipid and the enterocytes must respond to these dietary challenges.

To investigate the molecular details of TAG absorption and chylomicron assembly and how these are regulated, we have developed methods for the isolation of viable adult enterocytes from rabbit small intestine (19, 20). These cells synthesize apoB-48 without apoB-100 and, when incubated with lipid/bile salt micelles, secrete apoB-48 in TAG-rich particles of the density of chylomicrons. In the present study we have used newly developed methods for the separation of the secretory compartment (RER, SER, and Golgi) of isolated enterocytes in a single self-generating gradient to investigate the intracellular details of chylomicron assembly. Our results show that chylomicron assembly differs in several respects from VLDL assembly. ApoB-48 and TAG accumulate in the SER membrane and are transferred into the lumen in a microsomal triglyceride transfer protein (MTP)-dependent process, which appears to be rate limiting when rabbits are fed a low fat diet. In enterocytes from animals fed a high fat diet movement of the TAG from the SER to the Golgi lumen appears to be rate limiting.

MATERIALS AND METHODS

Materials

Optiprep (60% iodixanol) and Maxidens were from Lipotek (Peter Joste Enterprise Centre, Liverpool, UK; John Moores University, Liverpool, UK; or contact J.A.H.); protease inhibitor cocktail (Complete™ minus ethylenediaminetetraacetic acid) was from Boehringer Mannheim (Mannheim, Germany). Anti-TGN38 (*trans*-Golgi marker) was a gift from G. Banting (University of Bristol, Bristol, UK), anti-p58 (*cis*-Golgi SER network marker) was a gift from J. Saraste (University of Bergen, Bergen, Norway), and anti-protein disulfide isomerase (anti-PDI) was purchased from StressGen (Victoria, British Columbia, Canada). Secondary antibodies and other reagents were from Sigma (St. Louis, MO) or as described previously (21–28). The MTP inhibitor CCI7932 was a gift from M. Walker (Glaxo-Wellcome, UK).

Animals and diets

Dwarf lop rabbits (~6 months old, 2.56 ± 0.12 kg) bred in the University of Sheffield Field Laboratories were used for these studies. They were allowed free access to water and chow [2.5% fat (w/w), equivalent to 7% of the dietary energy content, average intake 95 g/day] and were maintained on a 12-h light/dark cycle. In some experiments, the diets were supplemented with sunflower oil to increase the TAG content to 7.5% (equivalent to 21% of the calorie content) for 2 weeks as described previously (20).

Isolation and incubation of enterocytes

Villous enterocytes were isolated from the small intestine of rabbits and resuspended in oxygenated Dulbecco's modified Eagle's medium (DMEM) at 37°C as described previously (19). To measure the synthesis and secretion of TAG or apoB-48, isolated cells (2 g in 3.6 ml of DMEM) were incubated with lipid/bile salt micelles (0.4 ml) containing [^{14}C]oleate (1 μCi/0.4 ml) or with [^{35}S]methionine (100 μCi) plus micelles (19, 20). Enterocytes were incubated for various times up to 120 min and pelleted by centrifugation at 800 *g* for 2 min. Chylomicrons were isolated from the incubation medium by centrifugation at 13,000 *g* for 20 min, or by adsorbtion onto PMH-Liposorb (Calbiochem, Nottingham, UK) (19, 20). In some experiments, enterocytes were incubated for 30 min with the radiolabeled substrates, isolated by centrifugation, and reincubated with unlabeled micelles, for various times.

Homogenization of isolated enterocytes and preparation of subcellular fractions

Subcellular fractions derived from the secretory compartment were prepared from isolated enterocytes by procedures similar to those we have previously used for hepatocytes (26, 29). Freshly prepared enterocytes, or enterocytes that had been incubated with bile salt/lipid micelles as described above, were washed in phosphate-buffered saline (PBS, pH 7.4; 5.0 ml/g cells) and were pelleted by centrifugation at 2,000 *g* for 5 min at 4°C. Cell pellets were resuspended in 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES, pH 7.8) at 4°C (2 g of cells wet weight in 10 ml) and protease inhibitor cocktail was added. The suspension was placed on ice for 5 min to allow the swelling of the cells, after which they were pelleted by centrifugation (10,000 rpm for 2 min). Seven milliliters of the supernatant was removed and mixed with 3.0 ml of 0.6 M sucrose in 10 mM HEPES (pH 7.8) at 4°C, and the cells were homogenized using 30 strokes of the tight-fitting pestle of a Dounce homogenizer (type A; Wheaton, Millville, NJ). The homogenate was centrifuged at 8,000 *g* for 20 min to remove cell debris and large granules. The supernatant was removed and recentrifuged at 150,000 *g* for 40 min at 4°C in a Beckman (Palo Alto, CA) 50.2Ti rotor. The supernatant was discarded and the microsomal pellet was resuspended in 3.5 ml of 0.25 M sucrose in 10 mM HEPES (pH 7.8) at 4°C, using the loose-fitting pestle of a Dounce homogenizer (type B; Wheaton). A volume of 3.2 ml of the microsomal suspension was mixed with 1.6 ml of Optiprep (final concentration, 20% iodixanol). A volume of 4.5 ml of the microsomes/iodixanol suspension was layered under 4.5 ml of 15% iodixanol (Optiprep diluted 1 to 4 in 0.25 M sucrose-10 mM HEPES, pH 7.8) in a Beckman Optiseal tube. The microsomal suspension was underlayered with a cushion of 30% iodixanol (1.8 ml) in 0.25 M sucrose, 10 mM HEPES (pH 7.8). The tubes were sealed and centrifuged at 363,000 g_{av} for 2 h in the Vti65.1 rotor. The gradients were collected, using the Beckman fraction recovery system, by upward displacement with Maxidens after piercing the bottom of the tube. Depending on the experiment, twenty 0.5-ml fractions or ten 1.0-ml fractions were collected, omitting the 30% iodixanol cushion, into Eppendorf tubes. The membrane and luminal contents of the fractions were separated by sodium carbonate treatment, as previously described for subcellular fractions from liver (26). Briefly, the fractions collected from iodixanol gradients, were diluted with 2 volumes of 0.25 M sucrose, to dilute the iodixanol. The vesicular fractions were pelleted by centrifugation at 18,000 *g* (30,000 *g*) for 60 min at 4°C. The pellets were resuspended in 100 mM sodium carbonate (pH 11.0) and left on ice for at least 30 min. The tubes were recentrifuged at 18,000 *g* (30,000 rpm) for 90 min to separate the membranes (pellets) and the luminal contents (supernatants).

Extraction and analysis of lipids

Cellular and secreted lipids were extracted and separated by high performance thin-layer chromatography (HPTLC) (19, 20,

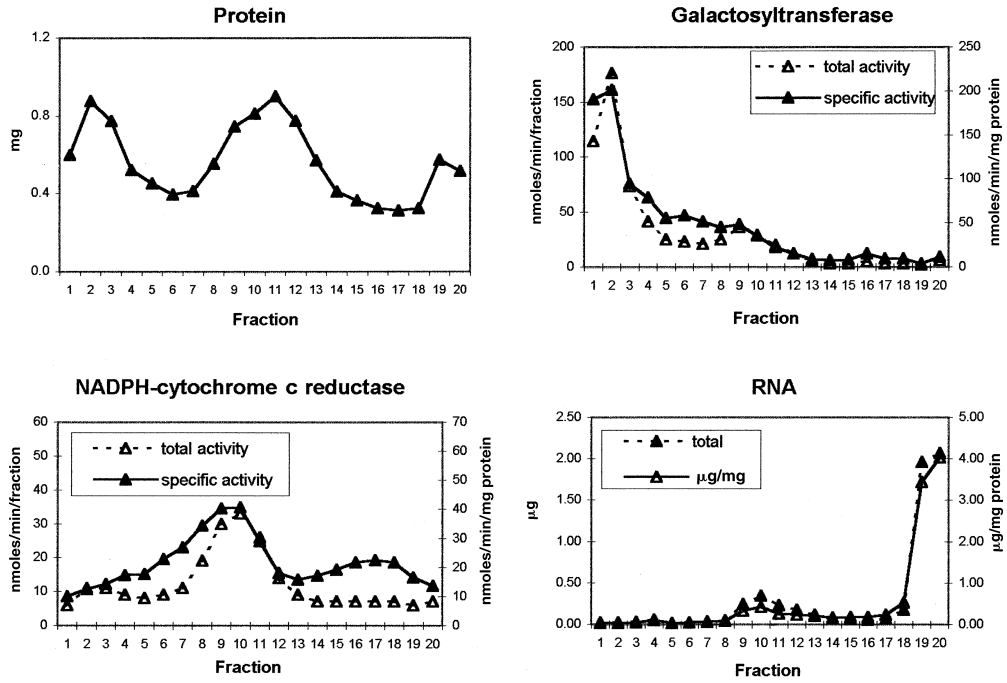

Fig. 1. Distribution of markers in iodixanol gradients. Total microsomes were prepared and separated into subfractions in iodixanol gradients as described in Materials and Methods. The gradients were collected in twenty 0.5-ml fractions, and the protein (mg/fraction), RNA (µg/fraction and µg/mg protein), NADPH-cytochrome-c_2 reductase (nmol/min/fraction and nmol/mg protein/min), and galactosyltransferase (nmol/min/fraction and nmol/mg protein/min) were determined as described in Materials and Methods.

24, 26). [^{14}C]oleate incorporation into separated lipids was determined with a Packard (Downers Grove, IL) InstantImager (2D counter) and the mass of each lipid was determined by laser densitometry (19, 20, 24, 26). The radiolabel in lipid bands was measured over a fixed time and the result is expressed as counts accumulated minus the blank measured on a part of the HPTLC plate without lipid.

Analysis of apoB-48

Incorporation of [^{35}S]methionine into cellular and secreted apoB-48 was determined as previously described (19, 20, 28). The radiolabel in the apoB-48 band was determined with the Packard InstantImager over a fixed time and is expressed as counts accumulated minus the blank measured over a part of the gel outside the protein lanes.

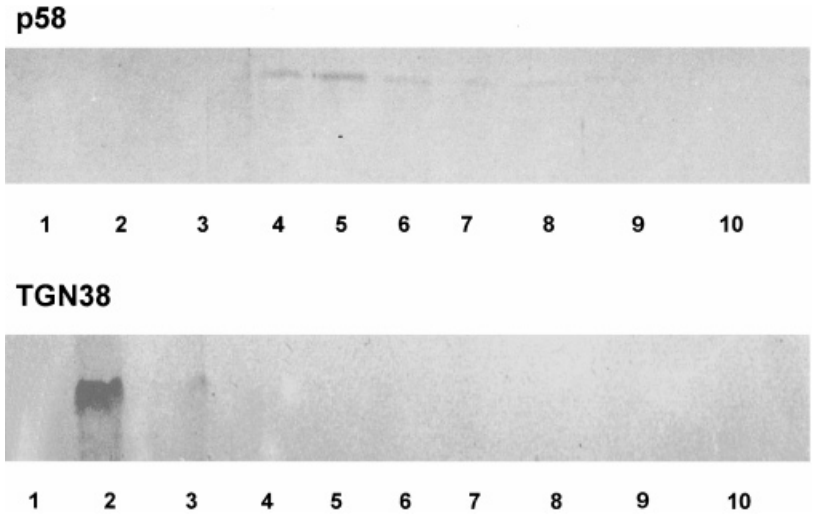

Fig. 2. Distribution of TGN38 and p58 in iodixanol gradients. Total microsomes were prepared and separated into subfractions in iodixanol gradients as described in Materials and Methods. The gradients were collected in ten 1.0-ml fractions, so that fraction 1 corresponds to fractions 1 + 2 in Fig. 1, and fraction 2 corresponds to fractions 3 + 4, and so on. Aliquots containing 100 µg of protein of each fraction were separated by SDS-PAGE and transferred onto nitrocellulose, and TGN38 or p58 in the fractions was detected by immunoblotting.

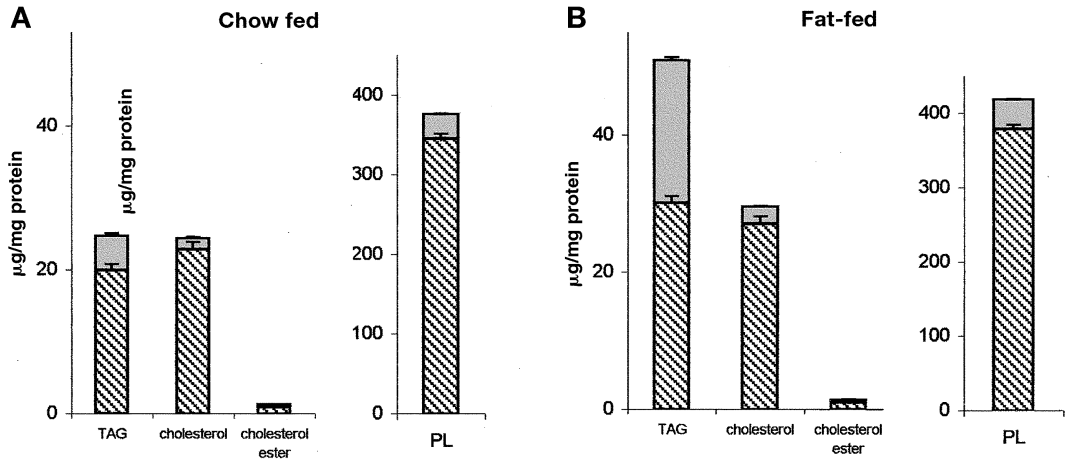

Fig. 3. Composition of microsomal lipids prepared from isolated enterocytes from chow- and fat-fed rabbits. Total microsomes were prepared from isolated enterocytes and separated into membrane and luminal content fractions, as described in Materials and Methods. The lipids were extracted and analyzed by HPTLC. The lipid composition (µg/mg of microsomal protein) is plotted for (A) fractions from enterocytes from chow-fed rabbits and (B) fractions from enterocytes from fat-fed rabbits. The mean values are plotted and the columns indicate the standard deviation for four determinations. The lower part of each column indicates the membrane lipid and the upper part the luminal content lipid.

Other analyses

UDPgalactose–glucose galactosyltransferase, NADPH–cytochrome-c_2 reductase, RNA, and protein, were assayed as described previously for liver fractions (27, 29). Acylcholesterol acyltransferase (ACAT) and diacylglycerol acyltransferase (DGAT) were assayed as described previously (26) TGN38, p58, and PDI were identified in subcellular fractions by immunoblotting after separation of the proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), using 3–20% gradient gels (19, 21, 22, 29).

RESULTS

Fractionation of the secretory compartment of enterocytes in self-generating gradients of iodixanol

Isolated enterocytes were homogenized and a total microsomal fraction was prepared from the homogenate by differential centrifugation. This fraction contained 33% of the galactosyltransferase (*trans*-Golgi marker) and 31% of the NADPH-cytochrome-c_2 reductase [endoplasmic

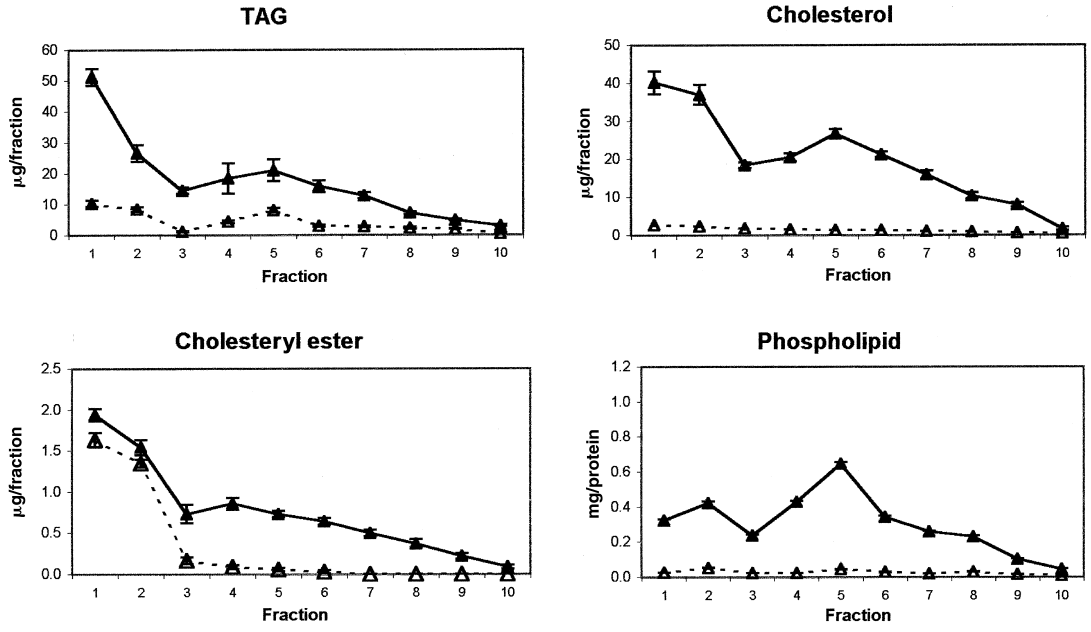

Fig. 4. Lipid composition of microsomal fractions prepared from isolated enterocytes of chow-fed rabbits. Total microsomes were prepared and separated in iodixanol gradients, which were collected in ten 1.0-ml fractions. Membrane and luminal contents were prepared from each fraction and the lipids were extracted and analyzed by HPTLC. The lipid (micrograms per fraction) is plotted against fraction number. The data plotted represent means ± standard deviation for four determinations. The closed triangles indicate membrane lipids and the open triangles and dotted lines indicate luminal lipids.

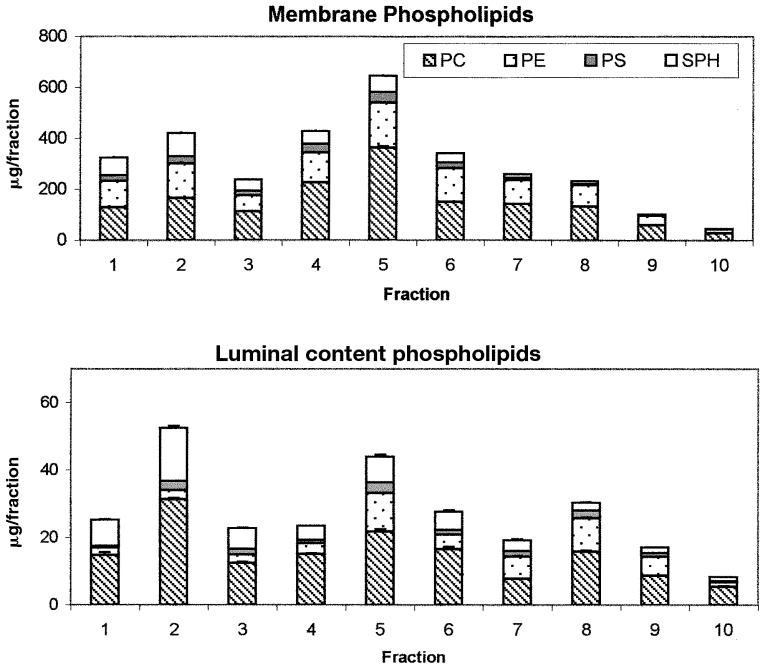

Fig. 5. Phospholipid composition of subcellular fractions of microsomes from isolated enterocytes of chow-fed rabbits separated on iodixanol gradients. Fractions were prepared and the lipids were extracted and analyzed as described in the legend to Fig. 4. The mean phospholipid composition for membranes (upper graph) and luminal contents (lower graph) is plotted. The columns indicate the standard deviation for four determinations. In many cases the standard deviations were small, so that the bars are not visible.

reticulum (ER) marker] of the homogenized enterocytes, no detectable succinic dehydrogenase (mitochondrial marker), and low acid phosphatase (lysosomal marker). The total microsome fraction was subfractionated in self-generating gradients of iodixanol, in which the microsomal protein distributed in three peaks (**Fig. 1**). One peak, fractions 1–4, at the top of the gradient contained most of the galactosyltransferase activity and immunodetectable TGN38, a *trans*-Golgi marker (**Fig. 2**); a second peak, fractions 8–14, in the middle of the gradient contained most of the NADPH-cytochrome-c_2 reductase, an ER marker (Fig. 1). p58, a *cis*-Golgi/smooth ER network marker, was detected by immunoblotting in the lighter part of this peak, 7–10 (Fig. 2). A third peak, toward the bottom of the gradient (fractions 17–20), also contained NADPH-cytochrome-c_2 reductase, and the membrane-bound ribosomes were concentrated in the denser part of this peak, above the cushion, in fractions 18–20 (29). The subfractionation of microsomes from enterocytes is similar to that found when we used these methods to prepare subcellular fractions from liver or isolated hepatocytes (26, 29). Thus, the self-generating gradient provides a snap-

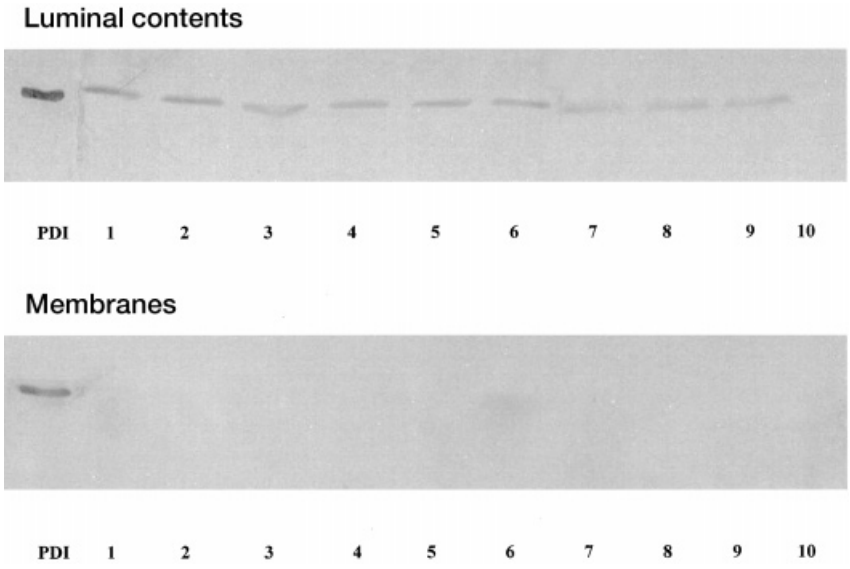

Fig. 6. Distribution of PDI in membrane and luminal content fractions separated in iodixanol gradients. Total microsomes were prepared and separated into subfractions in iodixanol gradients as described in Materials and Methods. The gradients were collected in ten 1.0-ml fractions and each fraction was separated into membrane and luminal contents. Aliquots equivalent to 100 µg of protein of the total fraction were separated by SDS-PAGE and transferred onto nitocellulose, and PDI in the fractions was detected by immunoblotting.

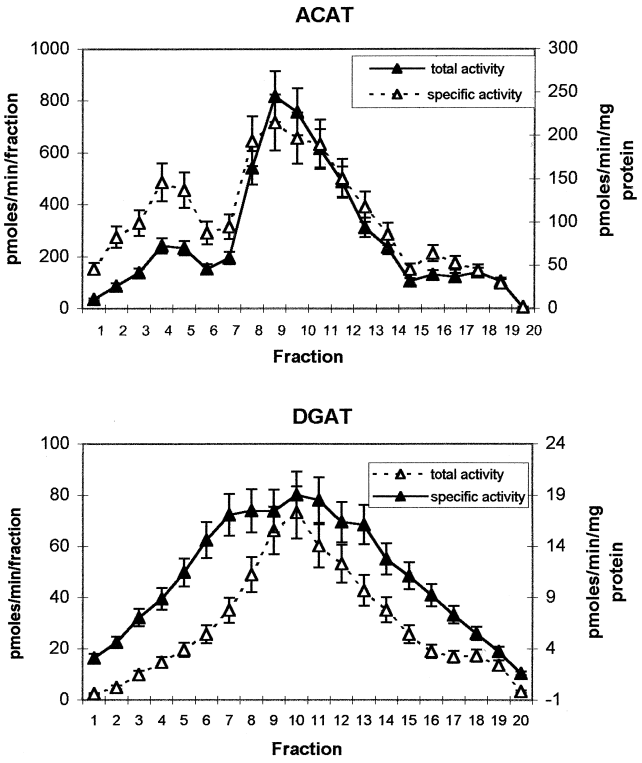

Fig. 7. Distribution of ACAT and DGAT activity in fractions separated in iodixanol gradients. Total microsomes were prepared and separated into subfractions in iodixanol gradients as described in Materials and Methods. The gradients were collected in twenty 0.5-ml fractions and the activity of ACAT and DGAT in each fraction was determined. ACAT activity (pmol/min/fraction and pmol/min/mg protein; top), and DGAT activity (pmol/min/fraction and pmol/min/mg protein; bottom), are plotted against gradient fraction. The results plotted represent the means of three determinations.

shot of the continuum of the secretory compartment. The vesicles involved in early events in secretion (RER-derived vesicles) are at the bottom of the gradient, those involved in intermediate events (SER/*cis*-Golgi network-derived vesicles) are in the center of the gradient, while those involved in the late events (Golgi-derived vesicles) are at the top of the gradient. Moreover, each of the major peaks can be collected in several subfractions, allowing finer resolution of secretory events.

Most of the lipids associated with the secretory compartment of enterocytes from chow-fed rabbits are membrane bound

During lipid absorption by enterocytes, TAG-rich droplets, chylomicrons, or their precursors, are observed within the lumen of the SER and the *trans*-Golgi (5–7). Therefore, it might be expected that the bulk of the TAG destined for secretion would be located in the lumen of the secretory compartment. However, we found that, in subcellular fractions prepared from enterocytes of rabbits allowed free access to chow, most of the TAG (72–92%) was associated with the membranes rather than the luminal contents (**Figs. 3** and **4**). The TAG content of the gradient fractions peaked in the SER and *trans*-Golgi with approximately 28% in the lumen of these fractions. There was little TAG in either the RER membrane or luminal contents. The data in Fig. 4 are plotted as the lipid per fraction, as we consider that this is most useful to show the distribution of the lipid mass through the secretory compartment; however, when the data are plotted as membrane composition (milligrams of lipid per milligram of protein) similar patterns of distribution were observed. Using the recovery of marker enzymes from the gradient (31% of the NADPH-cytochrome-c_2 re-

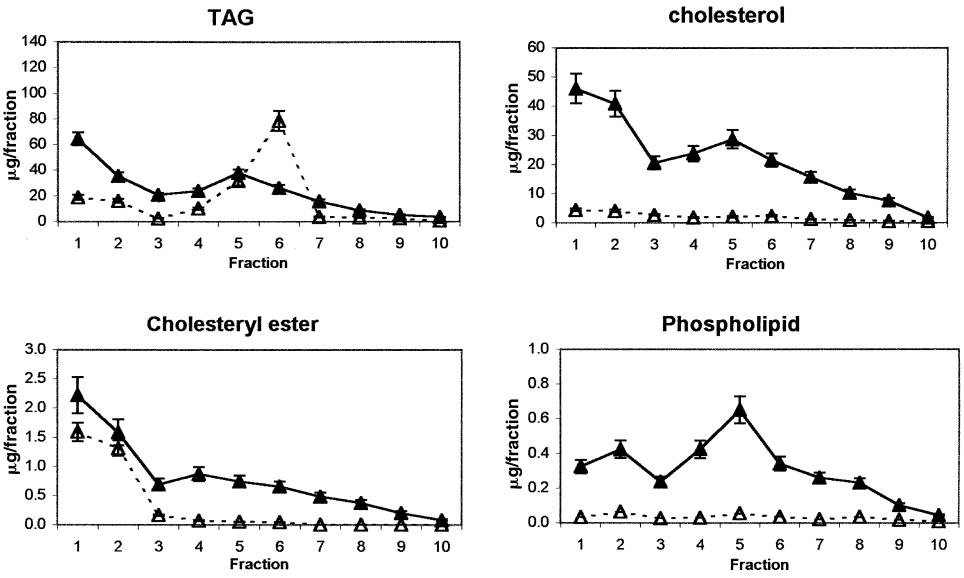

Fig. 8. Lipid composition of microsomal fractions from isolated enterocytes of fat-fed rabbits. As in Fig. 4, except that the microsomal fractions were prepared from the enterocytes of fat-fed rabbits. The solid triangles indicate membrane lipids and the open triangles and dotted lines indicate luminal lipids.

ductase and 33% of the galactosyltransferase) it can be calculated that the total TAG recovered in the total microsomes (349.07 ± 37.26 μg/g cells) accounts for 96.7% of the total cellular TAG (361.50 ± μg/g cells). Therefore there is little cytosolic TAG in enterocytes from chow-fed rabbits.

Cholesterol and phospholipid are components of chylomicrons and of membranes. Consistent with this, more than 90% of the cholesterol and phospholipid were recovered in the membranes of both total microsomes and gradient fractions (Figs. 3–5). The cholesterol content of the membranes increased through the gradient from RER to SER to Golgi, as we have found previously for similar fractions from hepatocytes (26). Cholesteryl ester was present in microsomes and gradient fractions in small amounts and was at the highest concentration in the Golgi vesicles, with approximately 50% in the luminal contents, suggesting that cholesteryl ester is transferred into the lumen of the secretory compartment separately from TAG. The phospholipid compositions of the membrane and luminal contents differed with the contents containing a greater proportion of phosphatidylcholine and sphingomyelin (**Fig. 5**).

To test the efficacy of our procedures for separating membranes and lumen content fractions, the presence of the luminal protein PDI in both fractions was detected by immunoblotting. This protein was found in the luminal content fractions throughout the gradient and was not detectable in the membrane fractions (**Fig. 6**) indicating that the protocol used for opening microsomal vesicles was efficient. Therefore, although enterocytes from chow-fed rabbits do secrete chylomicrons (19, 20), most of the TAG in the secretory compartment is membrane bound rather than luminal, suggesting that movement of this lipid into the lumen may be rate limiting in chylomicron assembly and secretion.

DGAT and ACAT activities are concentrated in the SER fraction

The activity of DGAT, which catalyzes the final step of synthesis of TAG, was concentrated in the SER peak, which also showed the highest specific activity, and was essentially absent from the Golgi and RER fractions (**Fig. 7**). ACAT, which synthesizes cholesteryl ester, was also at a high activity level in the SER, with a small peak in the Golgi. More than 75% of the total activity of each enzyme was recovered in the SER peak (fractions 8–14).

Feeding fat prior to isolation of enterocytes increases the TAG in the SER lumen

We have previously shown that feeding a diet supplemented with sunflower oil stimulates the ability of isolated enterocytes to secrete chylomicron TAG more than 80-fold and apoB-48 more than 18-fold (20). Enterocytes from sunflower oil-fed rabbits were therefore used to investigate the effect of stimulating fat absorption on the distribution of lipids in the secretory compartment. The TAG content of these cells increased to 1,105 ± 50 μg/g cells compared with 361 ± 16 μg/g cells from chow-fed rabbits and the TAG content of the total microsomes increased 2.5- to 3-fold. From the recovery of the marker enzymes in the microsomal fraction it was calculated that 60% of the cellular TAG is associated with the secretory compartment. Thus, feeding fat increases the cytosolic TAG from approximately 12 to 450 μg/g cells. The luminal content TAG increased 7-fold and the membrane TAG also increased about 2-fold (Fig. 3 and **Fig. 8**). The distribution of markers on the gradients was similar for fractions from enterocytes of fat-fed animals and chow-fed animals (data not shown). Feeding fat did not alter the cholesterol, cholesteryl ester, or phospholipid compositions of the microsomes and gradient subfractions apart from a small increase in the proportion of phospholipid in the luminal contents. These observations suggest that, when fat absorption is stimulated, movement of TAG in chylomicrons, or their precursors, from the SER

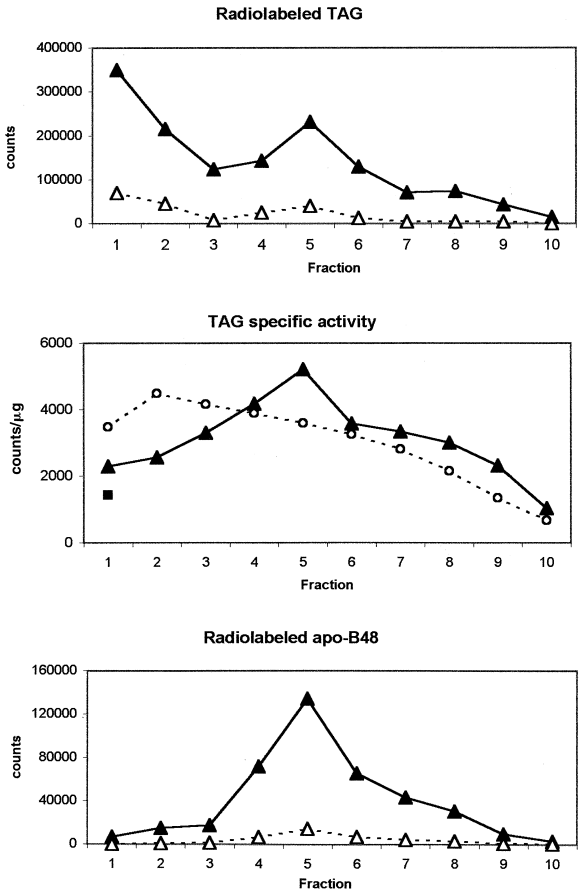

Fig. 9. Incorporation of [^{14}C]oleate into TAG and of [^{35}S]-methionine into apoB-48 of membrane and luminal contents of microsomal fractions separated in iodixanol gradients. Isolated enterocytes were incubated for 30 min with micelles containing [^{14}C]oleate or with [^{35}S]methionine plus micelles as described in Materials and Methods. The cells were isolated by centrifugation; total microsomes were prepared and separated in iodixanol gradients. The fractions were separated into membrane and luminal content subfractions and the incorporation of [^{14}C]oleate (counts/fraction) into TAG (top), the specific activity of TAG (counts/μg) (middle), and the incorporation of [^{35}S]methionine into apoB-48 (counts/fraction) (bottom) were determined as described in Materials and Methods. The data plotted are from a typical experiment. The solid triangles indicate membrane lipids and the open triangles and dotted lines indicate luminal lipids. The solid square indicates the specific activity of secreted TAG.

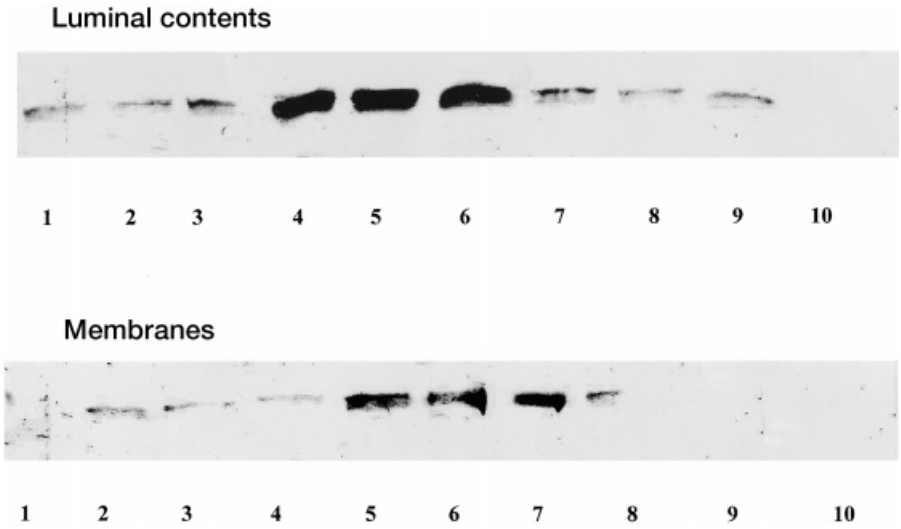

Fig. 10. Distribution of apoB-48 in membrane and luminal content fractions separated in iodixanol gradients. Total microsomes were prepared and separated into subfractions in iodixanol gradients as described in Materials and Methods. The gradients were collected in ten 1.0-ml fractions and were separated into membrane and luminal contents. Aliquots containing 100 μg of the membrane protein and all of the content protein from 1 mg of protein of the original microsomes were separated by SDS-PAGE and transferred onto nitrocellulose, and apoB-48 in the fractions was detected by immunoblotting.

lumen to the Golgi lumen may become rate limiting so that the lipid accumulates in the SER lumen.

Newly synthesized TAG and apoB-48 accumulate in the SER membrane and are subsequently transferred to the lumen and secreted

Incubation of isolated enterocytes with lipid/bile salt micelles stimulates secretion of chylomicrons (19). When enterocytes were incubated for 30 min with micelles containing [^{14}C]oleate, most of the newly synthesized TAG was associated with SER and the Golgi membranes, with less than 10% of the radiolabeled TAG of the total fractions in the luminal contents (**Fig. 9**). The specific activities of the TAG pools in SER membrane were similar or slightly greater than those of the luminal contents of the SER and the Golgi, consistent with the possibility that the membrane TAG is the source of the luminal TAG (Fig. 9). The specific activity of the TAG associated with the Golgi membranes and of the TAG secreted from the cells during the incubation period was lower than that in the SER membrane and lumen. This is probably due to dilution of newly synthesized TAG with unlabeled TAG present in the secretory compartment at the beginning of the incubation. To determine whether the newly synthesized TAG in the SER membrane is indeed transferred to the lumen and secreted, enterocytes were incubated with [^{14}C]oleate-containing micelles, isolated by centrifugation, and reincubated with unlabeled micelles. Microsomes were isolated at the beginning and end of the chase period and the radiolabel in the TAG of the membrane and luminal contents was determined. Under these conditions, the radiolabeled TAG in the microsome membranes and in the luminal contents fell, while that in the luminal contents and the secreted chylomicrons increased (Fig. 11C). The total secreted and microsomal radiolabeled TAG, corrected for recovery of markers, after 60 min of incubation was 95% of that at the beginning of the chase. Only 15% of the secreted radiolabeled TAG could not be accounted for by transfer of radiolabeled TAG from from the luminal contents. Therefore, membrane-bound TAG is also transferred to the secreted pool during the chase period.

After incubation with lipid/bile salt micelles and [^{35}S]methionine, radiolabeled apoB-48 exhibited a peak in the SER and was mainly recovered in the membrane fraction, with less than 10% in the lumen (Fig. 9). Immunodetectable apoB-48 was also present in all membrane and luminal content gradient fractions, with the highest concentration in the SER (**Fig. 10**). To avoid overloading the SDS gel, it was necessary to separate a smaller proportion of the membrane protein from each gradient microsomal fraction than from the luminal content fractions, which contain less protein than the membranes. In the gel illustrated, the proportion of the luminal content fraction used was 10 times that of the membrane fraction. Taking this into account, the amount of apoB-48 detected by immunoblotting approximated the distribution of radiolabeled apoB-48.

To determine whether radioabeled apoB-48 associated with the SER membrane is secreted, enterocytes were incubated with [^{35}S]methionine and micelles, isolated by centrifugation, and reincubated with an excess of unlabeled methionine and micelles. Microsomes were isolated at the beginning and end of the chase period and the radiolabeled apoB-48 was determined. During the chase period, the radiolabel in the membrane apoB-48 fell. The radiolabel in luminal apoB-48 also fell to a smaller extent, while secreted radiolabeled apoB-48 increased (**Fig. 11A**). Approximately 44% of the total radiolabeled apoB-48 was

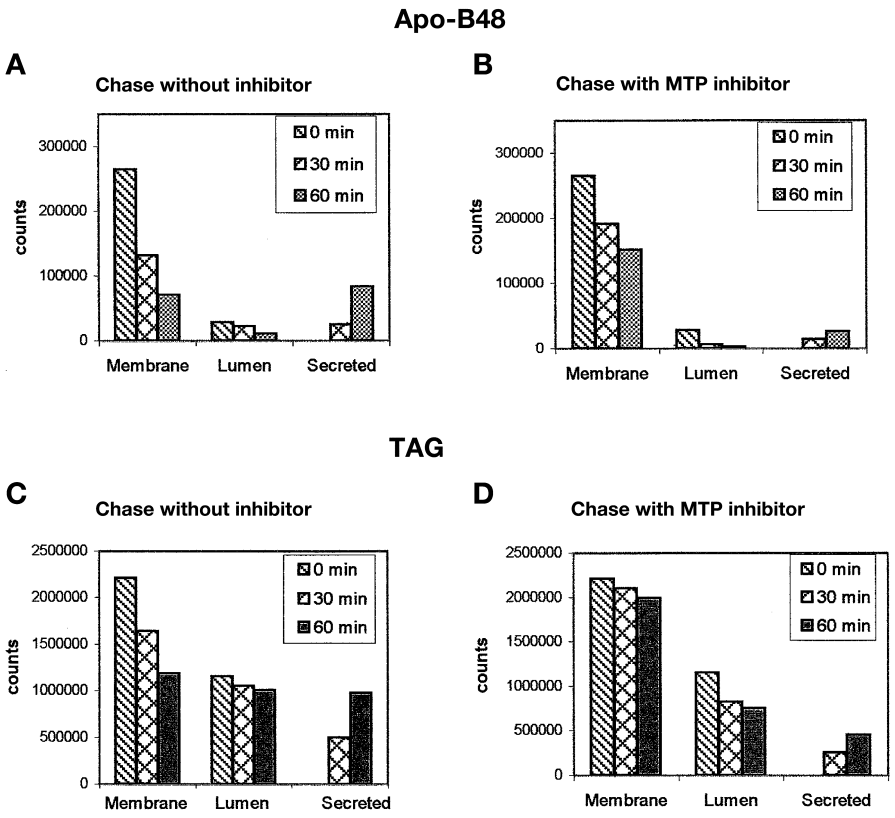

Fig. 11. Fate of newly synthesized apoB-48 and TAG in enterocytes incubated with and without MTP inhibitor. Isolated enterocytes were incubated for 30 min with micelles containing [^{14}C]oleate or with [^{35}S]methionine plus micelles, as described in Materials and Methods. The cells were isolated by centrifugation and reincubated (chase step) with unlabeled micelles and an excess of unlabeled methionine for 0, 30, and 60 min. At each time point cells were pelleted by centrifugation and secreted chylomicrons were isolated. The microsomal fraction was isolated from the cell pellets and separated into membrane and luminal content subfractions, and radiolabeled apoB-48 and TAG were determined as described in Materials and Methods. At the beginning of the chase step, cells were reincubated with MTP inhibitor CCI7932 (100 μg/ml), which was added as a solution in DMSO (final concentration, 0.5%) (B and D) or with 0.5% DMSO (A and C). The radiolabel in TAG or apoB-48 was normalized to that in a gram of hepatocytes. The data plotted are from a typical experiment.

lost during the chase. A similar loss of newly synthesized apoB-48 was found from the enterocytes incubated under the same conditions (data not shown) and is presumably due to intracellular degradation of apoB-48. Loss of radiolabeled apoB-48 from the luminal contents of the microsomes accounted for only 21% of that secreted. Therefore radiolabeled apoB-48 in the membrane is transferred to the lumen and secreted during the chase period.

Transfer of apoB and TAG from SER is inhibited by inhibition of MTP

MTP plays an important role in the initial steps in the assembly of VLDL in the RER of hepatocytes (30–32) and has also been implicated in secretion of apoB-48 and apoB-100 by Caco-2 cells and hamster intestine (33–35). To investigate whether MTP is involved in transfer of newly synthesized apoB-48 and/or TAG from the SER membrane to the secreted pool, the experiments described above were carried out with an MTP inhibitor added during the chase period. Under these conditions, secretion of radiolabeled apoB-48 by the isolated enterocytes was reduced by ~70%, and 95% of the secretion could be accounted for by loss of radiolabeled apoB-48 from the luminal contents of the microsomes (Fig. 11B). Thirty-eight percent of the radiolabeled apoB-48 was lost from the membrane fraction, similar to the level of degradation in the absence of the MTP inhibitor. Transfer of radiolabeled TAG from the microsomal membrane to the secreted pool was inhibited ~50% in the presence of the MTP inhibitor (Fig. 11D). The loss of radiolabeled TAG from the luminal contents accounted for 87% of that appearing in the secreted pool. Thus, inhibition of MTP does not prevent movement of newly synthesized apoB-48 or TAG from the lumen of the ER into the secreted pool, but inhibits the transfer of these chylomicron components from the membrane into the lumen.

DISCUSSION

The aim of this study was to elucidate the intracellular events in chylomicron assembly. These studies were car-

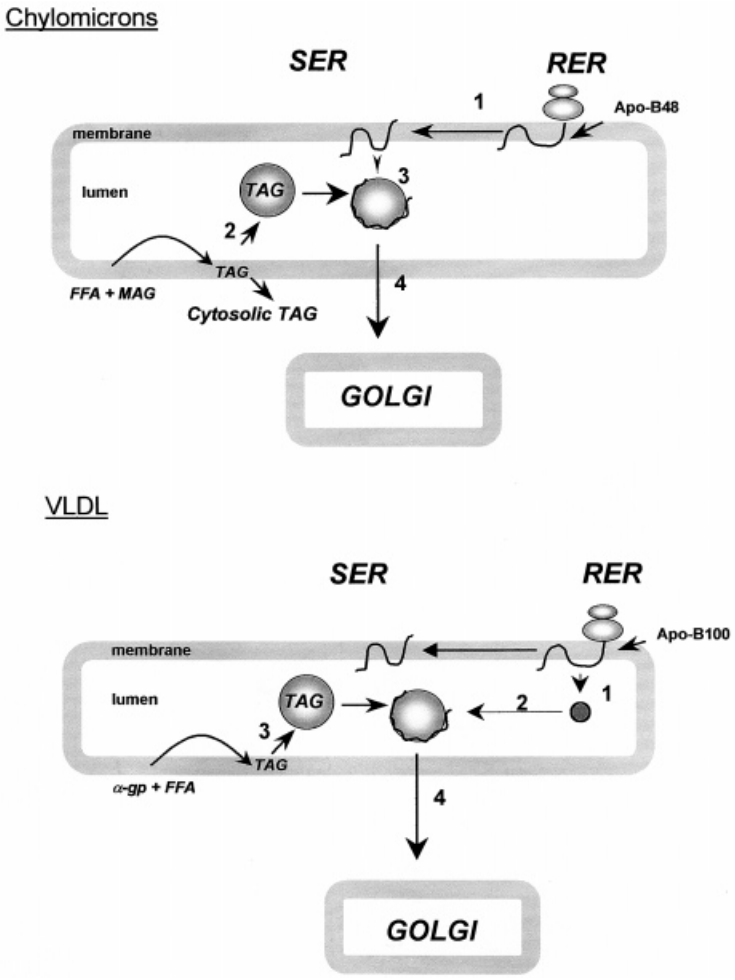

Fig. 12. Comparison of models for chylomicron and VLDL assembly. Top: A model for the assembly of chylomicrons in enterocytes, based on our data. Bottom: A model for VLDL assembly in hepatocytes, based on data from several laboratories (10–12, 23, 25, 30–32, 38, 39). In chylomicron assembly, apoB-48 synthesized in the RER moves as a membrane component to the SER (step 1). TAG is synthesized by DGAT in the SER. TAG and apoB-48 are transferred into the lumen in MTP-dependent steps 2 and 3. For two of the steps shown (2 and 3), however, the data are insufficient to say whether a single or multiple steps are involved in this transfer. In step 4, the chylomicrons move to the Golgi lumen. In rabbits fed a low fat diet, transfer into the SER lumen (step 2) appears to be rate limiting, while after fat feeding, assembly of chylomicrons in the SER lumen and movement from the SER to the Golgi lumen appear to be rate limiting; excess newly synthesized TAG also moves into the cytosol. In VLDL assembly, apoB-100 is synthesized in the RER and moves into the lumen in dense VLDL precursor particles (step 1). TAG is synthesized in the SER and is transferred into the SER lumen in apoB-deficient particles (step 3). The apoB-100-containing particles move into the SER and fuse with the TAG-rich particles to form VLDL (step 2), which move to the Golgi (step 4). Rate-limiting steps in VLDL assembly include the movement of apoB-100 into the RER lumen (step 1) and the movement of the dense particles from the RER to the SER lumen (step 2), and possibly formation of the TAG-rich particles (step 3).

ried out with freshly isolated villus enterocytes from adult rabbits, which provide a cell system as close to the in vivo situation as is practicable and allows studies to be carried out after dietary variations. To dissect the intracellular events in secretion, we have used a single-step self-generating gradient that separates the continuum of the secretory compartment of the isolated enterocytes, such that vesicles involved in early events in secretion are at the bottom of the gradient and those involved in late events are toward the top of the gradient. By using these techniques it has been possible to determine the steady state composition of the different components of the secretory compartment and the relationships between the intracellular pools of newly synthesized apoB-48 and TAG.

Our observations suggest that the SER is the main site of assembly of chylomicrons. Although apoB-48 must be synthesized by bound ribosomes, the mass of apoB-48 and the newly synthesized apoB are at a low concentration in the RER membrane and lumen and are concentrated in the SER membrane. The SER is also the main site of synthesis of TAG, which is concentrated in the membrane. In chase experiments, both newly synthesized TAG and apoB-48 move from the membrane into the secreted chylomicrons. In enterocytes from chow-fed rabbits, TAG and apoB-48 do not accumulate in the lumen of the SER or the Golgi, suggesting that the transfer from the SER membrane is rate limiting. Other investigators using different approaches have also concluded that the movement of TAG from ER to Golgi is a rate-limiting step in chylomicron assembly (35, 36), although those studies did not distinguish between membrane and luminal components.

There is a considerable amount of evidence for a two-step model for VLDL assembly in hepatocytes (10–12). The first step involves the transfer of apoB-100 from the RER membrane to the lumen for assembly into a lipid-poor VLDL precursor. MTP has been implicated in this step (30–32). The apoB-100 that is not transferred to the lumen is degraded and the main site of degradation is the RER membrane (23, 25). There is also significant intraluminal degradation of apoB-100 (23, 25). The second step is the acquisition of the full complement of lipid. TAG-enriched particles apparently form in the SER and these fuse with the VLDL precursors (38, 39). Pulse-chase experiments in McArdle cells have shown that this second step, addition of bulk lipids, does not involve MTP (40), although other studies using the same cell line have reached the opposite conclusion (41). Our results indicate that chylomicron assembly differs from VLDL assembly (see **Fig. 12** for a comparision). As described above, the SER appears to be the site of transfer of both apoB-48 and TAG

into the lumen of the secretory compartment. Our previous observations of rat and rabbit hepatocytes have shown that the RER membrane is the site of accumulation and of intracellular degradation of newly synthesized apoB-100 (23, 25). In contrast, in enterocytes newly synthesized apoB-48 is concentrated in the SER membrane, suggesting that this is the site of degradation. Our present results do not demonstrate whether complete chylomicrons are transferred into the lumen of the SER in a single step, or whether two or more steps are involved in chylomicron assembly. However, when the rabbits were fed a high fat diet prior to isolation of the enterocytes, the TAG content of the SER lumen increased more than 10-fold, suggesting that under conditions of markedly increased fat absorption a further luminal step in chylomicron assembly may determine the rate of secretion, while in enterocytes from animals fed low fat chow the transfer of TAG and/or apoB into the lumen determines the rate of secretion. A two-step assembly is also indicated by the fine structural studies of Young et al. (42), who found that, in apoB-deficient fetal mice, lipid droplets accumulated in the lumen of the SER of enterocytes. In addition, in adult rat enterocytes, lipid particles lacking immunocytochemically detectable apoB are detected in the SER lumen (6).

It has been suggested that, in VLDL assembly, TAG synthesis in the SER proceeds until this neutral lipid reaches the maximum stable level in the phospholipid bilayer. At this stage, phase separation occurs and TAG particles bud from the membrane. The factors that determine whether TAG moves into the lumen, for assembly into chylomicrons, or is transferred to the cytosol for storage, are not understood. However, our observation that feeding fat increases the enterocyte cytosolic TAG, compared with chow, suggests that the transfer of TAG to the SER lumen is saturatable. Hamilton and co-workers (42) have described a genetically modified mouse line that expresses apoB in the liver but not in the intestine. Chylomicron secretion is blocked in these animals. At 19 days of gestation, when lipoprotein secretion is established in wild-type mice, the enterocytes contain lipid particles in the SER. However, in sucklings, which have a high fat diet, the enterocytes of the apoB-deficient mice become filled with TAG droplets that are cytosolic rather than membrane bound. This also suggests that the transfer of TAG from its site of synthesis in the SER becomes saturated, and the TAG moves into the cytosol.

The MTP inhibitor CCI7932 inhibits secretion of chylomicrons from isolated enterocytes. Our results have pinpointed the site of inhibition as the transfer of newly synthesized apoB and TAG from the SER membrane to the lumen. Inhibition of MTP does not block secretion of apoB-48 or TAG from the lumen of the SER. This is consistent with the observation that in abetahypolipidemia, which is a defect in MTP, chylomicron secretion is inhibited and droplets of lipid accumulate in the cytosol [discussed in (ref. 43)]. A further human genetic defect, chylomicron retention disease, specifically affects chylomicron assembly and secretion and is characterized by accumulation of lipid droplets in the ER and Golgi of enterocytes (44). The molecular basis for chylomicron retention disease is not known. However, its existence is consistent with a second factor involved in the assembly of chylomicrons, which is active after transfer of apoB-48 and lipid into the SER lumen. Such a saturatable step is also suggested by our results.

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