Intracellular Assembly and Degradation of Apolipoprotein B-100-containing Lipoproteins in Digitonin-permeabilized HEP G2 Cells*

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Khosrow Adeli‡§, Margit Wettesten, Lennart Asp¶, Abbas Mohammadi‡, Joseph Macri‡, and Sven-Olof Olofsson†¶

From the ‡Department of Chemistry and Biochemistry University of Windsor, Windsor Canada and the ¶Department of Medical Biochemistry, University of Göteborg, 413 90 Göteborg, Sweden

Permeabilized Hep G2 cells have been used to investigate the turnover of apolipoprotein B-100 (apoB-100). When the cells were chased in the presence of buffer, there was no biosynthesis of apoB-100, nor was the protein secreted from the cells. Thus the turnover of apoB-100 in these cells reflected the posttranslational degradation of the protein. Pulse-chase studies indicated that apoB-100 was degraded both when associated with the membrane and when present as lipoproteins in the secretory pathway. Neither albumin nor α₁-antitrypsin showed any significant posttranslational intracellular degradation under the same condition. The kinetics for the turnover of apoB-100 in the luminal content differed from that of apoB-100 that was associated with the microsomal membrane. Moreover, while the degradation of the luminal apoB-100 was inhibited by N-acetyl-leucyl-leucyl-norleucinal (ALLN), this was not the case for the membrane-associated protein. Together these results suggest the existence of different pathways for the degradation of luminal apoB-100 and membrane-associated apoB-100. This was further supported by results from pulse-chase studies in intact cells, showing that ALLN increased the amount of radioactive apoB-100 that associated with the microsomal membrane during the pulse-labeling of the cells. However, ALLN did not influence the rate of turnover of the membrane-associated apoB-100.

The presence of an ATP-generating system during the chase of the permeabilized cells prevented the disappearance of pulse-labeled apoB-100 from the luminal lipoprotein-associated pool. The ATP-generating system combined with cytosol protected the total apoB-100 in the system from being degraded. The cells cultured in the presence of oleic acid and chased after permeabilization in the presence of cytosol and the ATP-generating system showed an increase in the amount of apoB-100 present on dense (“high density lipoprotein-like”) particles. This increase was linear during the time investigated (i.e., from 0 to 2 h chase) and independent of protein biosynthesis. Our results indicate that the dense particle was generated by a redistribution of apoB-100 within the secretory pathway and that it most likely was assembled from the membrane-associated form of apoB-100. These results indicate that the release of apoB-100 from this membrane-associated form to the microsomal lumen is dependent on cytosolic factors and a source of metabolic energy.

Apolipoprotein B (apoB) 1 assembles very low density lipoproteins (VLDL) in liver cells, a process that requires at least two steps. During the first step apoB is translocated to the lumen of the endoplasmic reticulum (ER), which appears to be coupled with a partial lipidation of the protein (1). In the second step the majority of lipids is added to this partially lipidated form of apoB, to form a full-size VLDL particle (2) (for review with references, see Innerarity et al. (3)). Both steps occur in primary hepatocytes (4) and in certain cell lines (such as McA-RH7777 cells) (2). However, Hep G2 cells are unable to make any significant amount of VLDL; instead these cells assemble a triacylglycerol-rich particle with the size and density of a low density lipoprotein (LDL) (5). It is therefore likely that these cells lack the second step. We will refer to these triacylglycerol-rich LDL-like particles as the LDL-VLDL particles.

Evidence to date suggests that modulation of the secretion of apoB-100 is posttranscriptionally regulated (see, for example, Refs. 6–9). One of the control points in this regulation appears to be the translocation of apoB-100 into the ER lumen (10–13). Thus at least a portion of apoB-100 that is associated with the ER membrane appears to be sorted to degradation (1, 14, 15). Moreover, apoB-100 that has been translocated into the ER lumen has been suggested to be the target for posttranslational degradation (15, 16). The process involved in sorting lipoproteins in the lumen to degradation appears to recognize the degree of lipidation of the particles (1, 17, 18). Thus, while the 200–300-Å LDL-VLDL particle that is assembled in the Hep G2 cells is secreted, the more dense apoB-100 lipoproteins present in the lumen of the secretory pathway appear to be retained and degraded in the cell (1, 6, 15).

The subcellular site where the posttranslational degradation of apoB occurs has thus far not been unequivocally identified, but is generally thought to be the ER or a closely associated compartment (10, 19–21), although studies in rat hepatocytes

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¶ To whom correspondence should be addressed: Dept. of Medical Biochemistry, University of Göteborg, Medicinaregatan 9, 413 90 Göteborg, Sweden. Tel.: 46-31-773 3485; Fax: 46-31-41 61 08.

1 The abbreviations used are: apoB, apolipoprotein B; ALLN, N-acetyl-leucyl-leucyl-norleucinal; CSK, cytoskeletal buffer; HDL, high density lipoprotein; LDL, low density lipoprotein; VLDL, very low density lipoprotein; LDL-VLDL, the triacylglycerol-rich LDL-size lipoprotein secreted from the Hep G2 cells; ER, endoplasmic reticulum; PIPES, 1,4-piperazinediethanesulfonic acid.
have suggested post-ER degradation of apoB (22–24). A compartment that is closely associated with ER would fit with recent data showing that other proteins (25) are sorted to degradation in a pre-Golgi, intermediate tubular compartment.

Permeabilized cells provide a good model for studies of apoB degradation and have provided important insights into the degradative mechanisms. An important advantage with permeabilized cells is that they allow for an easier identification of degradation intermediates including an abundant N-terminal 70-kDa degradation fragment (11, 21). Using permeabilized Hep G2 cells we have obtained results (21, 26) indicating that apoB degradation occurs in the ER, is temperature- and pH-dependent, and is stimulated by ATP, and that an ALLN-sensitive cysteine protease may be involved. Moreover, we showed that the 70-kDa fragment is present in the lumen of the secretory pathway and that the generation of this fragment is ALLN-sensitive (21).

The first step in the assembly of lipoproteins, i.e. the translocation of apoB into the ER lumen, appears to be of importance for the regulation of the secretion of apoB, and the amount of particles secreted depends on assembly and degradation. We have studied these processes in permeabilized Hep G2 cells, i.e. in cells that carry out only the first step in VLDL assembly. The data suggest that both the membrane-bound and lipoprotein-associated apoB-100 pools are targets for intracellular degradation. The apoB on luminal lipoproteins appears to be degraded by an ALLN-sensitive degradative mechanism and can be rescued from degradation by an ATP-generating system. To rescue the entire pool of apoB-100 in the cell, the ATP-generating system had to be combined with cytosol.

EXPERIMENTAL PROCEDURES

Materials—Hep G2 cells (ATCC HB 8065) were obtained from American Type Culture Collection. Fetal bovine serum (certified grade) and cell culture media were from Life Technologies Inc. (Paisley, UK). Culture dishes and flasks were obtained from Corning or Falcon. Digitonin (50% purity), oleic acid, leupeptin, pepstatin, ALLN, and other common laboratory reagents were from Sigma. Digitonin of a higher purity (100%) was obtained from Calbiochem. Ready Sufle was from Boehringer. Trasylol (aprotinin) was from Bayer Leverkusen, FRG. Ultra-pure electrophoresis reagents were from Bio-Rad. L-[35S]methionine (specific activity of >1000 Ci/ml), [35S]protein labeling mixture (Pro-mixTM; specific activity of >1000 Ci/ml), [14C]-methylated protein standards (rainbow markers), and Amplify were purchased from Amersham International. Monospecific apoB antibodies were in most cases prepared in the laboratory; however, such antibodies were also to a limited extent obtained from Medix-Biotech (Foster City, CA) and puriﬁed in the laboratory. Rabbit anti-goat IgG, α1-antitrypsin antiserum, and albumin antiserum were from DAKO (Gloustrup, Denmark) or from Sigma. Immunoprecipitin was obtained from Life Technologies Inc. (Paisley, UK). ENHANCE was from DuPont Canada (Toronto, ONT).

Cell Culture, Metabolic Labeling, and Permeabilization of Hep G2 Cells—Hep G2 cells were grown in 85- or 100-mm dishes at 37 °C, 5% CO2 in complete medium (i.e. modification of Eagle’s minimal essential medium, 10% fetal bovine serum). In the cases when the cells were cultured in the presence of oleic acid, the culture medium was supplemented with 360 μM oleic acid 48 h prior to the start of the experiment. Confluent Hep G2 cultures were incubated with methionine-free modiﬁed Eagle’s medium for 120 min, pulse-labeled (15–20 min) with 75–150 μCi/ml of [35S]methionine + cysteine (Pro-Mix TM) or [35S]methionine, washed three times, and chased for 10–30 min in culture medium supplemented with 10 mM methionine in order to fully elute labeled nascent polypeptides to obtain full-length apoB-100. The cells were then washed and incubated in 0.3 M sucrose, 0.1 M KCl, 2.5 mM MgCl2, 1 mM sodium-free EDTA, 10 mM PIPES, pH 6.8 (CSK buffer) containing 50 μg/ml of digitonin for 10 min at room temperature. Digitonin-treated cells washed three times in CSK buffer and were then used for the studies.

To supplement one 85- or 100-mm dish of permeabilized cells with cytosol the following protocol was used. Cytosol from one 85- or 100-mm dish of confluent Hep G2 cells was prepared as described in Find et al. (27) with the exception that the cytosol was not desalted. The ATP-generating system described in Find et al. (27) was used, i.e. 5 parts of 40 mM ATP was mixed with 5 parts of 200 mM creatine phosphate and 1 part of rabbit muscle creatine phosphokinase (100 units/ml). The cytosol was mixed with 110 μl of the ATP-generating system, and the mixture was diluted to a final volume of 2 ml with CSK buffer and added to the permeabilized cells after three washes with CSK buffer (see above).

Sucrose Gradient Ultracentrifugation of apoB-100-containing Lipoproteins from the Medium and the Lumen of the Microsomal Fraction—The luminal content of the total microsomal fraction was isolated as described previously (1, 6, 28, 29). Briefly, permeabilized cells incubated for 0–2 h were washed twice with 250 mM sucrose, 3 mM imidazole, pH 7.4, and once with 50 mM sucrose, 3 mM imidazole, pH 7.4. The cells were then scraped in 0.5 ml of 50 mM sucrose solution supplemented with a mixture of protease inhibitors (0.1 mM leupeptin, 1 mM phenylmethylsulfonyl fluoride, 100 kallikrein-inhibitory units/ml Trasylol, 1 μM pepstatin A, and 5 μM ALLN), and the total microsomal fraction was recovered as described previously (6, 28). The microsomes were subjected to carbonate extraction (30) with the modification described in Boström et al. (28). It has been demonstrated that the recovery of proteins weakly associated to the membrane could be increased by carrying out the sodium carbonate extraction in the presence of a high concentration of salt. During the establishment of this method (28) we investigated the effect of high concentration of salt for the recovery of apoB-100 with the membrane. The results indicated that there were no substantial effects of high concentration of salt on the recovery of apoB-100 from the membrane.

The luminal contents were fractionated by ultracentrifugation as described elsewhere (1). In short, the luminal contents were supplemented with protease inhibitors (0.1 mM leupeptin, 1 mM phenylmethylsulfonyl fluoride, 100 kallikrein-inhibitory units/ml Trasylol, 1 mM pepstatin A, and 5 mM ALLN) and then subjected to ultracentrifugation on a discontinuous sucrose gradient (1.5 ml 49%/3.0 ml 25%/2.0 ml 20%/3.2 ml sample/1.9 ml 5%/0.9 ml of 0% sucrose) at 35,000 rpm in a SW40 rotor for 65 h, at 12 °C. All solutions contained the protease inhibitor mixture as above. Gradients were fractionated into 1 ml fractions.

Immunoprecipitation and Polyacrylamide Gel Electrophoresis in the Presence of SDS—Cells lysates or fractions collected from sucrose gradients were immunoprecipitated with polyclonal antibodies to apoB-100, albumin, or α1-antitrypsin, as described earlier (31). The immunoprecipitated proteins were electrophoresed in 3–15% polyacrylamide gradient gels containing SDS, and the bands were visualized by fluorography, cut out of the gel, and digested, and radioactivity was counted (31).

RESULTS

Turnover of apoB-100 Lipoproteins in the Microsomal Lumen of Permeabilized Cells

Cells Cultured in the Absence of Oleic Acid—The cells were pulse-labeled for 15 min and chased for 30 min, in order to obtain full-length apoB-100. The cells were then permeabilized and chased for 0 or 120 min in the presence of CSK buffer (0.3 M sucrose, 0.1 M KCl, 2.5 mM MgCl2, 1 mM sodium-free EDTA, 10 mM PIPES, pH 6.8) alone or cytosol and an ATP-generating system.

The 2-h chase in buffer alone resulted in a decrease in the amount of apoB-100 radioactivity in the cell, thus the recovery (given as percent of the apoB 100 radioactivity that was present before the chase) was 57 ± 11% (mean ± S.D., n = 5). There was also a substantial decrease in the apoB-100 radioactivity present on lipoproteins in the microsomal lumen. Thus only half (recovery 48 ± 19%, mean ± S.D., n = 6) of the total apoB present in the lumen before the chase was recovered after the 2-h chase. Since no significant amount of apoB appeared in the medium under these conditions, it appears that total apoB-100, including apoB-100 present on lipoproteins in the secretory pathway, was degraded. This degradation was unique for apoB-100 since we did not detect any loss of pulse-labeled albumin (Fig. 1A) or α1-antitrypsin (Fig. 1B).

Gradient ultracentrifugation revealed that there was a loss of pulse-labeled apoB-100 both from the LDL-VLDL fraction and from the more dense (“HDL-like”) fraction (Fig. 2, −OA). There was an almost complete recovery (94 ± 36%; mean ±
FIG. 1. Turnover of pulse-labeled albumin (A) and α₁-antitrypsin (B) in the lumen (filled symbols) and membrane (open symbols) of the microsomes from permeabilized Hep G2 cells. Hep G2 cells, cultured in the absence of oleic acid, were pulse-labeled with 100 μCi/ml [³⁵S]methionine + cysteine for 10 min. The cells were then permeabilized (cf. “Experimental Procedures”) and chased for various times in the presence of CSK buffer (10 mM PIPES, pH 6.8, with 0.5 mM sucrose, 0.1 mM KCl, 2.5 mM MgCl₂, and 1 mM sodium-free EDTA). After the chase, the microsomal fraction was recovered, and the luminal content was extracted by sodium carbonate treatment. Albumin and α₁-antitrypsin were isolated from the luminal content as well as the membrane pellet by immunoprecipitation and analyzed by polyacrylamide gel electrophoresis in the presence of SDS, and the radioactivity was determined. The results are given as the recovery (percent) of radioactive protein present at 0-min chase (mean ± S.D., n = 3).

S.D., n = 4) of the apoB-100 radioactivity in the system (i.e. cells + medium) when the 2-h chase was carried out in the presence of cytosol and the ATP-generating system. Moreover, under these conditions there was a complete recovery of the apoB-100 radioactivity present in the secretory pathway (recovery 103 ± 40%; mean ± S.D., n = 5). Gradient ultracentrifugation revealed a significant loss of apoB-100 from LDL-VLDL, while the apoB-100 radioactivity in the HDL density range tended to increase.

The cytosol and the ATP-generating system induced a significant release of apoB-100 to the medium, and when the apoB-100 radioactivity of the LDL-VLDL fraction of the medium was included in the calculations, we observed a complete recovery of the apoB-100 radioactivity present in the LDL-VLDL fraction in the system (i.e. cell and medium).

Cells Cultured in the Presence of Oleic Acid—Culturing the cells in the presence of oleic acid before permeabilization did not influence the recovery of the total apoB-100 in the system (i.e. cell + medium) if the 2-h chase was carried out in the presence of CSK buffer alone (recovery 55 ± 17; mean ± S.D., n = 4). There was also a loss of apoB-100 radioactivity from the microsomal lumen (recovery 68 ± 6%; mean ± S.D., n = 3). Gradient ultracentrifugation (Fig. 2, +OA) showed that this decrease was mainly confined to the LDL-VLDL fraction, where the recovery was less than 50%. Even if the apoB-100 radioactivity that was recovered from the LDL-VLDL fraction of the medium was included in the recovery calculation, we did not observe a complete recovery of the apoB-100 radioactivity present in the LDL-VLDL fraction. The apoB-100 radioactivity present in the more dense fraction (the HDL-like fraction) did not, however, decrease during the 2-h chase.

FIG. 2. The recovery of pulse-labeled apoB-100 in LDL-VLDL particles as well as on dense particles (apoB-100 ‘‘HDL’’) recovered from the luminal content of microsomes from permeabilized Hep G2 cells after a 2-h chase in the presence of CSK buffer alone or buffer plus cytosol and an ATP-generating system (hatched bars). Hep G2 cells, cultured in the absence (−OA) or presence (+OA) of oleic acid, were pulse-labeled with 60 μCi/ml [³⁵S]methionine + cysteine for 15 min and chased for 30 min. The cells were then permeabilized and chased for 0 or 120 min in the presence of CSK buffer alone or CSK buffer plus cytosol (recovered from cells cultured in the absence (−OA) or presence (+OA) of oleic acid) together with an ATP-generating system. The microsomal contents were recovered by sodium carbonate extraction after a 0- or 120-min chase and analyzed by sucrose gradient ultracentrifugation. apoB was recovered from each fraction by immunoprecipitation and polyacrylamide gel electrophoresis in the presence of SDS, and the radioactivity was determined. The apoB-100 radioactivity present in the LDL-VLDL fractions as well as in the dense fractions (banding in the density range of HDL) was determined. Results are given as the relation (percent) between the radioactivity recovered after the 120-min chase of the permeabilized cells and the apoB-100 radioactivity present before this chase (mean ± S.D.; n = 5 for cells cultured in the absence of oleic acid; n = 4 for cells cultured in the presence of oleic acid). Total LDL-VLDL means that the LDL-VLDL particles recovered from the incubation medium have been taken into account in the recovery calculations. The amount LDL-VLDL present in the medium (expressed as percent of the total amount of LDL, i.e. cell + medium) was 6 ± 4% (mean ± S.D., n = 4) for cells cultured in the absence of oleic acid and chased in CSK buffer alone; 17 ± 4% (mean ± S.D., n = 5) for cells cultured in the absence of oleic acid and chased in cytosol and the ATP-generating system; 17 ± 9% (mean ± S.D., n = 4) for cells cultured in the presence of oleic acid and chased in the presence of CSK buffer alone; and 18 ± 9% (mean ± S.D., n = 4) for cells cultured in the presence of oleic acid and chased in the presence of cytosol and the ATP-generating system.
Including cytosol (from cells cultured in the presence of oleic acid) and the ATP-generating system during the 2-h chase resulted in a complete recovery (121 ± 31%; mean ± S.D., n = 4) of the total apoB-100 radioactivity in the system (i.e. cells + medium). There was an increase in the total apoB-100 radioactivity present on lipoproteins in the luminal content. Thus the amount of apoB-100 radioactivity observed in the lumen after the chase corresponded to 175 ± 47% of the pre-chase value (mean ± S.D., n = 4). This was due to an increase in the apoB-100 radioactivity present in the HDL-like fraction (Fig. 2, +OA), indicating that more such particles appeared in the secretory pathway during the chase. We also observed an almost complete recovery of the apoB-100 radioactivity present in the LDL-VLDL fraction provided that the radioactivity in the LDL-VLDL fraction of the medium was included in the calculation (Fig. 2, +OA).

The above results indicated that there was a generation of apoB-100 lipoproteins, in particular dense particles, in cells that had been chased in the presence of cytosol and the ATP-generating system. Although we regard it as less likely, it is possible that the addition of cytosol and ATP starts an incorporation of the remaining radioactive amino acids (or radioactive amino acids released during protein breakdown) into apoB and that reinitiation of apoB-100 synthesis may explain the increase in the apoB-100 lipoproteins in the luminal content. To address this possibility we carried out a control experiment in which the chase was carried out in the presence of cycloheximide and a surplus of cold methionine (in addition to cytosol and the ATP-generating system). The results indicated that approximately the same amount of apoB-100 radioactivity was recovered in the dense luminal fraction (particles banding in the density range of HDL) under the two conditions. Thus the ratio between apoB-100 HDL from cells chased in the absence or presence of cycloheximide was 0.9 (mean of two experiments). Also the recovery of the apoB-100 radioactivity from LDL-VLDL was the same under the two conditions (the ratio between LDL-VLDL radioactivity recovered in the absence or presence of cycloheximide was 1.0; mean of two experiments).

Effects of the ATP-generating System in the Absence of Cytosol

In these experiments we investigated the effect of buffer and the ATP-generating system during the 2-h chase after permeabilization. We used cells that had been cultured in the absence of oleic acid. To exclude the possibility that the ATP-generating system restarted the biosynthesis of radioactive apoB-100, we carried out the 2-h chase after permeabilization in the presence of cycloheximide and a surplus of unlabeled methionine. The results indicated that the ATP-generating system was not sufficient to protect the recovery of total apoB-100 in the system (recovery 60 ± 19; mean ± S.D., n = 4), and there was a difference in the recovery of total apoB-100 between permeabilized cells that were chased in the presence of the ATP-generating system and CSK buffer and those chased in the presence of the ATP-generating system and cytosol (see above; p < 0.05; two-tailed paired t test).

There was, however, a complete recovery of apoB-100 from the different lipoprotein fractions present in the secretory pathway (Fig. 3), provided that the LDL-VLDL fraction recovered from the medium was taken into account. The decline in total apoB-100 radioactivity in the system (i.e. cells + medium) when chased in the presence of the ATP-generating system could be explained by a loss of the membrane-associated apoB-100 radioactivity (recovery 54%; mean of two experiments).

The Generation of the Dense (HDL-like) Particle and the Turnover of ApoB-100 in the Microsomal Membrane

The results presented above indicate that supplementation of permeabilized cell with cytosol and the ATP-generating system induced the formation of the dense (HDL-like) apoB-100-containing fraction. The formation of this fraction occurred without any change in the total amount of apoB-100 in the system and was not influenced by cycloheximide. It is therefore likely that it is formed by a redistribution of apoB-100 within the microsome. In agreement with such a redistribution we found a loss of pulse-labeled membrane-associated apoB-100 during the 2-h chase in the presence of cytosol and the ATP-generating system. This loss was large enough to explain the accumulation of radioactive apoB-100 in the dense (HDL-like) fraction. This was shown by calculating the decrease in the membrane-associated apoB-100 radioactivity during the 2-h chase and relating that to the increase in apoB-100 radioactivity in the dense fraction during the same time period. The ratio (i.e. Δ apoB-100 radioactivity associated with the membrane/Δ apoB-100 radioactivity in the dense fraction) was 2.3 ± 1.7 (mean ± S.D., n = 5). Pulse-chase experiments revealed that the accumulation of apoB-100 radioactivity in apoB-100 HDL was almost linear with time during the 2-h chase (data not shown).

Accumulation of ApoB-100 in the Medium

ApoB-100 radioactivity accumulated in the medium during the 2-h chase of cells that had been cultured in the absence of oleic acid, but permeabilized and chased in the presence of cytosol and the ATP-generating system. When the cells were cultured in the presence of oleic acid, we detected a significant accumulation of apoB-100 both in the presence or absence of cytosol and the ATP-generating system. However, this accumulation increased severalfold in the presence of cytosol and the ATP-generating system (Fig. 4). After correcting for leakage from the cells (see below) we estimated that the presence of

![FIG. 3.](image)
cytosol and the ATP-generating system gave rise to a more than 3-fold increase in apoB secretion into the medium. It should, however, be noted that the rate of lipoprotein secretion from the cells was low compared to that of intact cells. Thus the percent of apoB-100 secreted (as the percentage of the total apoB-100 radioactivity present in the cell before the chase; mean ± S.D., n = 4) from cells cultured in the presence of oleic acid and chased in the presence or absence of cytosol and the ATP-generating system was 3 ± 0.9% and 0.9 ± 0.1%, respectively. The cells cultured in the absence of oleic acid and chased in the presence of cytosol and the ATP-generating system secreted 1.0 ± 0.6% of the total intracellular pool of radioactive apoB-100. There was no detectable accumulation of apoB-100 in the medium of cells that were cultured in the absence of oleic acid and chased in the presence of buffer alone.

To correct for leakage we estimated the amount of apoB-100 HDL in the medium (Fig. 4), since intact Hep G2 cells secrete only trace amounts of this lipoprotein. The percent of this leakage (the apoB-100 radioactivity in the HDL density region of the medium as a percentage of the maximal apoB-100 radioactivity in the HDL density region of the whole system) was 5.96 ± 2.33% (mean ± S.D., n = 5) for cells cultured in the absence of oleic acid and chased in the presence of cytosol and the ATP-generating system. The corresponding values for cells cultured in the presence of oleic acid were 4.5 ± 2.1 (n = 4; cells chased in the presence of buffer alone) and 5.7 ± 2.9 (n = 4; cells chased in the presence of cytosol and the ATP-generating system), respectively. To confirm the results we also followed the secretion of the precursor form of α₁-antitrypsin. This form should be processed to the mature form before being secreted. The results indicated that about 5% of the precursor form of α₁-antitrypsin was recovered in the medium.

A second control experiment was used to confirm that cytosol and the ATP-generating system restarted the intracellular transport. In this experiment we followed the processing of α₁-antitrypsin. The cells were pulse-labeled for 10 min and chased for 10 min before permeabilization. The labeled permeabilized cells were then chased for 2 h. The two α₁-antitrypsin bands (M and P) (32) were seen both when the cells were chased in the presence of cytosol and the ATP-generating system and in buffer alone (Fig. 5). Treatment of the immunoprecipitated α₁-antitrypsin with endoglycosidase H demonstrated that, when the chase was carried out in the presence of buffer alone, most of the band migrating as P was in the endoglycosidase H-sensitive form (Fig. 5, P1) (32). On the contrary, when the cells were chased in the presence of cytosol and the ATP-generating system, a substantial amount of the P band was endoglycosidase H-resistant (i.e. P2) (32). These results indicate that the addition of cytosol and the ATP-generating system restarted the intracellular transport.

**Intracellular Degradation of ApoB-100 in Permeabilized Hep G2 Cells**

The results presented above indicated that permeabilized Hep G2 cells that had been cultured in the absence of oleic acid neither secreted nor assembled any significant amount of lipoproteins during the 2-h chase in the presence of buffer alone. These cells could therefore be used to investigate the intracellular degradation of apoB-100. Thus we followed the turnover of apoB-100 in the microsomal lumen and membrane under these conditions. In these experiments, we pulse-labeled for 20 min and chased for 10 min, before the cells were permeabilized and chased for periods up to 2 h.

As discussed above (Fig. 1, A and B), neither albumin nor α₁-antitrypsin was to any significant degree degraded during the 2-h chase. Thus 103.1 ± 0.6% (mean ± S.D., n = 3) of luminal [³⁵S]labeled albumin detected at zero time was still present after a 2-h chase, while the percent of α₁-antitrypsin that remained after the 2-h chase was 85.6 ± 16.2% (mean ± S.D., n = 3). Fig. 1A also shows that, as could be expected for a secretory protein, the majority of albumin (96.7 ± 5.0% mean ± S.D., n = 3) was recovered from the luminal content, while only a minor fraction of albumin (3.3 ± 0.5%) was recovered from the membrane fraction. This was also the case for α₁-antitrypsin (Fig. 1B).

The results presented above (Fig. 2B) demonstrated that apoB-100, present on lipoproteins in the microsomal lumen, is degraded during the chase, indicating that apoB-100 was the target for intracellular degradation both when present on the dense particles (i.e. the particles that are retained and degraded in intact cells) and on the LDL-VLDL particles (i.e. the particles that are secreted from intact cells).

We have previously demonstrated that the membrane-associated apoB-100 is a target for degradation in intact Hep G2 cells (29). Here we compared the turnover of membrane-associated apoB-100 with the protein present in the microsomal lumen. The results (Fig. 6A) showed that pulse-labeled apoB-100 that was associated with the membrane fraction rapidly...
and almost totally turned over during the 2-h chase. The percent of apoB remaining in the membrane fraction after a 2-h chase was $9.0 \pm 2.5\%$ (mean $\pm$ S.D., $n = 8$). In comparison, the $^{35}$S-labeled apoB detected in the luminal fraction of the microsomes was more stable, although it was also significantly degraded over the 2-h chase. The percent of luminal apoB that remained after the 2-h chase was $34.6 \pm 9.1\%$ (mean $\pm$ S.D., $n = 11$) for luminal content.

The rate of degradation of the luminal and membrane-bound apoB pools were also compared in a pulse-chase experiment (Fig. 6B). Both membrane-bound and luminal apoB fractions were degraded over the 2-h chase but at different rates. Degradation rates particularly differed over the 1st h of chase with the degradation rate of membrane-associated apoB being much faster. Thus over 80% of this apoB-100 was lost during the 1st h of chase. In contrast, the luminal apoB pool was degraded at a slower rate with just over 40% degraded after a 1-h chase. The data indicate that both the luminal and membrane-bound pools of apoB are subjected to intracellular degradation; however, the degradation of the two pools occurs at different rates.

Concomitant with the disappearance of the apoB-100 lipoproteins from the microsomal lumen, degradation intermediates, including the earlier identified 70-kDa fragment, appeared (Fig. 7A). The 70-kDa fragment was mainly recovered from the HDL density range (data not shown). In contrast we could find only trace amounts of the 70-kDa fragment in association with the membrane fraction. Results from a pulse-chase experiment (Fig. 7B) indicated that the 70-kDa fragment accumulated in the microsomal lumen when apoB-100 was degraded.

**Luminal Degradation of ApoB Is Inhibited by ALLN**

ALLN slowed down the turnover rate of luminal apoB considerably (Fig. 8B). At 30 min of chase, control cells had retained only 56.9% of luminal apoB radioactivity at zero time, whereas ALLN-treated cells had retained 83.8% of luminal apoB. Although some of the luminal apoB was still degraded in ALLN-treated cells, the total apoB remaining was considerably higher in ALLN-treated cells compared to that in control cells (Fig. 8D). Interestingly, treatment with ALLN appeared to abolish the generation of the 70-kDa fragment in the luminal contents of permeabilized cells (Fig. 7A).

The effect of ALLN on the luminal apoB-100 was also investigated in an experiment in which the luminal apoB-100-containing lipoproteins were fractionated by the sucrose gradient. The results confirmed that ALLN protected the lipoprotein-associated apoB-100 from degradation. Moreover, these results
confirmed that treatment with ALLN abolished the generation of the 70-kDa fragment (data not shown).

In contrast the rate of the disappearance of the membrane-associated apoB-100 was not influenced by ALLN (Fig. 8A). Thus the percent of membrane-bound apoB remaining after a 2-h chase was 9.0 ± 2.5% (−ALLN, mean ± S.D., n = 8) and 7.3 ± 0.4% (+ALLN, mean ± S.D., n = 3) (not statistically different).

The Turnover of ApoB-100 in the Microsomal Membrane and Lumen of Intact Hep G2 Cells, Effects of ALLN

In these experiments we followed the turnover of the apoB-100 radioactivity in the membrane and lumen of the microsomes following a 15-min pulse with [35S]methionine + cysteine. The results demonstrated (both in the presence and absence of ALLN) a time-dependent decrease in radioactivity of the membrane-associated apoB-100 paralleled with a time-dependent increase in apoB-100 radioactivity of the lumen (Fig. 9A), indicating a precursor-product relationship (Fig. 9A).

ALLN treatment gave rise to a substantial increase in the accumulation of the membrane-associated apoB-100 radioactivity after the 15-min pulse (Fig. 9A). There was also a considerable increase in the accumulation of apoB-100 radioactivity in the lumen in particular in the HDL density fraction. Thus 61% of the apoB-100 radioactivity was present in the HDL fraction after the labeling period (0-min chase), while the corresponding figures after a 30- and 90-min chase were 83 and 64%, respectively. A similar distribution of the apoB-100 radioactivity in the lumen was observed in the experiment that was carried out in the absence of ALLN. Thus the recovery of the apoB-100 radioactivity in the HDL density fraction was 73% (0-min chase), 70% (30-min chase), and 64% (90-min chase). The observation that the major assembly product was present in the HDL density region could most likely be explained by the fact that the experiment was carried out in the absence of oleic acid (15).

Although ALLN influenced the amount of apoB-100 radioactivity that accumulated in association with the microsomal membrane, this protease inhibitor did not change the rate of turnover of this form of apoB-100 (given as percent apoB remaining as a function of time; Fig. 9B). The ALLN-induced increase in the amount of apoB-100 that associated with the membrane and was released to lumen was not influenced by the presence of cycloheximide during the chase (data not shown).

DISCUSSION

In the present study, we used permeabilized Hep G2 cells to investigate the turnover of apoB-100. The results indicated that apoB-100 was degraded both when associated with the membrane and when present on lipoproteins in the secretory pathway. The degradation appeared to be a specific process for apoB-100 since neither albumin nor α1-antitrypsin showed any significant posttranslational degradation under the same condition. The observation that albumin is not degraded under these experimental conditions is in agreement with previous results (21). The presence of cytosol and an ATP-generating
system during the chase of the permeabilized cells gave rise to a complete recovery of pulse-labeled apoB-100. Since the ATP-generating system by itself (in buffer) failed to prevent the turnover of the total apoB-100, it appears that factors in the cytosol could prevent the intracellular degradation of apoB-100.

We could also demonstrate that the posttranslational degradation of apoB-100 involved not only the membrane-associated form of apoB-100, as previously shown (1, 14, 16) but also the apoB-100 present on mature (secretion-competent) lipoproteins. To our knowledge this is the first report demonstrating the sensitivity of apoB-100 to degradation even when present on such secretion-competent particles in the lumen of the secretory pathway.

The disappearance of apoB-100 radioactivity from the LDL-VLDL particles as well as the dense HDL-like particles (apoB-100 HDL) could be prevented by the ATP-generating system (in buffer) and did not seem to require cytosol. This is interesting since it suggests the existence of different mechanisms involved in the protection of the lipoprotein-associated apoB-100 pool and the membrane-associated form of apoB-100. Our results would therefore indicate that cytosol is of importance for the stabilization of the membrane-associated form of apoB-100, i.e. apoB-100 that has not yet been assembled into lipoproteins (33). This is also an intriguing observation since it has previously been suggested that the membrane-associated form of apoB-100 is associated with the cytosolic chaperone hsp 70 (34).

Not only did the cytosol and the ATP-generating system prevent the disappearance of apoB-100 but there was also a considerable increase in apoB-100 present on dense HDL-like particles (apoB-100 HDL) in the secretory pathway. The nature of this dense particle is not fully understood. It is extracted by sodium carbonate from microsomes of Hep G2 cells (6), McA-RH7777 cells (2), and primary rat hepatocytes (4), but it is not secreted from these cells. Instead it appears to be retained and degraded in the cell (15, 17).

The increase in the apoB-100 radioactivity present in apoB-100 HDL during the chase in the presence of cytosol and the ATP-generating system occurred without any significant change in the total amount of apoB-100 radioactivity. Moreover, it could not be inhibited by cycloheximide. These results indicate that apoB-100 HDL is generated by a redistribution of apoB-100 within the microsome. We have previously demonstrated that apoB-100-containing lipoproteins could be assembled from the membrane-associated form of the apolipoprotein (33). It is therefore highly likely that the membrane-associated apoB-100 is the precursor for apoB-100 HDL. This is further supported by the observation that there was a decrease in the amount of membrane-associated apoB-100 that could account for the entire increase in the radioactive apoB-100 HDL during the 2-h chase of the permeabilized cells. Moreover, the pulse-chase studies in the intact cells supported a precursor-product relationship between membrane-associated apoB-100 and the assembled apoB-100-containing lipoproteins.

We have previously presented results indicating that apoB-100 and lipoproteins cotranslationally (1). To reconcile these results with those of the present study as well as our recent results (33) that demonstrate a posttranslational assembly of lipoproteins, we suggest that apoB-100 could during translation either form a lipoprotein that is released to the lumen of the secretory pathway or associate with the ER membrane in an assembly-competent form. This membrane-associated form of apoB-100 could acquire lipids posttranslationally to form lipoproteins. Such a mechanism is also supported by the results presented by other authors (35).

It is now well established from numerous studies in cultured cells that apoB-100 undergoes posttranslational degradation. Our previous results indicated that apoB-100 on the dense HDL-like particle (see above) is retained and most likely degraded in the cell. These results are confirmed in the present study. We have suggested that the HDL-like particle is retained and degraded in the cell because it is too small to allow apoB-100 to fold correctly (this hypothesis has been elaborated previously (3, 17, 18).

In contrast, there is no previous information on degradation of the mature secretion-competent LDL-VLDL in intact cells, while such a degradation is clearly demonstrated in the present study. This is an intriguing observation since the reason for sorting to degradation is not obvious. One possibility would be that the arrest of the intracellular transport would result in a general degradation of secretory proteins. This is, however, ruled out by the observation that neither albumin nor α1-antitrypsin is degraded under these experimental conditions. When evaluating the intracellular fate of the lipoproteins assembled in Hep G2 cells, it should be kept in mind that these cells do not assemble the normal apoB-100 assembly product, i.e. VLDL, but rather a particle with an LDL size that contains triglycerol as the major neutral lipid. Whether this LDL-like particle is more prone to interact with intracellular systems involved in the retention and degradation of lipoproteins remains to be addressed experimentally.

It has been shown that the protease inhibitor ALLN (14, 21) inhibits the degradation of apoB and promotes the secretion of the protein (14, 35). In our previous studies in permeabilized Hep G2 cells (21), ALLN was found to be the most potent inhibitor of apoB degradation with a dose-dependent effect on this degradation. It was found very effective in abolishing the degradation of apoB and the generation of apoB degradation fragments. Our current results indicate that ALLN inhibited the degradation of apoB-100 that was present on lipoproteins in the lumen of the secretory pathway.

Posttranslational degradation is of potential importance in the regulation of apoB-100 secretion. Our previous results (15) indicate that apoB-100 that is not assembled into a mature lipoprotein is degraded. Other observations (35–37) may support a more regulatory role for degradation in the process of apoB-100 secretion. Characteristic for this regulatory degradation is that it is inhibited by ALLN (14, 21, 35), and it appears to generate rather specific proteolytic intermediates such as a 70- and an 85-kDa protein. Our results may indicate that the regulated degradation occurs at least to a certain degree on lipoproteins in the secretory pathway. Thus in addition to the inhibition of the degradation with ALLN, we have also observed that the apoB-100 fragments, including the recently described 70-kDa protein, appear in association with the different lipoproteins in the lumen of the secretory pathway, in particular with the dense particles banding in the density range of HDL. The generation of these fragments was completely inhibited by ALLN. This clearly indicates that the generation of the luminal apoB fragments is ALLN-sensitive and further confirms that luminal degradation of apoB is inhibited by ALLN. In contrast, the degradation of the membrane-associated apoB-100 of the permeabilized cells was not sensitive to ALLN. We could, however, detect the 70-kDa fragment in the membrane pellet after the carbonate extraction. The levels appeared, however, to be significantly lower than those found in the luminal content. The reason for this could simply be that this fragment is only relatively weakly associated with the lipids and therefore is extracted with sodium carbonate, thus ending up in this extract together with the lipoproteins. It should, however, be kept in mind that small amounts of secretory proteins, which are present in the ER lumen, will associate with the membrane during the carbonate extraction (Fig. 1).
Thus a portion of apoB-100 as well as of the 70-kDa fragment that is associated with the microsomal membrane could be expected to represent a nonspecific association of material from the lumen. Moreover, the observation that ALLN clearly inhibits the appearance of the 70-kDa fragment while it does not interfere with the degradation of the membrane-associated apoB-100 makes it less likely that this fragment is derived from membrane-associated apoB-100.

Both in intact and permeabilized cells ALLN failed to influence the turnover of the membrane-associated form of apoB-100, i.e. it failed to influence the percent of the initial pool that was turned over with time. However, the experiments carried out in intact cells revealed that ALLN increased the amount of radioactive apoB-100 that was associated with the membrane during the pulse-labeling of the intact cells. This increase was in turn reflected in an increase in the assembly of apoB-100-containing lipoproteins. The observation indicates that ALLN acts at a step in the assembly process that is very close to the translation of apoB 100.

The results presented in this report indicate that there exists, in Hep G2 cells, two different pathways for the degradation of apoB-100, one that is specific for the assembled lipoproteins in the lumen of the secretory pathway and one that may have the membrane-associated protein as its target. Our results also demonstrate that the luminal apoB-100 is degraded via an ALLN-sensitive pathway that generates the types of degradation intermediates that previously have been suggested to be associated with the regulatory posttranslational degradation. In the case of membrane-associated apoB-100, ALLN appears to promote the formation of a larger preassembly pool, while it does not seem to interfere with the turnover of this pool.

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