The Assembly and Secretion of ApoB 100 Containing Lipoproteins in Hep G2 Cells

EVIDENCE FOR DIFFERENT SITES FOR PROTEIN SYNTHESIS AND LIPOPROTEIN ASSEMBLY*

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Pulse-chase studies combined with subcellular fractionation indicated that LpB 100 (i.e. the apoprotein B (apoB) 100 containing lipoproteins) was released to the lumen of the secretory pathway in a subcellular fraction enriched in smooth vesicles, and referred to as SMF (the smooth membrane fraction). The migration of apoB 100 during ultracentrifugation as well as kinetic studies indicated that the fraction was derived from a pre-Golgi compartment, probably the smooth endoplasmic reticulum (ER). Only small amounts of apoB 100 could be detected during these pulse-chase experiments in the subcellular fractions derived from the rough endoplasmatic reticulum (RER).

SMF contained the major amount of the diacylglycerol acyltransferase activity present in the ER, while the major amount of membrane bound apoB 100 was present in the RER.

Pulse-chase studies of the intracellular transfer of apoB 100 demonstrated the formation of a large membrane-bound preassembly pool in the ER, where no significant amount of apoB 100 radioactivity was present in the membrane of the Golgi apparatus. The maximal radioactivity of LpB 100, recovered from the ER or the Golgi lumen, was small compared with the radioactivity recovered from the ER membrane, indicating that the assembled LpB 100 rapidly leaves the cells. This in turn indicates that the rate-limiting step in the secretion of apoB 100 was the transfer of the protein from the ER membrane to the LpB 100 in the lumen. A portion of the intracellular pool of apoB 100 was not secreted but underwent posttranslational degradation.

Apolipoprotein B 100 is the quantitatively dominating protein component of the liver derived very low and low density lipoproteins (VLDL1 and LDL). The major apoB 100-containing lipoprotein that is secreted from the Hep G2 cells is a triacylglycerol-rich particle with a density and size of a LDL particle (1, 2). We have previously identified this particle (1) in the secretory pathway of the cells and partially characterized it. In the present paper we will refer to the apoB 100-containing lipoproteins that are assembled in and secreted from the Hep G2 cells as LpB 100.

The assembly of LpB 100 is a sequential process that starts with a cotranslational association between apoB 100 and the membrane of the endoplasmic reticulum (ER) (1, 3, 4). This interaction between apoB 100 and the ER membrane directs the protein to the cell structure that houses the enzymes involved in the synthesis of the neutral lipid (for review with references see Ref. 5). These enzymes are bound to the membrane of the ER (6–9), and the reactions leading to the formation of triacylglycerol and cholesteryl esters are carried out in the cytoplasmic leaflet of ER. It is possible that the interaction between apoB 100 and the neutral lipids, leading to the formation of lipoproteins, occurs within the ER membrane, and that the formed lipoproteins are transferred to the lumen of the secretory pathway during this assembly process (1, 5, 10, 11).

Our earlier results (1) indicate that apoB 100 is present in the ER membrane for 10–15 min before it leaves the membrane and appears in the lumen of the secretory pathway. These results may be compatible with a transfer of the protein from the site of protein synthesis to a site of lipoprotein assembly, where the protein is transferred to the lumen of the secretory pathway bound to lipoproteins. In the present paper we have further tested this possibility by investigating the relation between the compartment where LpB 100 is released to the lumen of the secretory pathway and the compartment where the protein is synthesized. We have also followed the intracellular transport of apoB 100 and the newly assembled apoB 100-containing lipoproteins.

EXPERIMENTAL PROCEDURES

Materials—Eagle's minimal essential medium was from Flow Laboratories (Irvine, United Kingdom) while Eagle's minimal medium without methionine was from Gibco. UDP-[14C]glucosamine, [14C]methylated protein standards ("rainbow standards"), [35S]methionine, [1-14C]-palmitoyl-CoA, and Amplify were purchased from Amer sham Int. (Amersham, United Kingdom). Ready Safe was from Beckman (Fullerton, CA). UDP-N-acetyl-[6-14C]glucosamine was from Du Pont-New England Nuclear. NADPH, cytochrome c, and Endoglycosidase H were from Boehringer Mannheim. UDP-galactose, UDP-GlcNAc, ATP, ovalbumin (Fraction V), Triton X-100, oleic acid ("cell culture grade"), fatty acid-free bovine serum albumin, palmityl-CoA, L-phosphatidylcholine (from egg yolk), 1,2-dioleoyl-sn-glycerol and L-phosphatidyl-L-serine (from bovine brain) were from Sigma. Immunoprecipitin was purchased from Bethesda Research

*This paper was supported by Grants 7142, 8862, 4531 from the Swedish Medical Research Foundation, the Heart and Lung Foundation, the Swedish Olo-Margarine Foundation for Nutritional Research, the Swedish Diabetes Society, the Göteborg Medical Society, and the Swedish Society for Medical Research. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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Received for publication, October 17, 1989.
Laboratories. Transylol (Aprotinin) was from Bayer Leverkusen (West Germany). Antibodies against \( \alpha \)-antitrypsin were from DAKO-PATTS (Copenhagen, Denmark). All chemicals used for SDS-polyacrylamide gel electrophoresis were from Bio-Rad. PAA 2–16% non-denaturating polyacrylamide gels were from LKB (Bromma, Sweden). All chemicals were of analytical grade.

The subcellular fractionation experiments were carried out on confluent Hep G2 cells cultured in 56 cm² culture dishes (1, 3). Oleic acid (1) was present in the culture medium during at least 48 h before the experiment was started.

The labeling as well as the chase of the cells were carried out as described earlier (1, 3). In some experiments, the chase was carried out in the presence of 10 \( \mu \)M cycloheximide. Control experiments showed that this concentration of cycloheximide gave total inhibition of the synthesis of apoB 100.

**Subcellular Fractionation**—The cells were harvested and homogenized as described earlier (1, 3). Subcellular fractionation was carried out on a sucrose gradient formed by overlaying a cushion of 0.5 M of 49% sucrose with a 3.7-ml linear sucrose gradient from 32 to 40%. The sample, in 1.0 ml, was layered on top of the gradient. All solutions used contained 3 mM Imidazol, pH 7.4, with 0.1 mM leupeptin, 1 mM phenylmethylsulfonyl fluoride, and 100 KIU/ml aprotinin. Centrifugation was carried out in a Beckman VTi 65.2 vertical rotor at 50,000 rpm for 3 h at +4 °C. The tubes were unloaded from the bottom into 24 fractions, and each fraction was assayed for marker enzymes. The NADPH-cytochrome c reductase activity (ER) and the galactosyltransferase activity (trans-Golgi) was analyzed by the methods described in Refs. 12 and 13 whereby the N-acetylglucosaminyl transferase activity (cis-Golgi) was assayed by the method described in Ref. 14. The isolated vesicles were disrupted by sodium carbonate treatment, and the separation of Na\(_2\)CO\(_3\) released material from the membranes was carried out as described (3).

**Endoglycosidase H (Endo H) Treatment of \( \alpha \)-Antitrypsin**—Hep G2 cells, previously incubated for 48 h with oleic acid, (see above) were labeled for 3 h with 250 \( \mu \)Ci of \( ^{35} \)S]methionine/56-cm² culture dish and subjected to subcellular fractionation as described above. \( \alpha \)-Antitrypsin was recovered from each fraction by immunoprecipitation and divided into two portions, one of which was incubated with Endo-H as described in Ref. 15. Both samples were analyzed by SDS-polyacrylamide gel electrophoresis, followed by autoradiography.

**Ultra-centrifugation of the Lipoproteins Present in the Luminal Content of the Subcellular Fractions**—Two ultracentrifugation methods were used to recover the apoB 100 lipoproteins (LpB 100) from the luminal content of the subcellular fractions. One of these methods was based on ultracentrifugation in a sucrose gradient. This method was a slight modification of the method published earlier (1). The luminal content of the recovered subcellular fractions was adjusted to a sucrose concentration of 95%. Three ml were layered on top of a 1.0-ml cushion of 49% sucrose with a 3.7-ml linear sucrose gradient from 32 to 40%. The sample, in 1.0 ml, was layered on top of the gradient. Ultracentrifugation was carried out in a Beckman SW 40 rotor at 35,000 rpm for 65 h at +4 °C. The tubes were unloaded from the bottom into 24 fractions, if not stated otherwise in the text. A dilution series of the labeled microsomes (1/2, 1/4, 1/8, 1/16, and 1/32) was created either in 1 mM Imidazol, pH 7.4, with 250 mM sucrose (Fig. 1A), or in unlabeled microsomes (Fig. 1B), and the apoB 100 radioactivity was determined in each sample as described above. The dilution series covered the amount of vesicles subjected to immunoprecipitation in the different experiments of this paper. The results (Fig. 1, A and B) showed a linear relation between the recovered apoB 100 radioactivity and the amount of labeled microsomes subjected to immunoprecipitation.

The variation within assay was 1.8% (mean of four different experiments) while the variation between assays was 3.9% (based on variation on 3 consecutive days).

A dilution series was also created for the labeled culture medium (1/2, 1/4, 1/8, 1/16, and 1/32) in unlabeled culture medium (Fig. 1C) and apoB 100 was recovered from each sample as described above and the radioactivity counted. The results (Fig. 1C) showed a linear relation between the recovered apoB 100 radioactivity and the amount of labeled medium subjected to immunoprecipitation. The dilution series...
of the labeled medium covered the amount of medium subjected to immunoprecipitation in the different experiments of this paper.

The variation within assay was 1.5% (mean of four different experiments) while the variation between assay was 3.1% (based on analysis on 3 consecutive days).

The analyses of the apoB 100 radioactivity of one experiment were carried out on the same day.

Electron Microscopy—The subcellular fractions were fixed in glutaraldehyde, post-fixed in osmium tetroxide and stained en bloc with 1% uranyl acetate in 70% ethanol (17). After dehydration in a graded series of ethanol solutions, the samples were embedded in agar resin (100 mesh cut into ultrathin sections on an LKB Ultrotome V). The samples were counterstained with alkaline bismuth solution (18) and examined in a Philips 400 transmission electron microscope.

The Determination of the Dioleoylphosphatidylcholine Activity—The assay was a modification of that reported by Polokoff and Bell (19). For one assay, 1.2 mg of phosphatidylcholine and phosphatidylserine were sonicated for 20 s under the conditions given above. The radioactive fraction was scraped off the thin layer plate and the radioactivity counted.

The reaction was stopped by transferring the incubation mixture to a two-phase system (20) and 0.1 mg of triolein was added as a carrier. The lipid phase was recovered and fractionated on thin layer chromatography using chloroform/acetic acid (96:4) as solvent. The triacylglycerol fraction was scraped off the thin layer plate and the radioactivity counted.

Electrophoresis—SDS-polyacrylamide gel electrophoresis was carried out in gradient (3-15%) gels as described earlier (1, 3). Autoradiography was carried out by fluorography using Amplify as fluorographic reagent. For electrophoresis in non-denaturing polyacrylamide gel electrophoresis, we used Pharmacia PAA 2-16% gradient gels under the conditions recommended by the manufacturer. The gels were autoradiographed, and the autoradiographs were scanned with a LKB Ultrascan XL laser densitometer.

Determination of Lipid-Phosphorus—Lipids were extracted as described in Ref. 21, and the lipid-phosphorus was determined by the method described in Ref. 22.

RESULTS

Characterization of the Subcellular Fractions—The subcellular fractions recovered from the gradient were characterized by marker enzymes for the endoplasmic reticulum (NADPH-cytochrome c reductase) and the Golgi apparatus (N-acetylglucosamine phosphotransferase (cis-Golgi) and galactosyltransferase (trans-Golgi). The results are shown in Fig. 2. The major peak of NADPH-cytochrome c reductase activity occurred between fraction 1 and fraction 5 with a peak maximum at fraction 3, while the cis-Golgi marker enzyme started to appear in fraction 13 (Fig. 2A). There was no distinct separation between the marker enzymes for cis- and trans-Golgi, indicating that these two Golgi subfractions do not separate from each other under the conditions used (Fig. 2A).

To identify the position of the medial-Golgi vesicles in the gradient, we followed the processing of the carbohydrate moiety of α1-antitrypsin (23). The obtained results (Fig. 2B) indicated that the first 10 fractions of the gradient contained the Endo-H-sensitive form of the protein, p1 (23), while the Endo-H-resistant form, p2 (23), generated by the action of mannosidase II in the medial-Golgi, is present in fractions 13 to 18 (Fig. 2B). The mature form, that is formed in the trans-Golgi, was present in fractions 10-22 (Fig. 2B).

In conclusion, our results indicated that fractions 1 to 10 were, to a major extent, derived from the ER compartments, while fractions 11-21 contained vesicles that were derived from the Golgi apparatus.

An analysis of the first 11 fractions by electron microscopy showed that fractions 1-5, comprising the peak of NADPH-cytochrome c reductase activity, were characterized by rough membranes, while fractions 6–11 (the region between the peak of the NADPH-cytochrome c reductase activity and the peak of the cis-Golgi marker) were enriched in smooth vesicles (Fig. 3).
The distribution of membrane-bound apoB 100 and the diacylglycerol acyltransferase activity over the first 12 fractions of the gradient (the fractions that sedimented faster than the cis-Golgi marker) were also investigated. The highest amount of membrane-bound apoB 100 radioactivity recovered after a 3-h labeling period was found in the most dense ER fractions; the distribution of the protein followed the distribution of the NADPH-cytochrome c reductase activity very closely (Fig. 4).

The diacylglycerol acyltransferase activity had a bimodal distribution (Fig. 4). One maximum coseminated with the maximal amount of NADPH-cytochrome c reductase, however, the major peak of activity was present between fraction 5 and fraction 9. This bimodal distribution was reproduced in three different experiments.

The Appearance of LpB 100 in the Secretory Pathway—Pulse-chase studies combined with subcellular fractionation was used to follow the appearance of LpB 100 in the secretory pathway. The cells were labeled with \[^{35}S\]methionine for 10 min and chased for 0, 5, 10, 15, 20, 30, and 40 min. After each chase period LpB 100 was isolated as the d < 1.065 g/ml supranatant from the lumen of the following fractions: the ascending part of the NADPH-cytochrome c reductase peak (this fraction will be referred to as RER:I (rough ER:I), the descending part of the same peak (RER:II) and the part of the gradient between the NADPH-cytochrome c reductase and the Golgi peak (this fraction will be referred to as SMF). For the localization of these fractions on the subcellular gradient see also Fig. 2A. ApoB 100 was recovered from LpB 100 and the radioactivity determined.

The maximal LpB 100 radioactivity was found after 30 min in all three fractions (Fig. 5A). Experiments with the total ER fraction (containing RER:I, RER:II, and SMF) showed a pulse maximum between 20 and 30 min (20, 20, and 30 min in three different experiments (not shown). The kinetics for the appearance of LpB 100 in the two portions of the NADPH-cytochrome c reductase peak (i.e. RER:I and RER:II) was very similar (Fig. 5A), with the exception of a significant increase in LpB 100 radioactivity at 30 min chase in RER:I.

The LpB 100 radioactivity increased much more rapidly during the first 20 min chase (i.e. before the pulse maximum was reached) in SMF than in the other two fractions (Fig. 5A). The increase in LpB 100 radioactivity during this period was 7 dpm/nmol lipid-phosphorus and min in SMF (as estimated from a linear regression analysis), while the corresponding figure for RER:I and RER:II was 1.4 and 1.6 dpm/nmol lipid-phosphorus and min, respectively. The radioactivity was normalized in this experiment to lipid-phosphorus. The conclusion from the experiment, however, was not dependent on this normalization. Thus, the increase in absolute LpB 100 radioactivity during the first 20 min of chase was 295 dpm/min in SMF while the corresponding values for RER:I and RER:II was 88 and 89 dpm/min, respectively.

The kinetics for the appearance of LpB 100 in SMF were also compared with that of the first portion of the Golgi peak (Fig. 5B). The kinetics differed between the two fractions, and a defined maximum was not reached in the Golgi fraction during the chase periods investigated in this experiment.

Together these results indicated that LpB 100 starts to appear in the SMF. This possibility was tested in two additional pulse-chase experiments.

In the first, the cells were labeled for 15 min and chased for 15 min. The appearance of LpB 100 in the three ER fractions was investigated by electrophoresis in non-denaturing polyacrylamide gels. The results (Fig. 6A) showed that the major amount of LpB 100 radioactivity was present in SMF (Fig. 6A, lane 3), while only small amounts or traces of LpB 100 radioactivity were seen in RER:I and RER:II, respectively. 15 min pulse and 15 min of chase correspond to the ascending portion of the curves seen in Fig. 5A, i.e. before the maximum was reached after 20–30 min of chase.

The size of LpB 100 recovered after 15 min of pulse and 15 min of chase from the ER fractions was very similar to that of the major apoB 100 lipoprotein secreted into the medium (Fig. 6B). In the second pulse-chase experiment, the cells were labeled for 15 min and chased for 0, 30, and 45 min. The luminal content of RER:I and RER:II as well as SMF were analyzed after each chase period by sucrose gradient ultracentrifugation in order to investigate the presence of apoB 100-containing lipoproteins with densities similar to the major apoB 100-
FIG. 6. A, polyacrylamide gradient electrophoresis of LpB 100 recovered from RER:I (lane 1), RER:II (lane 2), and SMF (lane 3) after a 15-min pulse with 1.5 mCi [35S]methionine/56 cm2 culture dish followed by a 15-min chase. The cells were subjected to subcellular fractionation after the chase period, and the d < 1.065 g/ml supernatant was isolated from the luminal content of the three fractions and analyzed by electrophoresis in 2-16% polyacrylamide gradient gels, followed by autoradiography. B, electrophoresis in polyacrylamide gradient gels of the d < 1.065 g/ml supernatant of the luminal content of SMF (cf. Fig. 2) recovered after 15 min of labeling followed by a 15-min chase. The cells were subjected to subcellular fractionation after the chase period, and the d < 1.065 g/ml supernatant recovered from RER:I (lane 1), RER:II (lane 2), and SMF (lane 3). The autoradiographs were scanned with a LKB Ultrascan XL laser densitometer.

containing lipoproteins secreted into the medium. The results (Fig. 7) showed that such a lipoprotein was formed in the SMF but not in the RER fractions during this pulse-chase experiment (compare Figs. 7 and 8).

The Intracellular Transfer and Secretion of LpB 100—Fig. 9 shows an experiment in which the cells have been cultured for 5 min with [35S]methionine and chased for 0, 5, 10, 15, 20, 30, 40, 50, 90, and 120 min. After each chase period, the completed apoB 100 protein was recovered from the membrane and from LpB 100 recovered from the ER and the Golgi as well as from the medium. RER:I and RER:II as well as SMF were included in the total ER fraction in these experiments.

The first apoB 100 radioactivity was recovered from the membrane of the ER. The radioactivity reached a maximum after 5-10 min, that is, before a substantial amount of apoB 100 radioactivity could be recovered from LpB 100 in the ER. The maximal radioactivity recovered in LpB 100 was relatively low both in the ER and the Golgi. The pulse maximum in the Golgi was reached after 40 min (three different experiments), while it was reached after 20-30 min (see above) in the ER.

The pulse maximum was two to three times higher in the Golgi than in the ER.

It could be argued that the low amount of LpB 100 recovered from the ER and Golgi lumen could be due to a loss of lipoprotein during the isolation procedure and therefore, be underestimated. The observation of a total recovery of apoB 100 (i.e. the sum of the apoB 100 radioactivity recovered from the membrane, from LpB 100, and from Fraction I (cf. Ref. 1) from the ER of 80% and from the Golgi of 97% provides evidence against any major loss of material after disruption of the vesicles. However, there could be a general loss of luminal content due to incomplete disruption of the vesicles. Control experiments published elsewhere (1, 3) have excluded this possibility. It is, however, possible that LpB 100 precipitates, or in other ways, interacts with the membrane during the disruption of the vesicle. Such mechanisms may direct apoB 100 radioactivity from the lipoprotein fraction to the membrane that is recovered as a pellet after the disruption. The observation of a small amount of apoB 100 radioactivity present in the Golgi membrane does not support this possibility (see also Ref. 3). A control experiment was also carried out in which cold ER and Golgi fractions were mixed with the labeled luminal content from the total microsomal fraction (1) or labeled medium. The mixture was subjected to the conditions used for the disruption of the vesicles. After separation of the membrane pellet from the supernatant, the apoB 100 radioactivity was determined in the two fractions. The results showed that 1 ± 0.5% (mean ± S.D., n = 6) of the apoB 100 radioactivity was recovered in the ER membrane pellet (i.e. 99 ± 0.5% (mean ± S.D., n = 6) was present in the supernatant). The corresponding values after the incubation with the Golgi membranes were 0.9 ± 0.3% and 99.1 ± 0.3% (mean ± S.D., n = 6). The recovery of apoB 100 in the d < 1.065 g/ml supernatant during the ultracentrifugation that followed the incubation with the membrane was 87 ± 15% (mean ± S.D., n = 7).

Together these results provide evidence against the possibility that the low recovery of LpB 100 from the ER and Golgi lumen is due to major losses of the lipoprotein during the preparation.

It should be pointed out that the results given in Fig. 9 are corrected for cross-contamination of the subcellular fractions (13%), the recovery of ER and Golgi markers (the recovery of the ER and Golgi markers were 70 ± 2.7 and 76.8 ± 5.0%, respectively (mean ± S.D., n = 5), as well as the recovery of apoB 100 (see above). We have, however, not corrected for the recovery of LpB 100 during the ultracentrifugation.

LpB 100 started to be secreted into the medium after 20-30 min of chase. The secretion rate of LpB 100 did not reflect the decay rate of apoB 100 in the ER membrane. This observation suggests that not all of the formed apoB 100 is secreted into the medium. One explanation may be a posttranslational degradation of apoB 100 (24). To further address this question, we carried out the experiments that are shown in Fig. 10A. The cells were labeled for 3 h with [35S]methionine and chased in the presence of cycloheximide for periods between 0 and 360 min. After each chase period, apoB 100 was isolated from the cells as well as from the medium, and the total recovery of apoB 100 from the system was determined. The results showed a total loss of apoB 100 from the system. The majority of this loss occurred during the first 120 min of chase. The recovery of apoB 100 was 50-60%.

These experiments were based on the isolation of apoB 100 both from the cells and from the medium. It is possible that the secreted apoB 100 that is accumulated in the medium is removed from the medium by, for example, the LDL receptor. This would give a false low estimation of the amount of apoB 100 that is secreted from the cells. This possibility was tested in the experiment shown in Fig. 10B. Hep G2 cells were labeled for 3 h with [35S]methionine. The medium was changed and the cells chased for 180 min. The chase medium,
FIG. 7. Sucrose gradient ultracentrifugation of the apoB 100 containing lipoproteins formed in RER: I (A), RER: II (B), and SMF (C) during a pulse-chase experiment. The Hep G2 cells were pulse-labeled with 2 mCi of [35S]methionine/56-cm² culture dish for 15 min and chased for periods of 0, 30, and 45 min. The luminal content was recovered from the ER fractions after each chase period and analyzed by sucrose gradient ultracentrifugation. The gradient (total volume 12.4 ml) was unloaded from the bottom into 24 fractions, and apoB 100 was recovered from each fraction by immunoprecipitation and SDS-polyacrylamide gel electrophoresis and the radioactivity counted. Only the portion of the gradient showing LpB 100 (cf. Ref. 1) is shown in the figure.

X̄ X̄ X̄, ApoB 100 radioactivity; X X X X X X, density.

FIG. 8. Sucrose gradient ultracentrifugation of apoB 100-containing lipoproteins secreted by the Hep G2 cells. Hep G2 cells were incubated for 3 h with 1 mCi of [35S]methionine/56 cm² culture dish. The medium was recovered and subjected to ultracentrifugation in a sucrose gradient (cf. "Experimental Procedures"). The gradient was unloaded into 24 fractions, and apoB 100 was recovered from each fraction by immunoprecipitation and SDS-polyacrylamide gel electrophoresis and the radioactivity counted. The sucrose gradient is indicated in the figure (—–––).
loss of apoB 100 occurred during the incubation with the Hep G2 cells (Fig. 10B).

DISCUSSION

This paper deals with the assembly and the intracellular transfer of the apoB 100-containing lipoproteins in the Hep G2 cells.

It is well known that apoB 100, synthesized in the Hep G2 cells, is mainly secreted bound to lipoproteins with a density and size similar to that of LDL (1, 2). However, contrary to LDL, the apoB 100-containing lipoproteins secreted from the HepG2 cells are rich in triglycerides (1, 2). Very small amounts of VLDL particles are secreted from the cells (1, 2).

In both the present as well as in an earlier paper (1), we have identified a lipoprotein in the secretory pathway of the cell with a size and density similar to those of the major apoB 100 lipoprotein secreted into the medium. This lipoprotein (LpB 100) has a lipid core composed mainly of triglycerides, although substantial amounts of cholesterol esters were also found (1).

Previous studies (1, 3, 4) have indicated that the assembly starts with a cotranslational integration of apoB 100 into the ER membrane. The protein resides in the membrane for 10–15 min before it is transferred from this “pre-assembly pool” to the lumen of the secretory pathway, where it could be recovered in lipoprotein form. This observation could suggest that apoB 100 is transferred from the site of synthesis to a specific site of assembly where the lipoproteins are formed and transferred to the lumen. This possibility was tested in the present study in which we investigated the relation between the compartment where LpB 100 was released to the lumen of the secretory pathway and the site of protein synthesis. We established a gradient for subcellular fractionation which allowed a partial separation between the major peak of NADPH-cytochrome c reductase activity containing rough ER and a fraction enriched in smooth vesicles. Both fractions sedimented faster than the markers for cis-, medial- and trans-Golgi.

In a series of pulse-chase experiments, we followed the appearance of LpB 100 in these rough and smooth membrane fractions. (During these experiments we divided the rough membrane fraction into two parts: the ascending (referred to as RERI) and the descending (referred to as RERII) portion of the NADPH-cytochrome c reductase peak).

The different pulse-chase experiments each indicated that LpB 100 started to appear in the lumen of the smooth membrane fraction. Relatively small amount of LpB 100 radioactivity were seen in the rough membrane fraction into two parts: the ascending (referred to as RERI) and the descending (referred to as RERII) portion of the NADPH-cytochrome c reductase peak.

The different pulse-chase experiments each indicated that LpB 100 started to appear in the lumen of the smooth membrane fraction. Relatively small amount of LpB 100 radioactivity were seen in the rough membrane fractions, RERI and RERII, during these pulse-chase experiments, with the exception of one experiment, which showed a significant increase in LpB 100 in RERI after 30 min of chase. The reason for this is not clear. There is, however, most likely a technical explanation, since we failed to detect any significant amount of LpB 100 radioactivity in RERI (or in RERII) in the two other pulse chase experiments. Both these studies indicated that LpB 100 started to appear in the smooth membrane...
fraction was also suggested from previous studies (1, 30).

The use of non-denaturing polyacrylamide gels to identify LpB 100 in the d < 1.065 g/ml supernatant in one of these experiments allowed us to rule out the possibility that the difference in recovery of LpB 100 from the different ER fractions was a function of the difference in immunoreactivity.

We believe that our results indicate that LpB 100 is formed in a discrete part of the secretory pathway. It should, however, be pointed out that we have not excluded the possibility that the failure to detect LpB 100 in the RER:1 and II could be due to a rapid degradation of LpB 100, exclusively occurring in the rough ER.

A posttranslational degradation of apoB has been described in the rat (24), and the results of the present paper as well as in a previous publication (1) indicated that such a degradation also occurs in the Hep G2 cells.

The site of the posttranslational degradation of apoB 100 has not been unequivocally identified, however, recent results (25), showing the presence of apoB fragments within the rough and the smooth ER but not in the Golgi, have been taken to suggest that the degradation of apoB 100 occurs in the ER but not in the Golgi. These observations are in agreement with the results that suggest that posttranslational degradation of other secretory proteins occurs in a pre-Golgi compartment possible in the ER (see for example Refs. 26–28).

Although the possibility of a posttranslational degradation of LpB 100 in the rough ER needs further investigations, our results indicate that the LpB 100 that enter the transport mechanism for secretion are assembled in a smooth membrane compartment. Thus, our results indicated that apoB 100 is transferred from the site of synthesis in the rough ER to this smooth membrane compartment. This smooth membrane compartment gave rise to vesicles that sedimented faster than the Golgi markers during the subcellular fractionation and we could demonstrate that the kinetics for the appearance of LpB 100 in the smooth membrane fraction differed considerably from that of the total Golgi fraction as well as from the most heavy portion of the Golgi fraction. In conclusion, our results thus suggest that the smooth membrane fraction is a pre-Golgi compartment, probably belonging to the smooth ER. These observations are in agreement with results from immunoelectronmicroscopy studies carried out in rat hepatocytes (29). These studies showed the presence of VLDL particles at the smooth-surfaced termini of the rough ER.

It is of interest to notice that the compartment where LpB 100 is released to the lumen of the secretory pathway is characterized by the major peak of diacylglycerol acyltransferase activity of the ER. This enzyme catalyzes the final and unique step in the triacylglycerol synthesis. Although it may be possible that the enzyme and the mechanism for the formation of lipoproteins are present in different vesicles, these results may suggest that the triacylglycerol synthesis has a central role in the assembly of lipoproteins. The importance of triacylglycerol synthesis for the assembly of lipoproteins was also suggested from previous studies (1, 30).

It should be pointed out that substantial diacylglycerol acyltransferase activity is also present in the RER:1 and RER:II. This observation may suggest that there are additional factors beside apoB 100 and the synthesis of triacylglycerol that are essential for the assembly process and that characterize the site of lipoprotein assembly. It should, however, be pointed out that we have not excluded the possibility that LpB 100 is also assembled in the rough ER but degraded in this compartment (see above).

Membrane-bound apoB 100 is another component that is needed for the assembly of lipoproteins. It differed in distribution from that of the diacylglycerol acyltransferase activity but appeared to follow the distribution of the NADPH-cytochrome c reductase activity in the ER. Thus, there was a gradient in membrane-bound apoB 100 from the rough ER to the smooth membrane compartment where LpB 100 is formed. This may simply reflect the amount of ER membranes present, a possibility that is supported by the observation that α1-antitrypsin appears to have the same distribution in the ER (cf. Fig. 2B). It is, however, possible that such a gradient may be formed if apoB 100 is allowed to diffuse from the site of synthesis toward the smooth membrane compartment where LpB 100 is formed and apoB 100 molecules leave the membrane.

The intracellular transfer of apoB 100 was characterized by a rapid appearance of the mature, completed apoB 100 polyepitope in the ER membrane, forming a relatively large preassembly pool. The maximal radioactivity recovered in LpB 100 during these pulse-chase studies was, however, low relative to the radioactivity recovered in the ER membrane. Since control experiments provided evidence against the possibility that this observation was due to a loss of LpB 100 during the preparation, these results indicated that there was a rapid turnover of the LpB 100 pool in the lumen of the secretory pathway. In particular, this appears to be the case for the LpB 100 pool in the ER.

The results from these kinetic studies indicated that apoB 100 remained for a relatively long time in the preassembly pool in the ER membrane, while the formed particles are relatively rapidly secreted. Thus, the rate-limiting step in the secretion of LpB 100 appears to be the transfer of apoB 100 from the preassembly pool to the LpB 100 in the ER lumen. These observations are in agreement with the results that indicate that the transfer of apoB 100 (24, 25), as well as proteins in general (31–33) out of the ER compartment is the rate-limiting step in the secretion.

There was, however, not a direct relationship between the rate of disappearance of apoB 100 from the preassembly pool and the rate of secretion of the protein into the medium. Thus, the rapid decrease of the apoB 100 radioactivity in the preassembly pool between 5 and 30 min was not reflected in a similar rate of LpB 100 secretion from the cell. This may suggest that a portion of the preassembly pool of apoB 100 is diverged from the secretory process. One possible explanation for such a diversion of apoB 100 may be a posttranslational degradation of a portion of the intracellular pool of the protein.

As discussed above, results from studies in cultured rat hepatocytes (25) as well as studies in Hep G2 cells (1) suggest that a portion of the formed apoB 100 undergoes a posttranslational degradation in the cell.

The possibility of such a posttranslational degradation of apoB 100 was also tested in the present study. Pulse-chase experiments indicated that there was a significant loss of apoB 100 radioactivity with time. The experiments were based on the recovery of apoB 100 not only from the cells but also from the medium, and one possibility that had to be addressed was that the loss of apoB 100 was due to a degradation, for example via the LDL receptor, of the LpB 100 that was secreted into the medium. Neither previous results (1) nor control experiments presented in this paper support such a possibility. Furthermore, the possibility that a degradation of the secreted LpB 100 could explain the observed loss of apoB 100 radioactivity was not consistent with the observation that...
the majority of the loss of the protein occurred at chase periods shorter than 120 min, while there was a small almost negligible additional loss of apoB 100 radioactivity when the chase periods were extended from 120 to 360 min, i.e. conditions when the length of exposure of the lipoproteins to the cells increased three times.

The results presented in this paper would therefore support the possibility of a posttranslational intracellular degradation of apoB 100 in the Hep G2 cells, in turn supporting the possibility that the diversion of apoB 100 from the secretory process was due to a posttranslational degradation of portion of apoB 100.

The rapid transfer of the assembled LpB 100 out of the ER would be in agreement with the proposed hypothesis for a bulk flow transfer of secretory products through the cell (31, 32, 34). According to this hypothesis, a protein leaves the ER compartment by the bulk flow transfer mechanism soon after its correct structure has been acquired and is then rapidly taken through the cell and secreted (31, 32). Our results thus show that apoB 100 follows these rules, keeping in mind that the assembly is a sequential event and that the formation of apoB 100 radioactivity when the chase experiment from the Golgi compartment was several fold higher than the ER. Our results indicate that this is not due to a different recovery of LpB 100 from the two fractions. The observation could be explained by a more rapid transfer of LpB 100 out of the ER compartment than the transfer of the lipoprotein through the Golgi apparatus. The reason for this is not known. However, as a speculative suggestion, the assembly of LpD 100 could occur in a smooth membrane compartment relatively close to the transport mechanism, thus minimizing the distance the lipoprotein has to travel before it is transferred out of the compartment.

The observations presented in this paper together with our earlier published results (1, 3) indicate that a sequential process leads to the assembly of lipoproteins. The process starts with a cotranslational integration of apoB 100 into the membrane of the endoplasmic reticulum. The protein is transferred bound to the membrane from the site of synthesis to the site of assembly, where it interacts with neutral lipids and forms lipoproteins. These lipoproteins are released from the lumen of the secretory pathway, reaching the bulk flow transfer of the cell, and secreted. This model would agree with the observation from immunoelectronmicroscopy made by other authors (29).

Acknowledgments—We would like to thank Anita Magnusson, Ann-Sofie Andersson, Daniela Landys, Aina Lidell, and Margareta Edvardsson for excellent technical assistance, Ewa Landegren and Agneta Ladström for typing the manuscript, and Maria Stern for correcting the language.

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J. Biol. Chem. 1990, 265:10556-10564.