Direct Evidence for a Two-step Assembly of ApoB48-containing Lipoproteins in the Lumen of the Smooth Endoplasmic Reticulum of Rabbit Enterocytes*

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The aim of this study was to investigate the types and characteristics of chylomicron precursors in the lumen of the secretory compartment of rabbit enterocytes. Luminal contents were separated into density subfractions in two continuous self-generating gradients of different density profiles. In enterocytes from rabbits fed a low fat diet, newly synthesized and immunodetectable apoB48 was only in the subfraction of density similar to that of chylomicrons; low density lipoproteins (light particles). After feeding fat, newly synthesized, and immunodetectable apoB48 was in both dense (phospholipid-rich) and light (TAG-rich) particles. Luminal TAG mass and synthesis increased after fat feeding and was only in light particles. Pulse-chase experiments showed that the luminal-radiolabeled apoB48 lost from the dense particles was recovered in the light particles and the secreted chylo-

microns. All of the light particle lipids (mass and newly synthesized) co-immunoprecipitated with apoB48. How-

ever, in the dense particles, there was a preferential co-precipitation of the preexisting rather than newly synthesized phospholipid. Assembly of apoB48-containing TAG-enriched lipoproteins is therefore a two-step process. The first step produces dense apoB48 phospho-
lipid-rich particles, which accumulate in the smooth endo-

plasmic reticulum lumen. In the second step, these dense particles rapidly acquire the bulk of the TAG and additional phospholipid in a single and rapid step.

Dietary lipids are digested in the small intestine, and the products are transferred across the brush border of the enter-
ocytes. Triacylglycerols (TAG) are resynthesized and assem-
bled into chylomicrons, which are released into the lamina propria and move via the lymph into the blood. The ability of enterocytes to assemble and secrete chylomicrons is modulated by a variety of factors including the amount and composition of the dietary fats (reviewed in Refs. 1–3).

Chylomicrons consist of droplets of TAG with some choles-
terol ester, stabilized by a shell of phospholipids, cholesterol, and protein (1–3). The major protein of chylomicrons is apoli-

poprotein-B48 (apoB48), a truncated form of apoB100 produced through post-transcriptional editing of the mRNA of apoB100, the characteristic protein of very low density lipoproteins (VLDL), which transport endogenous lipid from the liver (4–6). The intracellular events in the assembly of VLDL and chylo-

microns revealed by electron microscopic studies are basically similar (7–9). ApoB is synthesized by bound ribosomes in the rough endoplasmic reticulum (RER), the lipid components are synthesized in the smooth endoplasmic reticulum (SER), and assembly of the lipoprotein particle occurs within the lumen of the ER/Golgi compartment. There is evidence for a two-step assembly of VLDL in which small, dense apoB-containing particles are formed initially in the lumen of the RER and fuse with TAG-rich particles, which form separately in the SER lumen (10–12). Generally these observations on liver have been extrapolated to the enterocyte, and it has been assumed that chylomicron assembly follows a similar pattern (reviewed in Refs. 2 and 3). However, chylomicrons are considerably larger and contain more TAG than VLDL. Enterocytes require a far larger capacity for chylomicron assembly (to accommodate the variations in the amount of dietary fat) than the liver needs for VLDL assembly. Rat hepatocytes, which synthesize apoB48 as well as apoB100, do not secrete chylomicrons, while apobec-1 knockout mice do secrete apoB100 in chylomicrons (13, 14).

Thus, assembly of chylomicrons is not determined by the form of apoB produced but is a characteristic property of enterocytes. Consistent with this, there is at least one protein that is specifically essential for chylomicron and not VLDL formation and is absent in cholesterol retention disease (15).

Although there have been many studies of VLDL assembly in liver, isolated hepatocytes, and cultured cell lines, there have been few studies of chylomicron assembly. This is in part because there is no good cultured cell model that secretes chylo-

microns containing only apoB48. Important data have been obtained using CaCO2 cells (reviewed in Refs. 2 and 3); however, these cells secrete relatively dense lipoproteins that contain apoB100 in addition to apoB48. As we are interested in the effects of diet on fat absorption, under physiological conditions, we have recently developed a method for the preparation of viable isolated rabbit enterocytes, which synthesize apoB48 and lipid (but not apoB100) and assemble and secrete chylo-

microns (16–18). We have also developed methods for the separation of the components of the secretory compartment of en-
terocytes (RER/SER/Golgi) in a single self-generating gradient that have allowed us to dissect the intracellular events in

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1 The abbreviations used are: TAG, triacylglycerol; HDL, high density lipoprotein(s); LDL, low density lipoprotein(s); VLDL, very low density lipoprotein(s); ER, endoplasmic reticulum; SER, smooth endoplasmic reticulum; RER, rough endoplasmic reticulum; HPTLC, high performance thin layer chromatography; PC, phosphatidylcholine; PE, phosphatidylethanolamine.

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chylomicron assembly (18). We have shown that in enterocytes, although apoB48 is synthesized by bound ribosomes, it is not detectable in either the membrane or lumen of the SER; membrane-associated and luminal apoB48 and TAG are both concentrated in the SER (16). This is in contrast to the hepatocyte, in which luminal-dense apoB100-containing particles and TAG-rich particles form in the SER and RES, respectively. Our observations raise questions concerning the sequence of events in chylomicron assembly vis à vis VLDL assembly. Does this take place in one or more steps? To answer this question, we have now investigated the nature of the chylomicron precursors in the lumen of the secretory compartment. Our results are consistent with a two-step assembly of chylomicrons, which exhibits differences from VLDL assembly that may be important in the regulation of fat absorption.

**EXPERIMENTAL PROCEDURES**

**Materials**—Liposep (iodixanol solution for lipoprotein separation), Optiprep (iodixanol solution for cell fractionation), and Maxipans (inert dense displacement medium) were purchased from Lipotek Ltd., UK. Vivascin centrifugal concentrators (PES membrane, 100,000 molecular weight cut off) were purchased from Sartorius Ltd., Epsom, UK. Hybridoma cells producing anti-rabbit monoclonal antibody MAC 31 were a gift from Drs. Bowyer and Gherardi, Cambridge University, UK. All other reagents were as described previously (16–18) or from Sigma.

**Animals and Diets**—Dwarf lop rabbits (~6 months old, 2.56 ± 0.12 kg) were bred in the University of Sheffield Field Laboratories. Rabbits were maintained on a 12-h light/dark cycle and allowed free access to water and low fat chow (2.5% fat (w/w) equivalent to 7% of the dietary energy intake) (chow-fed). In some experiments, the diets were supplemented with sunflower oil to increase the TAG content to 7.5% (w/v), to representative energy (16) and phosphatidylethanolamine (PE) was determined by laser densitometry (HPTLC), and stained, and the mass of TAG, phosphatidylcholine (PC), and phosphatidylcholamine (PE) was determined by laser densitometry (16–18, 21, 22). Incorporation of [14C]oleate into newly synthesized TAG, PC, and PE was determined on the same HPTLC plates using a Packard InstantImager two-dimensional counter as described previously (21, 22).

**RESULTS**

**Rabbit Plasma Lipoproteins on a Self-generating Gradients**—Rabbit plasma lipoproteins were separated by self-generated gradients of iodixanol because the density of the iodixanol fractions and then mixed for 16 h at 4°C. Immunoprecipitated apoB48-containing particles attached to the agarose beads were pelleted by centrifugation at 1700 × g for 30 min at 4°C. Immunoprecipitates were washed by resuspension in TBS and recentrifugation. Controls were carried out using the same protocol without primary antibody or with an anti-actin monoclonal antibody (gift of Dr Lynda Partridge in this department). No Immunoprecipitation of apoB48 or lipids occurred in the absence of MAC31.

**Analysis of Lipids**—Lipids were extracted from luminal content subfractions, separated by high performance thin layer chromatography (HPTLC), and stained, and the mass of TAG, phosphatidylcholine (PC), and phosphatidylcholamine (PE) was determined by laser densitometry (16–18). The incorporation of [35S]methionine into the apoB48 band was determined using the Packard InstantImager as described previously (23–25).

**Isolation and Incubation of Enterocytes and Preparation of Subcellular Fractions**—Villous enterocytes were isolated from the small intestine of rabbits and incubated for 30 min with lipid and bile salt micelles containing [14C]oleate (4 μCi) or [35S]methionine (1 μCi) to radiolabel newly synthesized TAG and phospholipid and apoB48, respectively (16–18). The enterocytes were pelleted by centrifugation and homogenized, and total microsomes were prepared and separated into RER, SER, and Golgi fractions in self-generating gradients of iodixanol (18). In some experiments, enterocytes were incubated with labeled micelles for 30 min, isolated by centrifugation, and reincubated with unlabeled micelles for a range of times. The cells were reisolated by centrifugation (14). The media were transferred to centrifuge tubes, the Beckman MLA rotor, 0.5 ml of HEPES-buffered saline was carefully pipetted on top of the media, and the tubes were centrifuged at 50,000 rpm for 1 h. After centrifugation, the interface between the HEPES-buffered saline layer and the media was distinct, and the top 0.5 ml, which was milky, was carefully removed, the apoB48-containing lipoproteins were immunoprecipitated, and the apoB48 and lipids were determined (18). In preliminary experiments, it was demonstrated that the infranatant did not contain detectable radiolabeled or immunologically detectable apoB48.

**Preparation and Subfractionation of Secretory Compartment Luminal Contents**—Microsomal membrane and luminal content fractions were prepared by treatment by the method we developed for separation of human lipoproteins (19). The enterocytes were pelleted by centrifugation and the luminal contents were concentrated to 0.2 ml by centrifugation at 4000 × g for 2 h in Vivascin ultratransfer devices followed by resuspension for 4 ml with TBS (10 mM Tris-HCl and 150 mM NaCl, pH 7.4) and reconstituted as above. The final concentrates were resuspended to 1.12 ml with TBS and mixed with 4.28 ml of Liposep solution (12% iodixanol), and the chylomicron precursors were separated in self-generated gradients exactly as described above for plasma lipoproteins.

**Immunoprecipitation of ApoB48 from Luminal Content Subfractions**—ApoB48 was immunoprecipitated from luminal content subfractions using an anti-rabbit monoclonal antibody (MAC31), which recognizes the N terminus of apoB100 (20). Samples were concentrated to 0.2 ml in Vivascin centrifugal concentrators and mixed with 0.05 ml of MAC 31 for 4 h at 4°C, followed by the addition of 0.2 ml of anti-rat IgG agarose, and then mixed for 16 h at 4°C. Immunoprecipitated apoB48-containing particles attached to the agarose beads were pelleted by centrifugation at 1700 × g for 30 min at 4°C. Immunoprecipitates were washed by centrifugation and recentrifugation. Controls were carried out using the same protocol without primary antibody or with an anti-actin monoclonal antibody (gift of Dr Lynda Partridge in this department). No immunoprecipitation of apoB48 or lipids occurred in the absence of MAC31.

**RESULTS**

**Rabbit Plasma Lipoprotein Classes Separate on the Basis of Size and/or Density in Self-generating Gradients of Iodixanol**—Self-generating gradients of iodixanol have been used for the separation of human plasma lipoproteins (19) and therefore potentially provide a simple procedure for the determination of the density distribution of luminal lipoprotein precursors in the secretory compartment of enterocytes. In initial experiments, rabbit plasma was used to establish the protocol. After centrifugation in two-step iodixanol gradients, rabbit plasma VLDL floated to the top of the gradient (Fig. 1, fraction 1), LDL peaked in the upper half of the gradient (Fig. 1, fractions 3–5), and HDL peaked in the lower half of the gradient (Fig. 1, fractions 7–10). Because of their low density (<1.000 g/ml), chylomicrons move to the top of the gradient with the VLDL; however, chylomicrons were not detectable in the rabbit plasma. The densities of the fractions in which the lipoproteins were collected were 1.032–1.21, 1.015–1.032, and 1.002–1.009 g/ml for HDL, LDL, and VLDL, respectively. It must be emphasized that this is the density of the iodixanol fractions and that the lipoproteins have slightly different densities in salt gradients because the salt removes water from the particles (19).

**Lipoprotein Precursors Are in Particles of High (~HDL) and Low Density (~Chylomicron/VLDL) in the Lumen of the Secretory Compartment**—We have previously shown that in enterocytes from rabbits fed low fat chow, most of the intracellular TAG is associated with the SER membrane, and apoB48 is both membrane-bound and in the luminal contents (18). After feeding fat, the membrane-bound TAG remains relatively unchanged, but there is a large increase in the ER luminal TAG; however, there was little change in the apoB48 (18). To deter
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Separation of rabbit plasma lipoproteins in 9–12% iodixanol gradients. Rabbit plasma (total of 1.25 ml) was separated in self-generating gradients of iodixanol and collected in 10 fractions (fraction 1 is the light end of the gradient) as described under "Experimental Procedures," using 9–12% two layers. Aliquots of the fractions were separated on agarose gels, and the cholesterol and TAG content was determined in other aliquots. The top figure shows the distribution profile of cholesterol (μmoles/fraction) (left-hand axis) and the distribution profile of TAG (μmoles/fraction) (right-hand axis). The middle figure illustrates an agarose gel; 1–10 are gradient fractions, and plasma is an aliquot of total plasma separated in a separate gel. The position of the lipoproteins, HDL, LDL, and VLDL, and the origin, where chylomicrons remain, are indicated. The bottom figure shows the density profile of the gradient determined from the refractive indices of the fractions collected (19). The results plotted illustrate a separation of one plasma sample. However, similar patterns were observed in many repeated analyses.

FIG. 1. Separation of rabbit plasma lipoproteins in 9–12% iodixanol gradients. Rabbit plasma (total of 1.25 ml) was separated in self-generating gradients of iodixanol and collected in 10 fractions (fraction 1 is the light end of the gradient) as described under "Experimental Procedures," using 9–12% two layers. Aliquots of the fractions were separated on agarose gels, and the cholesterol and TAG content was determined in other aliquots. The top figure shows the distribution profile of cholesterol (μmoles/fraction) (left-hand axis) and the distribution profile of TAG (μmoles/fraction) (right-hand axis). The middle figure illustrates an agarose gel; 1–10 are gradient fractions, and plasma is an aliquot of total plasma separated in a separate gel. The position of the lipoproteins, HDL, LDL, and VLDL, and the origin, where chylomicrons remain, are indicated. The bottom figure shows the density profile of the gradient determined from the refractive indices of the fractions collected (19). The results plotted illustrate a separation of one plasma sample. However, similar patterns were observed in many repeated analyses.

Determine the nature of the chylomicron precursors under these different dietary conditions, we analyzed the ER luminal contents from rabbit enterocytes fed a low fat chow diet and a high fat diet for 3 or 6 days. Rabbit enterocytes were incubated with micelles containing [35S]methionine (to radiolabel newly synthesized phospholipids) and [14C]oleate (to radiolabel newly synthesized lipid). The microsomal luminal contents were prepared and separated in the gradient developed for the separation of plasma lipoproteins. In luminal gradient fractions from chow-fed rabbit enterocytes and fat-fed for 6 days were incubated with micelles containing [35S]methionine for 30 min. Microsomes were isolated, the luminal contents were prepared and separated in self-generated gradients of iodixanol, and the gradient fractions were analyzed as described under "Experimental Procedures." The density distribution of radiolabeled apoB48 was plotted for the three dietary states ±SD (n = 3). A, the density distribution of radiolabeled apoB48 is plotted for the three dietary states ±SD (n = 3). B, immunodetectable apoB48 in the gradient fractions from chow-fed and fat-fed for 6 days is illustrated.

FIG. 2. Density distribution of apoB48 in luminal gradient fractions. Isolated enterocytes from rats fed chow or fat for 3 and 6 days were incubated with micelles containing [35S]methionine for 30 min. Microsomes were isolated, the luminal contents were prepared and separated in self-generated gradients of iodixanol, and the gradient fractions were analyzed as described under "Experimental Procedures." A, the density distribution of radiolabeled apoB48 is plotted for the three dietary states ±SD (n = 3). B, immunodetectable apoB48 in the gradient fractions from chow-fed and fat-fed for 6 days is illustrated.

Phosphatidylcholine (~80% of the phospholipid mass) and phosphatidylethanolamine were the only detected phospholipids in the luminal contents, and they showed similar density distributions in the gradient fractions (Figs. 4 and 5). Newly synthesized phospholipids were at a low concentration in the fractions from chow-fed rabbit enterocytes, and the amount of phospholipid in each fraction was only significant in the densest fractions from chow-fed rabbit enterocytes, and the amount of phospholipid in each fraction was only significant in the densest fraction 10, which also contained the highest concentration of apoB48 (Figs. 4A and 5A). After feeding fat, the mass of phospholipid increased in the denser fractions 7–10, coincident with the immunodetectable apoB48. Newly synthesized phospholipids showed a different distribution from phospholipid mass after feeding fat with a greater incorporation of radiolabel into fraction 1.

As the amounts of material in gradient fractions are small, especially in the samples from chow-fed rabbit enterocytes, gradient fractions were pooled into light (fractions 1–3), intermediate (fractions 4–7), and dense (fractions 8–10) to investigate the association between luminal phospholipids and apoB48. In the fat-fed enterocytes, all of the mass of the phospholipids and the newly synthesized phospholipids were co-immunoprecipitated with apoB48 in the luminal light and intermediate fractions (Figs. 4B and 5B). This was also the case.

The luminal TAG content was low in gradient fractions from enterocytes of chow-fed rabbits and only detectable in the top fraction 1 of the gradient (Fig. 3A). After feeding sunflower oil, there was an increase in both the newly synthesized TAG and the mass of TAG in this light gradient fraction (Fig. 3A). The increase was related to the length of time the high fat diet was fed from 0 to 3 and from 3 to 6 days. Almost 100% of the TAG mass and the radiolabeled TAG in the luminal light fractions 1–2 was co-immunoprecipitated with apoB48 (Fig. 3B), indicating that under all dietary conditions investigated, luminal TAG is only in apoB48-containing particles of the density of VLDL/cholesterol.

A

B

Chow-fed

Fat-fed

Density of gradient fractions

Density of gradient fractions

Chow-fed

Fat-fed
in fractions from chow-fed enterocytes, although the amounts of phospholipid were very small, and accurate measurement was not possible. In contrast, although a large fraction of the mass of the phospholipids (70–80%) in the dense fractions co-immunoprecipitated with apoB48, only ~30% of the newly synthesized radiolabeled phospholipids were co-precipitated (Figs. 4B and 5B). The specific activity of phospholipids that co-immunoprecipitated with apoB from the dense fractions was ~8-fold lower than that of phospholipids in the supernatant. Therefore, preformed phospholipids are preferentially incorporated into the dense apoB48-containing particle, whereas newly synthesized phospholipids are incorporated into the light particles.

The lipid composition of luminal gradient fractions was not altered by feeding fat. The light fraction contained 68.6 ± 0.3%, 23.7 ± 0.5%, and 7.8 ± 1.1% (n = 3) TAG, phosphatidylcholine, and phosphatidylethanolamine, respectively. The dense fraction contained 13.3 ± 1.1%, 67.3 ± 0.4%, and 19.0 ± 0.2% TAG, PC, and PE, respectively. The TAG content of the light particles compared with the phospholipid content is ~20% less than that usually reported for chylomicrons. However, to measure the very small amounts of lipids in the luminal content fractions, it was necessary to use a very sensitive technique, HPTLC to separate the lipids followed by staining with cupric acetate, which reacts with double bonds in the acyl moieties, followed by quantification by laser densitometry (21, 22). Tri-
olein was used as the standard TAG. As the hepatic TAG contains a mixture of fatty acids of different degrees of saturation, this would stain to a smaller extent per molecule than triolein, thus underestimating the amount of TAG.

The Dense ApoB48-containing Particles Float at a Similar Density to HDL on Denser Iodixanol Gradients—In iodixanol solutions, proteins have a density of $1.26 \text{ g/ml}$ (26) and would be expected to form a pellet in the 9–12% gradients used to separate luminal contents. However, using this gradient, we cannot exclude the possibility that some apoB48 in the dense fractions is free or denatured protein. Therefore, in order to check that the apoB48-containing dense fractions behave as

Fig. 4. Density distribution of PC in luminal gradient fractions. The experiment was conducted as in the legend for Fig. 3. A, the density distributions of radiolabeled PC respectively, are plotted for the three dietary states $\pm$SD ($n = 3$). B, fractions 1–3, 4–7, and 8–10 from microsomes from chow-fed and fat-fed for 6 days were pooled, and the apoB48 was immunoprecipitated. The percentage of radiolabeled PC and PE mass co-immunoprecipitating with apoB (%co-ppt) is plotted.
lipoprotein particles, the luminal contents from chow-fed enterocytes were also separated in 15% iodixanol gradients in addition to 9–12% gradients. Under these conditions, plasma HDL move to the middle of the tube, peaking in fractions 5–7, which have densities of 1.055–1.069 g/ml, while LDL move to the top of the tube in fractions of densities 1.024–1.039 g/ml (Fig. 6). VLDL are lost because they are considerably lighter than the density of the top fraction, which they move through, and adhere to the shoulders of the Optiseal tubes. In these gradients, the apoB48-containing fractions, which are at the bottom of the 9–12% gradient, moved to the middle fractions of densities 1.039–1.061 g/ml, overlapping the densities of HDL in the same gradients (Fig. 7). The small amount of TAG in the dense particles, both the mass and the radiolabeled, comigrated with the apoB48 in the gradient, as did most of the phospholipid. However, some of the phospholipid also remained in the bottom dense fractions, suggesting that there may be phospholipid-protein complexes denser than those containing apoB48 in the enterocyte secretory compartment. As apoB48 was not detected in the densest fractions of this gradient, we conclude that there is no lipid-free apoB48 in the luminal contents.

Chylomicron Precursors, ApoB48 and TAG, Are Both Located in the SER Lumen—To determine whether the luminal and dense and light apoB48-containing particle precursors are in separate intracellular compartments, the distributions of luminal contents from chow-fed enterocytes were also separated in 15% iodixanol gradients in addition to 9–12% gradients. Under these conditions, plasma HDL move to the middle of the tube, peaking in fractions 5–7, which have densities of 1.055–1.069 g/ml, while LDL move to the top of the tube in fractions of densities 1.024–1.039 g/ml (Fig. 6). VLDL are lost because they are considerably lighter than the density of the top fraction, which they move through, and adhere to the shoulders of the Optiseal tubes. In these gradients, the apoB48-containing fractions, which are at the bottom of the 9–12% gradient, moved to the middle fractions of densities 1.039–1.061 g/ml, overlapping the densities of HDL in the same gradients (Fig. 7). The small amount of TAG in the dense particles, both the mass and the radiolabeled, comigrated with the apoB48 in the gradient, as did most of the phospholipid. However, some of the phospholipid also remained in the bottom dense fractions, suggesting that there may be phospholipid-protein complexes denser than those containing apoB48 in the enterocyte secretory compartment. As apoB48 was not detected in the densest fractions of this gradient, we conclude that there is no lipid-free apoB48 in the luminal contents.

Fig. 5. Density distribution of PE in luminal gradient fractions. The experiment was conducted as in the legend for Fig. 3. A, the density distributions of radiolabeled PE, respectively, are plotted for the three dietary states ±SD (n = 3). B, fractions 1–3, 4–7, and 8–10 from microsomes from chow-fed and fat-fed for 6 days were pooled, and the apoB48 was immunoprecipitated. The percentage of radiolabeled PE mass co-immunoprecipitating with apoB (%co-ppt) is plotted.
nal apoB and TAG in subfractions of ER separated in self-generating gradients were determined. In gradient fractions from chow-fed enterocytes, both radiolabeled apoB48 and immunodetectable apoB48 were mainly in the SER lumen (fractions 4–8) and not detected in fraction 10, which contains most of the RER (18) (Fig. 8). Therefore, the dense particles that predominate under these dietary conditions are in the SER lumen. After feeding a high fat diet for 6 days, distribution of apoB48, detected by immunoblotting under the same conditions, was not altered (not illustrated); however, there was a large increase in the newly synthesized TAG and TAG mass in the SER lumen (Fig. 8). The TAG-rich light particles are therefore also in the SER lumen.

**Luminal ApoB48-containing Dense Particles Are Precursors of Light Particles and Secreted Chylomicrons**—To determine the relationship between the luminal-dense apoB48 particles and the light particles and whether these are precursors of secreted lipoproteins, isolated enterocytes were incubated (pulse) with radiolabeled [35S]methionine for 30 min followed by reincubation (chase) with unlabeled micelles for 45 min (Fig. 9). The time selected for the chase was determined in preliminary experiments in which we showed that the secretion of radiolabeled apoB48 and loss of luminal apoB48 were linear for 60 min (23). At the end of the pulse, the luminal-radiolabeled apoB48 was in the dense particles. At the end of the chase, radiolabeled apoB48 in the dense particles decreased, and radiolabeled apoB48 in the light particles and in the lipoproteins secreted into the incubation medium increased. 92% of the radiolabeled apoB48 initially in the dense particles was recovered with 23% of this in the light particles and the remainder in the medium. The secreted lipoproteins were collected from the incubation medium by flotation into a buffer of density >1.006 using a relatively short centrifugation time. These conditions are generally used for the flotation of chylomicrons from plasma. We therefore conclude that the secreted apoB48 is predominantly in chylomicrons.

**DISCUSSION**

The aim of this investigation was to determine the nature of the chylomicron precursors in the ER lumen of enterocytes to formulate a model for assembly. Our previous observation, that both TAG and apoB48 enter the lumen of the secretory compartment in the SER, had raised the possibility that chylomicrons form in a single step (18). However, the present results show that there are apoB48-containing particles of density similar to that of HDL in the ER lumen and that these are precursors of secreted lipoproteins. After feeding fat, luminal apoB48 also appears in particles of the density of chylomicrons/VLDL, consistent with a two-step model, and these light particles accumulate in the SER lumen. This is consistent with the observations of Mansbach and co-workers (27–29), who showed that in chylomicron secretion, there is a rate-limiting step between the ER and Golgi. This step is apparently saturated under conditions of fat feeding.
The small intestine secretes particles of the density of VLDL in the fasted state and of the density of chylomicrons in the fed state (1). The methods we have used previously and in the present investigation to isolate secreted lipoproteins from the incubation medium or from plasma are those normally used to prepare chylomicrons (16–19). A short centrifugation step is not sufficient to float most VLDL, which are usually isolated by adjusting the density of plasma to 1.019 g/ml followed by 18–24 h centrifugation at >100,000 g. However, we cannot exclude the possibility that isolated enterocytes secrete a range of particles from the density of light VLDL to that of chylomicrons. However, all of the particles contain apoB48. Similarly the luminal light particles are in the fraction of density 1.0019–1.0086 g/ml. However, as plasma VLDL are also recovered in this fraction, it is possible that the luminal contents contain particles of the density of VLDL as well as of chylomicrons.

There are several possible sequences of events by which chylomicrons may be formed in the second step (reviewed in Ref. 3). The dense particles may fuse with a preformed TAG-rich light particle, or there may be core expansion by sequential or continuous addition of lipid to the dense particles. In the latter case, it would be expected that a range of TAG-containing particles of intermediate density would be produced. However, our results reveal only dense and light apoB48-containing particles and light TAG-containing particles. There is some phospholipid in the intermediate density fractions from fat-fed enterocytes. However, TAG in these fractions is almost undetectable, and apoB48 is at a low concentration. Thus, when the chylomicron production rate is increased, there may be some intermediate density particles relatively enriched in phospholipid, but these contain only a small fraction of the luminal apoB48. The results indicate that the bulk of the TAG must be acquired by the dense particles in one step. All of the lipid in the light particles co-immunoprecipitates with apoB48, indicating that transfer of TAG into the SER lumen must be either coincident with or followed rapidly by fusion with dense particles. A large proportion of the apoB48 in the SER is membrane-bound (17). This may include dense particles in the process of fusion with TAG droplets in the membrane. Indeed, Rustaeus et al. (30) have shown that in McA7777 cells, part of the membrane-associated apoB is not integrated and is removed by mild detergent treatment. There is evidence from studies of genetically manipulated mice (deficient in intestinal apoB) that chylomicron-sized particles lacking apoB48 can form in the ER lumen of the enterocytes in addition to the large accumulation of cytosolic TAG droplets (31). These ER droplets are not observed in the suckling or adult mice in which TAG only accumulates in the cytosol (32). The reason for these conflicting observations is not clear; however, the enterocytes of fetal mice are essentially fasted and are not involved in the active absorption of dietary fat. In the normal adult enterocytes, TAG transfer into the lumen may be facilitated by fusion with apoB-containing dense particles but occur to a low extent in the absence of apoB.

We have previously calculated that in chow-fed enterocytes, ~97% of the cellular TAG is recovered in the microsomal membrane (17, 18). During a 90-min incubation, secreted TAG is equivalent to ~40% of the initial cellular TAG (17, 18). The microsomal membrane therefore contains sufficient TAG to account for that secreted. In the fat-fed enterocytes, ~60% of the cellular TAG is recovered in the microsomal membranes, and ~40% is recovered in the cytosol (17, 18, 33). During a 90-min incubation, secretion of TAG accounts for about 60% of the cellular TAG. It is possible in this case that cytosolic TAG contributes to secreted TAG in addition to that in the membranes. The TAG content of the SER membrane is ~33 μg/mg of phospholipid in chow-fed enterocytes and ~66 μg/mg of phospholipid in the fat-fed enterocytes (18). This is sufficient to dissolve in the membrane bilayer (34). However, TAG may be sequestered in regions of the membrane and separate by phase partition for incorporation into luminal lipoproteins. We have previously suggested (18) that the transfer of TAG from the SER membrane into the lumen for incorporation into light particles may be rate-limiting in the chow-fed enterocytes so that light particles are immediately secreted. The present results are consistent with this as only dense particles are present in the lumen of the chow-fed enterocytes. In the enterocytes from fat-fed rabbits, light particles are detected in the luminal fractions. As we have suggested before (18), the movement of chylomicron precursors from the SER to the Golgi may also be
saturatable under conditions of increased chylomicron assembly and secretion. The mechanisms involved in the transfer of TAG remain to be elucidated.

Diacylglycerol acyltransferase, the enzyme involved in the final step of TAG synthesis, has recently been shown to be active at both the cytosolic and luminal side of the ER membrane (35). This raises the possibility that triglycerides destined for secretion and for the cytosol are synthesized at different sites. However, transfer of TAG into the ER lumen is saturatable, and under conditions of high fat load, TAG is also transferred to the cytosol, so the two enzyme activities must be coordinately regulated (17). Luminal transfer does not appear to be limited by the availability of “acceptor” dense particles, which are present under all dietary conditions investigated. Transfer of the membrane-bound TAG to the apoB48-containing particle may involve a number of factors including microsomal triglyceride transfer protein (MTP), which has been implicated in intestinal fat absorption (36). We have previously shown that inhibition of MTP reduces the transfer of SER membrane-bound TAG into the lumen and its subsequent secretion (18). Although in vitro MTP transfers lipid by a shuttle mechanism, it does form a complex with apoB48 (37–39) and thus may facilitate the association of the apoB48 in the dense particles with TAG-rich droplets.

ApoB is an extremely hydrophobic protein and must be stabilized during its translocation into the ER lumen and in the dense particles. Our observations suggest that phospholipids, particularly PC, may fulfill this role. This is consistent with the observation that the initial steps in lipoprotein formation involve binding of phospholipid by the N terminus of apoB (40). We did not detect other lipid classes in the dense particle fractions, although it is possible that small amounts are present below the limits of detection by HPTLC. In our short term experiments, stabilization of apoB48 in the dense particles uses preformed rather than newly synthesized phospholipids. However, newly synthesized TAG and phospholipids appear in the light particles. Luchoomun and Hussain (41) have also shown that chylomicrons secreted by CaCO2 cells tend to contain preexisting phospholipids, whereas newly synthesized TAG is preferentially secreted. Our results suggest that this is due to compartmentalization of the lipids during the assembly process.

In summary, this study shows directly that chylomicron assembly in adult enterocytes involves formation of dense apoB48-containing particles, which are stabilized by phospholipids. In contrast to VLDL assembly in hepatocytes, these particles accumulate in the SER lumen. The bulk of the chylomicron TAG is acquired in a single step, which also occurs in the SER lumen. However, we did not detect TAG-rich apoB48-deficient particles, suggesting that in enterocytes, the second assembly step may involve either the budding of TAG-containing particles into the lumen followed by rapid fusion with dense particles or that assembly may take place at the SER membrane surface. The regulation of the second step is important in the determination of the size, composition, and rate of production of chylomicrons.

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FIG. 9. Fate of newly synthesized luminal apoB48 in dense particles in isolated enterocytes. Isolated enterocytes from fat-fed rabbits were incubated with [35S]methionine for 30 min (pulse). The cells were isolated by centrifugation and reincubated with unlabeled micelles and an excess of unlabeled methionine (18) for 45 min (chase). At the end of the pulse and the chase periods, the cells were pelleted by centrifugation, and secreted lipoproteins were isolated from the incubation medium. Luminal content fractions were prepared and separated in 9–12% density gradients, and radiolabeled apoB48 in the fractions was determined as described under “Experimental Procedures.” The radiolabeled apoB48 in the luminal fractions and the medium was normalized to 1 g of enterocytes. The data plotted are from a typical experiment. A, distribution of radiolabeled apoB48 at the end of the pulse and the end of the chase. B, sum of the radiolabeled apoB48 in the luminal fractions at the end of the pulse and the end of the chase and recovered from the media as described under “Experimental Procedures.”
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