A role for palmitoylation in the quality control, assembly and secretion of apolipoprotein B

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ApoB (apolipoprotein B)-containing lipoprotein particles, such as chylomicrons, very-low-density and low-density lipoprotein particles, transport triacylglycerol and cholesteryl esters in the bloodstream. A palmitoylation site was previously mapped to Cys-1085 in a functional truncated apoB variant (apoB-29) and abolished by mutagenesis. This Cys-1085Ser mutation resulted in secretion of smaller and denser lipoprotein particles containing 80 % less cholesteryl ester and triacylglycerol than wild-type controls. We show that palmitoylation of apoB-29 occurs in the ER (endoplasmic reticulum), stimulates the ER–Golgi transport rate of apoB-29 almost 2-fold, doubles the secretion efficiency of wild-type apoB-29 in comparison with (Cys-1085Ser)apoB-29 and reduces significantly the association of wild-type apoB-29 with calnexin in comparison with (Cys-1085Ser)apoB-29. While

non-palmitoylated apoB-29 co-localized extensively with constitutively secreted transferrin, wild-type apoB-29 did so only partially and was enriched in ER extensions. Our results suggest that palmitoylation of apoB regulates the biogenesis of nascent apoB-containing lipoprotein particles by concentrating apoB in a specialized ER compartment and by stimulating dissociation from constituents of the ER quality-control machinery. This reduced interaction would lead to a faster ER–Golgi transit time and a higher secretion efficiency of wild-type apoB-29. Palmitoylation could regulate the amount of apoB available for secretion of neutral lipids.

Key words: apolipoprotein B, endoplasmic reticulum, Golgi apparatus, palmitoylation, secretion.

INTRODUCTION

ApoB (apolipoprotein B)-100 and apoB-48 are important components of low-density lipoprotein particles, intermediate-density lipoprotein particles and both VLDL (very-low-density lipoprotein) particles and chylomicron remnants [1,2]. Interestingly, all of these apoB-containing lipoprotein particles are considered to be atherogenic since their plasma concentrations have been shown to correlate positively with the trend to develop atherosclerosis [1]. Since apoB is required for the assembly of many atherogenic lipoproteins, it is important to understand its biosynthetic processes in order to design strategies that will allow a reduction of the concentration of circulating apoB, thereby reducing the levels of cholesterol and triacylglycerols in the blood.

Like most secreted proteins, apoB biogenesis starts in the ER (endoplasmic reticulum); unlike other secreted proteins it needs the association of lipids to form a mature lipoprotein particle. One proposed model for the assembly of lipoprotein particles involves a two-step process [3–5]. In the first step, a small, lipid-poor and dense apoB-containing lipoprotein particle precursor is formed during the translation and translocation of apoB into the rough ER lumen. ApoB, complexed with small amounts of triacylglycerol and cholesteryl esters, forms the precursor to an apoB-containing VLDL lipoparticle in a process that is thought to be dependent on the activity of microsomal triacylglycerol-transfer protein, an ER-resident protein [6]. If neutral lipids are not available or microsomal triacylglycerol-transfer protein activity is impaired, the newly synthesized apoB protein associates with the inner leaflet of the ER membrane where it potentially waits to be lipidated [7]. If lipidation does not take place, apoB is ubiquitinated, retrotranslocated to the cytosol and eventually degraded by proteasomes [8].

ApoB has been shown to interact with several molecular chaperones, including CLX (calnexin) and calreticulin, during its synthesis and maturation in the ER compartment [9]. Such molecular chaperones are known to associate with nascent proteins and contribute to their proper folding into a mature form. The two ER-resident molecular chaperones, CLX and calreticulin, are unique in binding selectively to glycoproteins with N-linked oligosaccharides [10]. Irreversibly misfolded or aggregated proteins are degraded via the ubiquitin/proteasome pathway [11].

Alternatively, in the second step of this model, the apoB-containing VLDL precursor fuses with a neutral lipid droplet to yield a mature VLDL particle. This particle then reaches an ER exit site and moves along the secretion route [12]. Borchardt and Davis [13] demonstrated that the exit from the ER is the rate-limiting step in the apoB-secretion process. Interestingly, it has not yet been established whether the lipid-loading process occurs exclusively at the smooth ends of the rough ER [3,14] or if it also continues in the Golgi apparatus [15–19].

ApoB-100 is a 512 kDa single polypeptide that is modified by disulphide bond formation, glycosylation, phosphorylation and palmitoylation [1]. Despite the fact that apoB was shown to be palmitoylated [20–25], the involvement of palmitoylation in the structure and/or function of this lipoprotein has not been fully investigated. Previous studies performed by this laboratory showed that apoB-29, a functional truncated form of apoB that comprises the N-terminal 29 % of the protein, is palmitoylated on its only free cysteine (Cys-1085) and that palmitoylation at Cys-1085 was involved in the assembly of the hydrophobic neutral lipid core of the lipoprotein particle. Indeed, secreted lipoprotein particles containing non-palmitoylated apoB-29 with a Cys-1085 → Ser mutation [(Cys-1085Ser)apoB-29] were shown to contain five times less neutral lipids (cholesteryl esters and

Abbreviations used: apoB, apolipoprotein B; CLX, calnexin; endo H, endoglycosidase H; VLDL, very-low-density lipoprotein; ER, endoplasmic reticulum; DMEM, Dulbecco's modified Eagle's medium; DSP, dithiobis(succinimidylpropionate).

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triacylglycerols) than those containing palmitoylated apoB-29, whereas phospholipid levels were not affected. Using indirect immunofluorescence, under steady-state conditions most of the palmitoylated apoB-29 appeared to be found in large vesicular structures containing endogenous apoB-48 and apoB-100 and corresponding to a subcompartment of the ER, while non-palmitoylated (Cys-1085Ser)apoB-29 appeared to be concentrated in the Golgi compartment [25]. Although cells do not naturally synthesize apoB-29, this truncated form of apoB constitutes a functional model to study the role of palmitoylation in the biogenesis and secretion of apoB-100, since it may represent an early stage intermediate in the assembly of lipoprotein particles.

Since very little is known about the role of palmitoylation of secreted proteins, we wanted to investigate further the role of palmitoylation in the assembly and secretion apoB. In this work, we show that palmitoylation of apoB occurs early in its biogenesis, stimulates the ER–Golgi transport rate of apoB-29, doubles apoB-29 secretion efficiency and appears to be involved as a quality-control step favouring dissociation of apoB-29 from the ER chaperone CLX [26]. In addition, non-palmitoylated apoB-29 co-localized extensively with the constitutively secreted protein transferrin, while palmitoylated apoB-29 showed only partial colocalization and appeared to be enriched in extensions of the ER. Thereby, our results suggest an important role for palmitoylation in the quality control, assembly and secretion of apoB-containing lipoprotein particles.

MATERIALS AND METHODS

Cell lines, antibodies and reagents

McArdle RH-7777 rat hepatoma cells stably expressing human wild-type apoB-29 and human (Cys-1085Ser)apoB-29 were engineered in a previous study [25] and human apoB-18 was obtained from Dr Zemin Yao (University of Ottawa, Ottawa, Ontario, Canada). These stable cell lines were maintained at 37 °C in a humidified incubator with a 7 % CO₂ atmosphere and grown in DMEM (Dulbecco's modified Eagle's medium) containing 100 units/ml penicillin G (sodium salt), 100 mg/ml streptomycin sulphate, 400 µg/ml Geneticin and supplemented with 10 % fetal bovine serum, 10 % horse serum (Life Technologies, Grand Island, NY, U.S.A.). Tran³⁵S-Label ³⁵S-labelled *Escherichia coli* Hydrolysate Labelling Reagent, containing 70 % L-[³⁵S]methionine, was purchased from ICN Biomedicals (Irvine, CA, U.S.A.). [¹²⁵I]Iodopalmitate was prepared as described in [27]. Sheep anti-(human apoB) antiserum was purchased from Roche Molecular Biochemicals (Darmstadt, Germany). Endo H (endoglycosidase H) was purchased from New England Biolabs (Beverly, MA, U.S.A.). Trypsin (0.25 %, w/v) was purchased from Life Technologies. 1D1 monoclonal antibody, which recognizes amino acids 474–579 at the N-terminus of human apoB protein, was purchased from Heart Institute Research Corporation (Ottawa, Ontario, Canada). Rabbit anti-CLX antibody was from StressGen Biotechnologies Corp. (Victoria, BC, Canada). Rabbit anti-giantin antibodies were a kind gift from Dr Ed Chan (University of California San Diego, CA, U.S.A.). Texas Red-coupled donkey anti-mouse IgG and FITC-coupled rabbit anti-rat IgG were from Jackson ImmunoResearch Laboratories (West Grove, PA, U.S.A.). The reducible cross-linker DSP [dithiobis(succinimidylpropionate)] was from Pierce Biotechnology (Rockford, IL, U.S.A.). All other reagents used were purchased from Sigma (St. Louis, MO, U.S.A.) with the exception of electrophoresis reagents, which were from Bio-Rad (Hercules, CA, U.S.A.).

Metabolic labelling of McArdle RH-7777 cells stably expressing human apoB-29 and immunoprecipitation of apoB-29

Approx. 3.25×10^6 McArdle RH-7777 rat hepatoma cells stably expressing either wild-type human apoB-29 or human (Cys-1085Ser)apoB-29 were seeded on to 100 mm dishes and grown overnight at 37 °C as described above. Cells were washed twice with methionine/cysteine-deficient DMEM and incubated with 2.5 ml of methionine/cysteine-deficient DMEM containing 280 µCi of Tran³⁵S-Label for 15 min or incubated for 1 h with DMEM supplemented with 10 µg/ml fatty-acid-free BSA and then pulsed for 15 min with 250 µCi of [¹²⁵I]iodopalmitate in 2.5 ml of DMEM supplemented with 10 µg/ml fatty-acid-free BSA [27]. After incubation and removal of pulse medium, the cells were quickly washed twice with warm PBS, and 4 ml of DMEM supplemented with 200 µM methionine or 2.5 ml of DMEM supplemented with 10 µg/ml fatty-acid-free BSA was added. Cells were incubated at 37 °C for 0, 15, 30, 45, 60, 90, 120 and 240 min. At the indicated times, medium was removed and lipoproteins were solubilized for 24 h at 4 °C in the presence of 0.1 % SDS/RIPA buffer [1 mM EDTA, 1 mM dithiothreitol, 1 % (v/v) Triton X-100, 1 % (w/v) sodium deoxycholate, 150 mM NaCl, 50 mM Tris/HCl, pH 8.0] containing 1 mM PMSF and 40.5 µM leupeptin. Cells were lysed with hot (75 °C) 1 % SDS/RIPA buffer, solubilized 24 h at 4 °C and diluted to a final concentration of 0.1 % SDS/RIPA buffer. After solubilization, an aliquot was removed in order to determine the amount of total proteins present in each sample. ApoB lipoproteins were collected from the media or cell lysates by immunoprecipitation using Roche's anti-(human apoB) polyclonal serum (20 µl; 2 h at 4 °C with agitation) or mouse monoclonal 1D1 anti-(human apoB) (8 µg; 3 h at 4 °C with agitation). In immunoprecipitations, 100 µl of a slurry made of 20 % (v/v) Protein A–Sepharose CL-4B (Amersham Biosciences, Uppsala, Sweden) and 30 % (v/v) Sepharose CL-4B (Sigma) was added to solubilized supernatants and cell lysates and incubated for 2 h at 4 °C with agitation. Tubes containing the reactions were centrifuged for 15 min at 1400 g to isolate the Sepharose beads containing the immune complexes. The beads were further washed five times with 0.1 % SDS/RIPA buffer. In order to elute the apoBs, the beads were heated at 75 °C for 15 min in the presence of either endo H denaturing buffer (0.5 % SDS, 1 % β-mercaptoethanol and 10 mM Tris/HCl, pH 7.4) or loading buffer (5 % β-mercaptoethanol, 8 M urea, 10 mM Tris/glycine, pH 8.3, 2 % SDS and 10 % glycerol), spun down for 1 min at maximum velocity in a table-top microcentrifuge and the supernatants recovered.

ApoB-29 secretion analysis

Aliquots of the apoBs eluted from the immune complexes originating from cell lysates and media were separated by electrophoresis [28], dried and exposed to a phosphorimager screen. Intensities of the bands corresponding to apoB-29 obtained on a Storm 840 phosphorimager were quantified using the ImageQuant software (Amersham Biosciences). The values obtained were normalized by the total amount of proteins at each given time and then expressed as percentage of total arbitrary units at $t = 0$.

Quantification of apoB-29 bound to CLX using DSP cross-linking

The cross-linking experiments using the reducible cross-linker DSP were performed as described by Linnik and Herscovitz [9], except that DSP was used at a 10 times higher concentration and that all buffers contained the freshly added protease inhibitors 1 mM PMSF and 20 µg/ml leupeptin. Approx. 3.06×10^6 cells

were seeded on to 100 mm dishes grown overnight and incubated for 1 h in the presence of Met-/Cys-deficient DMEM. Cells were pulsed for 15 min with 200 μ Ci/plate of Tran³⁵S-Label in 3 ml of Met-/Cys-deficient DMEM and chased for 0, 15, 30, 45 and 60 min with regular DMEM supplemented with 200 μ M Met. At each time point, the cells were washed twice with cold PBS and incubated with 3 ml of a 2 mM solution of DSP in PBS (pH 7.4) for 2 h on ice. The cross-linking reaction was stopped by addition of 150 μ l of 1 M Tris/HCl, pH 7.4, for 10 min on ice. Cells were lysed with 1 ml of lysis buffer (50 mM Hepes, pH 7.4, 5 mM EDTA, 5 mM EGTA, 0.15 M NaCl and 2 % CHAPS) and the lysates were pre-cleared with 100 μ l of a 50 % slurry of Protein A-Sepharose in water for 45 min at 4 °C with agitation. After centrifugation (2 min at 16 000 g), CLX was immunoprecipitated from the supernatants using 8 μ l of a rabbit anti-CLX antibody for 4 h at 4 °C with agitation. Immune complexes were recovered by incubation with 100 μ l of a 50 % slurry of Protein A-Sepharose in water for 3 h at 4 °C with agitation. The beads were recovered by centrifugation and extensively washed as described by Linnik and Herscovitz [9], and CLX with its cross-linked proteins was recovered by boiling the beads for 5 min at 100 °C in 200 μ l of 1 % SDS in PBS, pH 7.4. The supernatant was recovered by centrifugation and diluted 5-fold with 1 % Triton X-100 in PBS, pH 7.4, and apoB-29 was recovered by immunoprecipitation with 20 μ l of 1D1 monoclonal antibody overnight at 4 °C with agitation followed by Protein A-Sepharose treatment as described above and washed three times with 1 ml of 1 % Triton X-100/0.5 M NaCl in PBS, pH 7.4.

To release apoB-29 from CLX, DSP cross-linker was reduced by boiling the beads for 5 min at 100 °C in 100 μ l of modified loading buffer (10 % β -mercaptoethanol, 8 M urea, 10 mM Tris/glycine, pH 8.3, 2 % SDS and 10 % glycerol). The samples were separated using Tris/Tricine SDS/PAGE [28] and transferred to a PVDF membrane. In order to quantify and confirm the presence of total CLX and apoB-29 in each lane, the bands corresponding to CLX and apoB-29 were identified by Western blot/ECL using a rabbit anti-CLX antibody and 1D1 monoclonal antibody respectively (results not shown). The ECL signal was then quenched by drying the membranes overnight and the dried membranes were exposed to a phosphorimager screen in order to quantify the ³⁵S-labelled apoB-29 released from CLX. The relative specific radioactivity (³⁵S-signal/ECL signal) activity of each apoB-29 band was calculated and plotted.

Endo H cleavage assay

After the 15 min radioactive methionine/cysteine pulse, apoBs were immunoprecipitated from media and cell lysates and eluted from Protein A-Sepharose as described above, combined and incubated overnight at 37 °C in the presence of 500 units of endo H in endo H reaction buffer (50 mM sodium citrate, pH 5.5). The digestion products were then separated by electrophoresis [28] and the gel was processed as described above. The data were expressed as percentage of endo H-sensitive apoB-29 in arbitrary units.

Microsome preparation

Microsomes were prepared using hypotonic cell lysis according to Berthiaume et al. [29]. Briefly, monolayers of McArdle RH-7777 cells expressing either wild-type human apoB-29 or human (Cys-1085Ser)apoB-29 were harvested, swelled in MgRS hypotonic buffer (10 mM NaCl, 1.5 mM MgCl₂ and 10 mM Tris/HCl, pH 7.4) for 15 min on ice and disrupted in a Dounce homogenizer.

Following the addition of 0.4 vol. of 2.5 $\times$ MS solution (525 mM mannitol, 175 mM sucrose, 12.5 mM Tris/HCl, pH 7.4, and 12.5 mM EDTA) to preserve organelle integrity, the cells were homogenized five more times. The post-nuclear supernatant (1000 g) was further centrifuged at 16 000 g for 20 min at 4 °C yielding a microsome-enriched pellet.

Permeabilization of microsomes by Na₂CO₃ treatment

Microsomes were permeabilized in cold 100 mM Na₂CO₃, pH 11.0, at a concentration of 2 mg of protein/ml, incubated on ice for 15 min and centrifuged at 120 000 g for 10 min at 4 °C in a Beckman TLA 100.4 rotor (Beckman Instruments, Fullerton, CA, U.S.A.) as described by Michalak et al. [30]. The supernatant was diluted in 1 $\times$ MS solution, separated into 1 mg aliquots, frozen in liquid nitrogen and stored at -80 °C. Sodium carbonate treatment does not affect the integrity of lipoprotein particles and is routinely used to prepare microsome extracts containing lipoprotein particles prior to density-gradient analysis in VLDL secretion experiments [31–33].

Trypsin digestion of microsomes

Sodium carbonate supernatant aliquots (1 mg) were thawed on ice and adjusted to pH 8.0 with 1 M KH₂PO₄, pH 4.0. The aliquots were incubated with 10 μ g of trypsin at 37 °C, and at various times 0.1 mg aliquots were taken out. Reactions were stopped by the addition of 1 vol. of 2 $\times$ sample buffer (0.25 M Tris, pH 6.8, 8 % SDS, 40 % glycerol, 0.1 % Bromophenol Blue, 100 mM dithiothreitol, 1 mM PMSF and 40.5 μ M leupeptin) and frozen immediately. Samples were separated by electrophoresis [28].

Fractionation of cellular membranes on a sucrose gradient

Fractionation of cellular membranes was carried out using the established procedure of Evans [34]. Microsomes from McArdle RH-7777 cells expressing either wild-type human apoB-29 or human (Cys-1085Ser)apoB-29 were resuspended in 1 ml of a 10 % (w/v) sucrose solution in 10 mM Tris/HCl, pH 7.4, buffer containing 20 μ g/ml leupeptin and layered on top of a discontinuous sucrose gradient consisting of (from bottom to top) 1.5 ml of 70 % (w/v), 2 ml of 55 % (w/v), 5 ml of 39 % (w/v), 2 ml of 27 % (w/v) sucrose in 10 mM Tris/HCl, pH 7.4, buffer. The gradient was then topped with 0.5 ml of buffer and centrifuged at 100 000 g in a Beckman SW 41 Ti rotor (Beckman Instruments) at 4 °C for 5 h. After centrifugation, 1 ml fractions were collected (from top to bottom) and PMSF was added to a 1 mM final concentration. Total proteins were quantified for each fraction. Fractions were kept at -20 °C.

Analysis of apoB-29 content of density gradient fractions using slot blot

Aliquots (20 μ l) of each fraction were diluted to 1 ml in 10 mM Tris/HCl, pH 7.4, buffer containing 1 mM PMSF and 20 μ g/ml leupeptin, and blotted on to a 0.45 μ m nitrocellulose membrane (Bio-Rad; previously equilibrated for 10 min in the same buffer) using a Bio-Rad Slot Blot apparatus. After blotting, membranes were blocked for 1 h in 1 $\times$ Blotto solution [5 % (w/v) non-fat dried milk in PBS] and probed for human apoB using the 1D1 anti-(human apoB) antibody as described earlier in Zhao et al. [25]. This 1D1 antibody does not cross-react with endogenous rat apoB [25].

Immunoblots

After electrophoresis [28], proteins were transferred on to an Immobilon-P PVDF membrane (Millipore Corporation, Bedford, MA, U.S.A.) for 2 h at 0.5 A in a Bio-Rad Trans Blot apparatus (Bio-Rad). Membranes were probed with the indicated antibodies and processed for ECL Plus detection (Amersham Biosciences).

Indirect immunofluorescence

McArdle RH-7777 cells stably expressing either wild-type human apoB-29 or (Cys-1085Ser)apoB-29 were processed for indirect immunofluorescence and observed by confocal laser scanning microscopy as described in Zhao et al. [25]. To detect apoB-29 and transferrin, 1D1 mouse monoclonal anti-(human apoB) and rat anti-transferrin (ICN) antibodies were used at a dilution of 1:1000 in 1 × Blotto. After incubation, cells were washed four times with PBS and incubated with Texas Red-conjugated goat anti-mouse IgG and with FITC-conjugated rabbit anti-rat IgG, both diluted 1:200, in 3 % BSA for 1 h at 37 °C in a humidified chamber. Cells were mounted in Prolong Antifade Solution (Molecular Probes, Eugene, OR, U.S.A.) and observed by confocal laser scanning microscopy.

Electrophoresis

Proteins were separated using discontinuous Tris/Tricine gels made of a 3 % acrylamide stacking gel in which there are 3 % total monomers and 3 % cross-linkers (3 %T/3 %C), and a 5.5 % acrylamide separating gel in which there are 5.5 % total monomers and 3 % cross-linkers (5.5 %T/3 %C) [28].

Protein quantitation

The protein concentration of samples was determined using the BCA Protein Assay Reagent (Pierce) following the manufacturer's instructions. BSA was used as standard.

Statistical analysis

Two-way ANOVA, followed by the Student–Newman–Keuls method for multiple comparisons, was used to detect significant differences in interactions of palmitoylated and non-palmitoylated apoBs with CLX.

RESULTS

In order to study the role of palmitoylation in apoB biogenesis, we compared various quality-control aspects of protein secretion using palmitoylated and non-palmitoylated apoB-29 variants. We therefore measured the apoB-29 secretion rate and efficiency, the rate of ER–Golgi transport of apoB-29 (by an assay of the acquisition of endo H resistance) and the interactions of apoB-29 with the molecular chaperone CLX. To do so, McArdle RH-7777 cells stably expressing wild-type apoB-29 or (Cys-1085Ser)apoB-29 [25] were labelled with [³⁵S]methionine/cysteine for 15 min (pulse) and the residual intracellular and secreted labelled apoB-29s were collected by immunoprecipitation and quantified by phosphorimaging (Figure 1). While the rates of disappearance of both residual apoB-29s inside the cells were very similar, there was a 2-fold decrease in the (Cys-1085Ser)apoB-29 secretion efficiency compared with that of the wild-type apoB-29 (26 ± 0.4 and 54 ± 1.2 %, respectively, $n = 4$).

Figure 1 Human (Cys-1085Ser)apoB-29 is secreted less efficiently than the wild-type apoB-29 from McArdle RH-7777 cells

[³⁵S]Cys/Met pulse-chase analysis of McArdle RH-7777 rat hepatoma cells stably expressing either human wild-type apoB-29 (●) or (Cys-1085Ser)apoB-29 (○) performed as described in the Materials and methods section. At the indicated times, apoB-29s from the medium (A) as well as from the cells (B) were recovered by immunoprecipitation, solubilized in electrophoresis sample buffer, boiled and separated by Tris/Tricine SDS/PAGE. The specific radioactivity of samples collected at each time point was calculated. The results are expressed as a percentage of total activity at $t = 0$ (means $\pm$ S.E.M.; $n = 4$) as a function of time.

This decrease in (Cys-1085Ser)apoB-29 secretion efficiency suggests that the non-palmitoylated form of the protein is more actively degraded than the wild type, perhaps due to a longer residence time in the ER.

In order to investigate this possibility, we sought to measure differences between the rates of ER–Golgi transport of wild-type and (Cys-1085Ser)apoB-29. To do so, we established the kinetics of acquisition of endo H resistance using [³⁵S]methionine/cysteine metabolically labelled McArdle RH-7777 cells stably expressing wild-type or (Cys-1085Ser)apoB-29s. While in the ER, proteins are modified with a high-mannose oligosaccharide moiety; once they are transported to the Golgi, the high-mannose moiety is trimmed, fucosylated and complex oligosaccharides are added to yield what is known as a hybrid oligosaccharide [35]. Both ER and medial Golgi forms of the protein are of similar apparent molecular mass. To differentiate the form of a protein that is present in the ER from the one in the Golgi apparatus, proteins are harvested and subjected to endo H cleavage. While endo H is able to trim the high-mannose moiety from a protein that is trafficking through the ER, it is unable to trim the fucosylated hybrid oligosaccharides that modify proteins moving along the Golgi apparatus. After the endo H cleavage reaction has been performed, these endo H-sensitive (faster electrophoretic mobility, originating from the ER) and endo H-resistant (slower electrophoretic mobility, originating from the Golgi) forms of a protein can be distinguished by SDS/PAGE and their proportions quantified by densitometry or phosphorimager analysis.

Figure 2(A) shows that, at time 0, both wild-type apoB-29 and (Cys-1085Ser)apoB-29 ³⁵S-labelled proteins appear uniquely in

Figure 2 Human (Cys-1085Ser)apoB-29 is retained longer in the ER than wild-type apoB-29

³⁵S-labelled apoB-29 from McArdle RH-7777 cells stably expressing either human wild-type (WT) apoB-29 or (Cys-1085Ser)apoB-29 was recovered during a pulse-chase experiment by immunoprecipitation at different times as described in Figure 1. ApoB-29s from cells and media were combined and incubated overnight in the presence or absence of endo H. (A) Digestions were separated by Tris/Tricine SDS/PAGE and the radioactivity incorporated into apoB-29 was detected by phosphorimager analysis. (B) Endo H-sensitive and -resistant bands were quantified using the ImageQuant program and their ratios are plotted as a percentage of endo H-sensitive apoB-29 (means $\pm$ S.E.M.) at each time point. The experiments were done in duplicate and repeated three times. $\circ$, (Cys-1085Ser)apoB-29; $\bullet$, wild-type apoB-29.

their cleaved endo H-sensitive (lower apparent molecular mass) ER form. At 15 min of chase, higher-apparent-molecular-mass, endo H-resistant, apoB-29 forms start to appear. At 30 min of chase, approx. 60 % of the wild-type ³⁵S-apoB-29 and 40 % of ³⁵S-(Cys-1085Ser)apoB-29 appear in the higher-apparent-molecular-mass, endo H-resistant form, and as such are believed to have reached the Golgi. This indicates that the time required to transport 50 % of apoB-29 from ER to Golgi seems to be shorter than 30 min for the wild type and significantly longer for the non-palmitoylated (Cys-1085Ser)apoB-29-expressing cells. Figure 2(B) represents the quantification of the endo H-sensitive apoB-29 expressed as the percentage of the total amount of signal at each time point, normalized by total amount of proteins before immunoprecipitation, for three independent experiments. This plot confirms that the apparent half-life ($t_{1/2}$ the time at which 50 % of the apoB-29 reaches the Golgi) for wild-type apoB-29 is shorter than that for (Cys-1085Ser)apoB-29. From a logarithmic replot of these data (results not shown), we calculated a $t_{1/2}$ for wild-type apoB-29 of 26 ± 4.4 min and a $t_{1/2}$ for (Cys-1085Ser)apoB-29 of 46 ± 1.9 min. Therefore palmitoylated apoB-29 was transported from the ER to the Golgi apparatus almost twice as fast as non-palmitoylated (Cys-1085Ser)apoB-29.

Figure 3 Both wild-type and Cys-1085Ser human apoB-29 are apparently folded in a similar fashion

Microsomes obtained from McArdle RH-7777 cells stably expressing either human wild-type (WT) apoB-29 or (Cys-1085Ser)apoB-29 were permeabilized with 0.1 M Na₂CO₃, pH 11.0, and centrifuged to yield an insoluble pellet and a soluble supernatant enriched in luminal proteins. (A) Luminal/supernatant aliquots (1 mg) were subjected to kinetic partial trypsin digestion and, at the indicated times, 0.1 mg aliquots were taken, separated by Tris/Tricine SDS/PAGE and immunoblotted using anti-(human apoB) antibody. (B) The bands corresponding to full-length apoB-29 were quantified by densitometry and plotted as the logarithm of the percentage of arbitrary units (a.u.) at $t = 0$. Experiments were done in duplicate and repeated three times; the most representative results are shown. $\circ$, (Cys-1085Ser)apoB-29; $\bullet$, wild-type apoB-29.

In order to test whether the ER exit rate differences might be due to differences in protein folding, we assessed the overall folding state of both wild-type and (Cys-1085Ser)apoB-29s using partial proteolytic cleavage kinetics. To do so, the luminal content of microsomes prepared from McArdle RH-7777 cells stably expressing either wild-type or (Cys-1085Ser)apoB-29s was subjected to kinetic partial proteolytic digestion with trypsin. The rationale behind this experiment is that if both proteins are folded in a similar fashion, they will expose the same proteolytic sites. Therefore, they should be degraded at the same rate and display similar proteolytic cleavage intermediates. Figure 3(A) demonstrates the high level of similarity of the proteolytic patterns as assessed by the number and size of cleavage intermediates and the speed at which these intermediates appear or disappear (Figure 3B). These results suggest that, in the luminal fraction, both wild-type and (Cys-1085Ser)apoB-29 proteins appear to be folded in a highly similar fashion.

To investigate the influence of palmitoylation in the sorting dynamics of apoB-29, we quantified the association of both wild-type and (Cys-1085Ser)apoB-29s with CLX using cross-linking followed by co-immunoprecipitation as described by others [6,9,26]. The data from Figure 4 suggest that the loss of palmitoylation at Cys-1085 of apoB-29 results in an increased association with chaperone CLX ($P < 0.05$). This increased interaction could explain the increased ER retention time and lower

Figure 4 Association of wild-type and (Cys-1085Ser)apoB-29s with CLX

McArdle RH-7777 rat hepatoma cells stably expressing either human wild-type (Wt) apoB-29 or (Cys-1085Ser)apoB-29 were pulse-labelled for 15 min with [³⁵S]Cys/Met medium and chased for 1 h. At the indicated times cells were cross-linked *in situ* and then lysed. CLX was immunoprecipitated (IP) and apoB-29 was recovered from the immune complexes using 1D1 antibodies, solubilized in electrophoresis buffer, boiled, separated by Tris/Tricine SDS/PAGE and transferred on to a PVDF membrane, and CLX as well as apoB-29 were identified by Western blot (results not shown). **(A)** The membranes were then dried and the radioactivity incorporated into the bands corresponding to apoB-29 was detected using phosphorimager analysis. **(B)** The specific activity of samples collected at each time point was calculated and the results expressed as the percentage of total activity at *t* = 0 (means ± S.E.M.). Closed bars, wild-type apoB-29; open bars, (Cys-1085Ser)apoB-29. Experiments were performed three times; * significant differences between wild-type and (Cys-1085Ser)apoB-29s at the indicated times, *P* < 0.05.

ER–Golgi transport rate of (Cys-1085Ser)apoB-29 seen in Figure 2 in comparison with wild-type apoB-29. Thus it appears that palmitoylation of apoB-29 might be involved in the dissociation of apoB-29-containing lipoprotein particles from CLX in the ER.

Using indirect immunofluorescence, palmitoylated and non-palmitoylated apoB-29s showed apparent acylation-dependent differences in subcellular localization [25]. In order to investigate whether palmitoylation affects the relative distribution of apoB-29 between the ER and Golgi apparatus, microsomes from cells stably expressing wild-type or (Cys-1085Ser)apoB-29 were separated on a discontinuous sucrose gradient. The presence of apoB-29 along the gradient was analysed (Figure 5) and showed that the lack of palmitoylation does not affect the proportions of apoB-29 accumulated along the main compartments of the secretory pathway. Therefore it appears that palmitoylation only affected the localization of apoB-29 within the ER itself, as suggested in Zhao et al. [25], or that our cellular fractionation method is unable to successfully separate the compartments containing the two forms of apoB-29 on the basis of density.

We compared the localization of various apoBs with that of the constitutively secreted protein transferrin using indirect immunofluorescence and confocal microscopy (Figure 6). While wild-type apoB-29 localized in large vesicular structures distri-

buted throughout the cytosol, (Cys-1085Ser)apoB-29, apoB-18 and transferrin were localized in larger perinuclear structures (identified as the Golgi apparatus; results not shown). The merged images in Figure 6 show that wild-type apoB-29 displays a low degree of co-localization with transferrin while both (Cys-1085Ser)apoB-29 and apoB-18 co-localize to a high degree with transferrin. These results indicate that non-palmitoylated apoB-29 and apoB-18 appear to be secreted in a constitutive fashion like transferrin. Thus these non-palmitoylated apoB constructs appear to lack the concentration step at the ER extensions prior to secretion. Therefore it appears that palmitoylation might confer a signal responsible for increasing the partitioning of apoB-29 to these intra-ER sites.

In order to identify the apoB-acylation site, we metabolically labelled McArdle RH-7777 cells stably expressing wild-type apoB-29 with [¹²⁵I]iodopalmitate for 15 min (pulse), and studied the dynamics of apoB-29 covalent acylation during the chase period by performing acquisition of endo H-cleavage-resistance assays. Figure 7 shows that apoB-29 is palmitoylated in both endo H-sensitive (ER) and endo H-resistant (Golgi) bands at times 0 and 15 min. This suggests that not only is wild-type apoB-29 palmitoylated early in its biosynthesis in the ER, but also that this process is likely to be dynamic and also occurs during the transit of the protein through the Golgi apparatus. This result links the site of palmitoylation of apoB-29 to the site of interaction with chaperones/glycoprotein-folding sensors and is therefore consistent with a role for protein palmitoylation in the quality control and secretion of apoB.

DISCUSSION

Lack of palmitoylation of apoB-29 resulted in changes in its subcellular distribution under steady state and a five-times reduction in triacylglycerol and cholesteryl ester content in lipoprotein particles containing mutated apoB-29 in comparison with those containing wild-type apoB-29 [25]. We now show that palmitoylation of apoB occurs in the ER compartment; this increases the ER–Golgi transfer rate of apoB-29 as well as apoB-29 secretion efficiency, and is probably achieved at the molecular level by decreasing the interaction time of the palmitoylated apoB-29 protein with the ER chaperone CLX. The longer interaction of the non-palmitoylated form of apoB-29 with CLX might explain the increased degradation as well as the increased ER residence time of this protein. Our data therefore demonstrate the involvement of protein palmitoylation as an active participant in the quality control of a secreted protein, apoB, and suggest that it may also act as a positive transport signal along the secretory pathway.

Since the increase in the retention time of non-palmitoylated apoB-29 in the ER was by no means due to aggregation, nor to a severe difference in folding, palmitoylation therefore appears to be involved in the quality control of the nascent apoB-containing lipoprotein particle assembly prior to its secretion. In addition, to apparently stimulate the dissociation of wild-type apoB-29 from CLX, apoB-29 palmitoylation might also act as a specialized ER transport signal, since it also stimulates ER–Golgi transport and favours concentration of wild-type apoB-29 to extensions of the ER. In agreement with this possibility is the fact that lipoprotein particles containing non-palmitoylated apoB-29, which have lower neutral lipid content, do not co-localize with palmitoylated endogenous apoB in these ER extensions [25]. This suggests that palmitoylation may act as an intra-organelle sorting signal directing the protein towards specific regions of the secretory pathway where the lipid loading machinery resides.

Figure 5 Lack of palmitoylation does not alter the relative apoB-29 distribution between the ER and Golgi compartments

Microsomes prepared from McArdle RH-7777 cells stably expressing either wild-type (WT) apoB-29 or Cys-1085Ser mutant human apoB-29 [(C1085S) apoB-29] were separated on a discontinuous sucrose gradient as described in the Materials and methods section. (A) Aliquots from fractions were slot-blotted on to nitrocellulose and apoB-29 was immunodetected using 1D1 anti-(human apoB). (B) The slot-blots were quantified by densitometry, normalized by the amount of total protein present in each sample and plotted as a percentage of total specific activity (means $\pm$ S.E.M.). Golgi-containing fractions or ER-containing fractions were determined by the presence of established organelle markers using immunoblotting; giantin (GNT) for Golgi (C) and CLX for ER (D). (E) ApoB-29 specific activities from Golgi- and ER-containing fractions were added and expressed as a percentage of total specific activity (means $\pm$ S.E.M.) as a function of fraction number. Experiments were done in duplicate and repeated twice. $\circ$ /Open bars, (Cys-1085Ser)apoB-29; $\bullet$ /closed bars, wild-type apoB-29; $\triangle$, fraction density.

Palmitoylation of apoB-29 could thus stimulate lateral diffusion of the protein within the ER. Lending credibility to this possibility is the fact that non-palmitoylated CCR5, a G-protein-coupled receptor, was shown to have a significantly reduced ER lateral diffusion coefficient in comparison with wild-type CCR5 [36].

The fact that non-palmitoylated apoB-29 co-localized extensively with transferrin and appeared to be concentrated in the Golgi is probably due to the lower relative amount of Golgi membranes in comparison with ER membranes present in the cells (ER/Golgi membrane ratio of 10:1) [37]. Interestingly, the non-palmitoylated apoB-29-containing lipoprotein particles appeared to be sorted without the concentration step in ER extensions and secreted in a more constitutive fashion, like transferrin, again suggesting a key role for palmitoylation in subcellular localization and assembly of apoB.

In contrast, recent results by Vukmirica et al. [38] claimed that there was a lack of evidence that palmitoylation of apoB-48 is required for lipoprotein secretion. They based their conclusion on the fact that a non-palmitoylated form of apoB-48 resulted in no significant differences in apparent intracellular localization or buoyant density of apoB-48-containing lipoprotein particles but had only a minor effect of the order of a 10–20% reduction of secretion efficiency [38]. Paradoxically, Vukmirica et al. [38] also showed a linear dependence of the number of palmitoyl moieties present on apoB-48 and the secretion efficiency of their various palmitoylated apoB-48 species. Interestingly, in that same figure, the percentage of secreted apoB-48s are shown as approx. 54 and 32% for wild-type and non-palmitoylated apoB-48^{Cys-1085-1635} respectively. Therefore, these results are in agreement with the results obtained with our apoB-29 constructs ($54 \pm 1.2\%$ and

Figure 6 Lack of palmitoylation alters the apparent intracellular localization of human apoB-29

Co-localization of transferrin with human wild-type apoB-29, human (Cys-1085Ser)apoB-29 and human apoB-18 in McArdle RH-7777 rat hepatoma cells. Wild-type human apoB-29 (MH-WT apoB-29), (Cys-1085Ser) human apoB-29 [MH-apoB-29 (Cys-1085Ser)] or human apoB-18 (MH-apoB-18) were detected using 1D1 anti-(human apoB) monoclonal antibody and goat anti-mouse IgG conjugated with Texas Red (red, left-hand column). Transferrin (green) was detected with rabbit polyclonal anti-(rat transferrin) and goat anti-(rabbit IgG) antibodies conjugated with FITC (green, central column). The merged images are presented in the right-hand column. Yellow indicates apparent co-localization of red and green signals. Scale bar, 10 μ m.

Chase (min)	0		15		30		45		60	
EndoH	+	-	+	-	+	-	+	-	+	-
apoB-29										

Figure 7 Palmitoylation of Cys-1085 in apoB-29 occurs at an early stage of synthesis

McArdle RH-7777 cells stably expressing wild-type apoB-29 were metabolically labelled with [¹²⁵I]iodopalmitate and wild-type apoB-29 was recovered at the indicated times by immunoprecipitation as described in the Materials and methods section. ApoB-29 was eluted from the immunocomplexes as described in the Materials and methods section, combined with endo H reaction buffer and incubated overnight in the presence or absence of endo H. Samples were separated by Tris/Tricine SDS/PAGE, blotted on to a PVDF membrane and exposed for 30 days to radiographic film.

26 ± 0.2 % for palmitoylated and non-palmitoylated apoB-29 respectively), since in both cases an approx. 40 % reduction was observed, which we believe to be important and highly significant. One of the more significant effects published by Vukmirica et al. [38] is that there was a 2–3-fold increase in the proportion of non-palmitoylated apoB-48 bound to microsomal membranes in comparison with wild-type apoB-48. This significant result is also consistent with our data that demonstrate a significant increase in binding of non-palmitoylated apoB-29 to CLX, an integral membrane protein. Therefore, palmitoylation may be involved in the regulation of the interaction/association of apoBs with the inner membrane leaflet of the lumen along the secretory pathway, per-

haps by regulating interaction with one or more molecular chaperones.

The timing of apoB-29 palmitoylation is consistent with its role in the folding, sorting, processing and transport of apoB, since it occurs early in apoB biosynthesis, and also occurs in a dynamic fashion in the Golgi and possibly further along the secretory pathway. Also supporting the possibility of the involvement of palmitoylation as a positive transport signal are several other observations published by other groups on proteins present at the cell surface. Some examples are: haemagglutinin of type A influenza viruses [39], the cytoplasmic domain of the HIV-1 envelope glycoprotein (gp160) [40], the mannose-6-phosphate receptor [41], the human transferrin receptor [42] and many G-protein-coupled receptors [43–45]. In all of these cases, lack of palmitoylation resulted in impaired trafficking, as shown by decreased cell-surface expression and increased intracellular accumulation. Thus loss of palmitoylation of these proteins interfered with their maturation and proper localization processes.

Altogether, and in addition to our findings on apoB palmitoylation, these observations support an important and perhaps ubiquitous role for palmitoylation as a positive signal for transport along the secretory pathway [46]. Palmitoylation might therefore be responsible for the regulation of spatio-temporal functional properties of several receptors, signalling proteins and secreted proteins such as apoB via an as-yet unidentified mechanism. Alternatively, the non-palmitoylated cysteine residue could play an active role, via its free thiol, in ER retention and binding to ER-localized chaperones [47,48]. In such models, the location and regulation of putative palmitoyl acyltransferase or

acyl-protein thioesterase activities would be critical for proper control of the acylated protein's movement along the secretory pathway.

Interestingly, it is well established that the availability of neutral lipids regulates the secretory pool of apoB and that in the absence of neutral lipids most apoB is degraded [49–53]. Since palmitoyl-CoA is the substrate for enzymes such as palmitoyl-diacylglycerol acyltransferase and acyl-CoA cholesterol acyltransferase involved in neutral lipid synthesis, as well as for the palmitoyl acyltransferase that palmitoylates apoB, an emerging concerted model could link increased levels of palmitoyl-CoA (above the K_m for these enzymes) to increased activity of the enzymes synthesizing neutral lipids and the palmitoyl acyltransferase that palmitoylates apoB. The ensuing increased availability of neutral lipids as well as increased palmitoylation of apoB would stimulate the rate of apoB transport from ER to Golgi, thereby reducing apoB degradation and increasing secretion efficiency overall.

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