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Palmitoylation of Apolipoprotein B Is Required for Proper Intracellular Sorting and Transport of Cholesteroyl Esters and Triglycerides

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Apolipoprotein B (apoB) is an essential component of chylomicrons, very low density lipoproteins, and low density lipoproteins. ApoB is a palmitoylated protein. To investigate the role of palmitoylation in lipoprotein function, a palmitoylation site was mapped to Cys-1085 and removed by mutagenesis. Secreted lipoprotein particles formed by nonpalmitoylated apoB were smaller and denser and failed to assemble a proper hydrophobic core. Indeed, the relative concentrations of nonpolar lipids were three to four times lower in lipoprotein particles containing mutant apoB compared with those containing wild-type apoB, whereas levels of polar lipids isolated from wild-type or mutant apoB lipoprotein particles appeared identical. Palmitoylation localized apoB to large vesicular structures corresponding to a subcompartment of the endoplasmic reticulum, where addition of neutral lipids was postulated to occur. In contrast, nonpalmitoylated apoB was concentrated in a dense perinuclear area corresponding to the Golgi compartment. The involvement of palmitoylation as a structural requirement for proper assembly of the hydrophobic core of the lipoprotein particle and its intracellular sorting represent novel roles for this posttranslational modification.

INTRODUCTION

Apolipoprotein B (apoB) is an essential component of chylomicrons, very low density lipoproteins (VLDL), and low density lipoproteins (Havel and Kane, 1995; Davis and Vance, 1996). These plasma lipoprotein particles are important agents responsible for the transport of hydrophobic, water-insoluble lipids in the bloodstream. Interestingly, the plasma concentrations of low density lipoproteins, intermediate density lipoproteins, and VLDL correlate positively with the propensity to develop atherosclerosis (Havel and Kane, 1995). ApoB is one of the largest known single polypeptide chains (4536 amino acids or 512 kDa). In humans, there are two natural forms of apoB found in lipoprotein particles: apoB-100 is synthesized by the liver, whereas apoB-48 is synthesized by the intestine through a process of RNA editing (Boström *et al.*, 1990; Innerarity *et al.*, 1996) (note that apoB-48 represents the N-terminal 48% of apoB). In rats, both forms of apoB are synthesized in the liver and the intestine. Likewise, rat hepatoma cells express both apoB-48 and apoB-100.

In some ways, the biosynthesis of apoB is similar to that of most secreted proteins. ApoB is translated on ribosomes bound to the endoplasmic reticulum (ER) and is cotranslationally translocated across the ER membrane to the lumen and transported through the secretory pathway (Palade, 1975). Unlike other secretory proteins, though, the secretion of a mature form of apoB requires cotranslational or posttranslational assembly with triacylglycerols (TG), cholesteroyl esters (CE), and phospholipids to produce TG-rich lipoprotein particles. The assembly of apoB-containing lipoproteins has been proposed to occur via a two-step mechanism. In the first step, the initial lipoprotein particle is formed in the rough ER (Borén *et al.*, 1994). In the second step, expansion of the lipid core of the lipoprotein occurs in the rough ER and the smooth ER or in the Golgi (Alexander *et al.*, 1976; Higgins, 1988). Several lines of evidence suggest that this process occurs predominantly in the rough ER (Alexander *et al.*, 1976; Rusiñol *et al.*, 1993) or, more accurately, at the smooth-surfaced terminal ends of the rough ER cisternae or at the junction between the rough and smooth ER compartments (Alexander *et al.*, 1976). In addition, elegant kinetic studies by Borchardt and Davis (1987) on the secretion rate of apoB demonstrated that exit from the ER

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Abbreviations used: apoB, apolipoprotein B; CE, cholesteroyl esters; EPO, erythropoietin; ER, endoplasmic reticulum; IgG, immunoglobulin G; PAT, protein fatty acyl transferase; PDI, protein disulfide isomerase; PVDF, polyvinylidene difluoride; ρ_{peak}, density of the peak fraction; TG, triacylglycerol; TR, Texas Red; VLDL, very low density lipoproteins; WT, wild-type.

compartment is the rate-limiting step in hepatocytes. Furthermore, they showed that the rate of intracellular transport of apoB-100 was approximately two times slower than that of albumin, suggesting that a processing step unique to apoB was occurring in the ER (Borchardt and Davis, 1987). The final lipoprotein particle product of this complex biosynthetic process is often depicted as a lipid-containing sphere made of a polar shell containing amphipathic protein, unesterified cholesterol, and phospholipids, with a neutral lipid core rich in TG and CE (Davis and Vance, 1996).

Many studies have shown that the regulation of apoB secretion is posttranslational and depends on the availability of neutral lipids. Under physiological conditions in which the availability of neutral lipid substrates is limiting, the secretion of apoB is reduced and a large proportion of nascent apoB will be degraded (Borchardt and Davis, 1987; Davis *et al.*, 1990). On the other hand, when the availability of neutral lipids is increased, the secretion of apoB lipoproteins is also increased. It is believed that apoB that fails to engage in lipoprotein assembly is inefficiently translocated and undergoes transmembrane integration (Davis *et al.*, 1990; Fisher *et al.*, 1997; Mitchell *et al.*, 1998). The transmembrane form of apoB is still competent for assembly with lipid and translocation into the ER lumen (Sakata *et al.*, 1993). Furthermore, transmembrane apoB was recently shown to remain in close proximity to the translocon, where it potentially waits to be lipidated and translocated (Mitchell *et al.*, 1998). In the prolonged absence of assembly with neutral lipids, apoB is proteolytically degraded (reviewed by Yao *et al.*, 1997) in the secretory pathway (Davis *et al.*, 1990; Dixon *et al.*, 1991; Furukawa *et al.*, 1992; Wang *et al.*, 1995) or by the ubiquitin-proteosome pathway after retrograde transport of apoB to the cytosol (Fisher *et al.*, 1997). Undoubtedly, translocation of apoB plays a key role in the regulation of lipoprotein particle secretion. To further illustrate this point, several domains of apoB known as pause-transfer sequences have been shown to transiently uncouple translocation of apoB in cell-free systems (Chuck and Lingappa, 1992, 1993; Nakahara *et al.*, 1994; Hegde and Lingappa, 1996) and thus could participate in the translocational regulation of lipoprotein assembly. Despite this knowledge, the molecular switch that links the availability of newly synthesized neutral lipids and the targeting of apoB lipoprotein for either secretion or proteolytic degradation has remained elusive.

During its complex biosynthesis, apoB is further modified by disulfide bond formation, glycosylation, phosphorylation, and fatty acylation (Havel and Kane, 1995; Davis and Vance, 1996). Earlier studies have demonstrated that apoB was covalently modified by the fatty acid palmitate via a thioester bond (Hoeg *et al.*, 1988; Huang *et al.*, 1988; Kamanna and Lee, 1989; Lee and Singh, 1990; Lee, 1991). Typically, protein palmitoylation is a reversible posttranslational modification that occurs on cysteine residues and is recognized to be an essential component of the dynamic membrane targeting of several signal-transducing proteins (Schlesinger, 1993; Casey, 1995; Milligan *et al.*, 1995; Dunphy and Linder, 1998). Proper palmitoylation of signaling proteins, such as the nonreceptor protein tyrosine kinases, the 224 subunits of heterotrimeric G-proteins, and some Ras protooncogenes, is critical for their function. Mutations preventing palmitoylation of these signaling proteins abolished or impaired proper membrane targeting and biological function. The role of protein palmitoylation has been studied extensively in signal-transducing proteins, but its involvement in the structure or function of a secreted lipoprotein has not yet been investigated.

In the present work, we mapped a palmitoylation site in apoB and studied the contribution of a single palmitoylated cysteine in the structure and function of apoB lipoprotein. We found that palmitoylation of apoB plays two critical roles in lipoprotein biogenesis: first, a structural role in the assembly of the neutral hydrophobic core of the lipoprotein particle; second, a positional role in the intracellular trafficking of apoB lipoprotein. These exciting new roles for protein palmitoylation add to its established role in reversible membrane targeting of signal-transducing proteins.

MATERIALS AND METHODS

Cell Lines, Antibodies, and Reagents

McArdle-RH7777 rat hepatoma cells were maintained in 10% FBS and 10% horse serum in DMEM (Life Technologies, Grand Island, NY) with 100 U/ml penicillin G sodium, 100 mg/ml streptomycin sulfate (Life Technologies) and passed twice per week. HepG2 human hepatoma cells were maintained in 10% FBS in modified Eagle's medium (Life Technologies) with the antibiotics mentioned above. Cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2. McArdle-RH7777 cells stably expressing apoB-18, apoB-31, and apoB-37 were kind gifts from Drs. Zemin Yao (University of Ottawa, Ontario, Canada) and Stephen H. Young (University of California, San Francisco).

Na[^{125}I] (2.14 Ci/mmol) and [^{3}H]glycerol (3.5 Ci/mmol) were purchased from Amersham–Pharmacia (Arlington Heights, IL). [^{125}I]Iodopalmitate was radiolabeled as described by Berthiaume *et al.* (1995) without HPLC purification of the final product. Donkey anti-rabbit immunoglobulin G–Texas Red (IgG-TR) and anti-rabbit IgG-FITC, donkey anti-mouse IgG-TR and anti-mouse IgG-FITC secondary Abs, normal donkey serum, and IgG-free, protease-free BSA were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). The 1D1 mouse monoclonal anti-human apoB antibody, which recognizes an epitope on the N terminus of human apoB (amino acids 474–539) (Pease *et al.*, 1990), was a kind gift of Drs. R.W. Milne and Y.L. Marcel (University of Ottawa). This mAb did not cross-react with rat apoB. The SY rabbit anti-mouse apoB-100 antibody used in immunocytochemistry was made with the use of a C-terminal epitope antigen of mouse apoB in a transgenic rabbit expressing human apoB-100. This SY antibody detected endogenous rat apoB-100 in Western blot and immunofluorescence protocols but did not react with mouse or rat apoB-48 or cross-react with human apoBs. This antibody was a kind gift of Dr. Stephen H. Young. The RD rabbit antibody recognizing both forms of rat apoB (B-48 and B-100) and not human apoB was a gift of Dr. Roger A. Davis (University of Colorado Health Science Center, Denver, CO). Rabbit anti-protein disulfide isomerase (PDI) antibody was a kind gift from Dr. Marek Michalak (University of Alberta, Edmonton, Alberta, Canada). Rabbit anti-α-mannosidase II was from Dr. Kelley Moremen (University of Georgia, Athens, GA). All other reagents were of the highest purity available and were generally purchased from Sigma (St. Louis, MO).

Metabolic Labeling

McArdle-RH7777 rat hepatoma cell lines expressing various human C-terminally truncated apoBs and HepG2 human hepatoma cells expressing apoB-100 were metabolically labeled with [^{125}I]iodopalmitate (Berthiaume *et al.*, 1995). After a 4-h incubation with 50–100 μCi per 100-mm dish, the radiolabeling media containing the [^{125}I]iodopalmitate were removed and cells were washed twice with culture media and allowed to secrete radiolabeled apoB–con-

taining particles for 16 h in serum-free media. Media containing secreted apoB were harvested, and detached cells were removed by low-speed centrifugation. Media were concentrated by ultrafiltration, delipidated by extraction with chloroform:methanol (1:3, vol/vol), dissolved in sample buffer containing 4% SDS, 40% glycerol, 65 mM DTT, and 0.5 M Tris-HCl, pH 6.5, and separated on 5% SDS-PAGE. The proportions of chloroform and methanol in the organic extraction were critical to remove the various types of glycerolipids bound to apoB particles before electrophoresis. This ensured minimal nonspecific binding of radiolabeled lipids to the various apoBs. Similar amounts of secreted lipoproteins (as judged by titration on a pilot Coomassie-stained gel) were separated by SDS-PAGE and electroblotted onto Immobilon-P polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA). Western blot analysis of various secreted apoB constructs was performed with the use of the mouse monoclonal 1D1 anti-human apoB antibody followed by ECL detection (Amersham–Pharmacia). Incorporation of [^{125}I]iodopalmitate into various secreted apoB proteins was visualized by phosphorimaging and autoradiography of the PVDF membrane.

Hydroxylamine Hydrolysis of apoB-29 Metabolically Labeled with [^{125}I]Iodopalmitate

After metabolic labeling of cells stably expressing apoB-29 with [^{125}I]iodopalmitate and SDS-PAGE analysis of the secreted apoB-29, the apoB-29 samples were blotted onto PVDF membranes. The membranes were soaked in either 1 M neutral hydroxylamine or 1 M Tris-HCl, pH 7.0, for 72 h (Schlesinger, 1993). After hydrolysis, membranes were rinsed several times in PBS and processed for Western blot and ECL. Dried membranes were subjected to phosphorimaging and autoradiography.

Hydrolysis of [^{125}I]Iodopalmitate-radiolabeled apoB-29 and TLC

After SDS-PAGE analysis of [^{125}I]iodopalmitate-labeled apoB-29, the acrylamide gel was subjected to autoradiography at −80°C to identify the gel band corresponding to apoB-29. The corresponding gel band was cut out, crushed with a glass rod, and hydrolyzed with 0.5 ml of 0.5 M KOH for 16 h at 4°C. The hydrolysate was then acidified to pH 5.0 with HCl, and the suspension was extracted twice with 1 ml of chloroform. Chloroform extracts were pooled, reduced to a small volume under a nitrogen stream, and analyzed by TLC on silica gel 60 plates (EM Separation Technology, Gibbstown, NJ). TLC plates were developed with a heptane:isopropylether:glacial acetic acid (60:40:4, vol/vol) mobile phase. [^{125}I]Iodopalmitate was used as a standard. Dried chromatograms were subjected to autoradiography.

Mutagenesis and Establishment of McArdle-RH7777 Cell Lines Stably Expressing Truncated apoB-29s

Cys-1085 in human apoB-29 cDNA was substituted by a serine residue with the use of the Quik-change protocol (Stratagene, La Jolla, CA). In the Quik-change mutagenesis protocol, copies of the template are made in a non-PCR mechanism with the use of high-fidelity thermostable Pfu DNA polymerase, minimizing second-site mutation. Methylated parental DNA is removed by *Dpn*I digestion. DNA containing the mutation is then transformed into bacteria. Five mutant cDNA clones were sequenced by automated dideoxy DNA sequencing (University of Alberta Biochemistry DNA Core Facility) to confirm the presence of the mutation. We subcloned the five independently sequenced cDNAs containing the mutation encoding for the Cys1085Ser substitution in apoB-29 and the corresponding wild-type (WT) apoB-29 cDNA into the mammalian expression vector pCMV5 (Andersson *et al.*, 1989). McArdle-RH7777 cell lines stably expressing the apoB-29s were established by cotransfecting the pCMV5 expression vectors containing either WT or Cys1085Ser mutant apoB-29 cDNAs with pSV2Neo vector encoding neomycin resistance (Clontech, Palo Alto, CA) and selecting for neomycin-resistant cells (Sambrook *et al.*, 1989; Blackhart *et al.*, 1990; Yao *et al.*, 1991). Neomycin-resistant clones secreting apoB-29 proteins were identified by Western blot analysis of culture supernatants.

Nondenaturating Gel Electrophoresis

Media from overlaying stably transfected McArdle-RH7777 cells grown on a 100-mm dish were concentrated by ultrafiltration, and aliquots were loaded on nondenaturing 3–10% polyacrylamide gradient gel and separated by electrophoresis, as described by McLeod *et al.* (1993). ApoBs were detected by Western blot as described above with the use of the 1D1 mAb.

Isopycnic Density Gradient Analysis of Secreted Lipoproteins and TLC of Lipids Bound to apoB Lipoproteins

Stably transfected McArdle-RH7777 cells were radiolabeled for 4 h with [^{125}I]iodopalmitate as described above. Media were concentrated by ultrafiltration, and 0.7 g of KBr was added to 1 ml of concentrated medium containing similar amounts of apoB-29 lipoproteins (as judged by titration of various aliquots on a pilot Coomassie-stained polyacrylamide gel). After dissolution, additional saline solution (0.9% NaCl) was added to complete the volume to 5.0 ml. After centrifugation for 1 h at 100,000 × *g* at 4°C in a Ti90 vertical rotor (Beckman, Fullerton, CA), 20 fractions of 250 μl each were collected from the bottom of the centrifugation tube. The density corresponding to each fraction was measured gravimetrically. Lipids corresponding to these fractions were extracted with chloroform:methanol (1:3, vol/vol) and analyzed by TLC, according to the procedure of Yao and Vance (1988), followed by autoradiography. Lipoproteins from each fraction were concentrated with the use of Cab-o-sil beads (Sigma, St. Louis, MO) and separated on 5% SDS-PAGE followed by Western blot analysis (Yao *et al.*, 1991, 1992; McLeod *et al.*, 1993).

Immunocytochemistry

McArdle-RH7777 cells stably expressing either apoB-29 or apoB-29(Cys1085Ser) were fixed for 10 min in 4% paraformaldehyde in PBS at 25°C and then permeabilized for 2 min at 25°C with 0.2% Triton X-100 in PBS. To detect human apoB-29s, permeabilized cells were incubated for 1 h at 37°C in a humidified atmosphere with the mouse monoclonal 1D1 anti-human apoB antibody in 1× Blotto. Goat anti-mouse IgG conjugated with TR (Molecular Probes, Eugene, OR) was then incubated as described above at 4 μg/ml. Between all steps, cells were washed four times with PBS during a 5-min period. Endogenous rat apoB-100 was detected with the use of the SY rabbit polyclonal anti-mouse apoB-100 antibody. Anti-rabbit IgG-FITC conjugate (Molecular Probes) was incubated as described above to detect the rabbit anti-mouse apoB-100 antibody. For colocalization of various apoBs with the ER marker PDI (Ferrari and Söling, 1999) and with the Golgi compartment marker protein α-mannosidase II (Velasco *et al.*, 1993), primary rabbit anti-PDI and rabbit anti-α-mannosidase II antibodies were used, followed by anti-rabbit IgG-FITC conjugate. Cells were mounted in Prolong antifade solution (Molecular Probes) and observed by confocal laser scanning microscopy with the use of a Zeiss (Thornwood, NY) 510 fluorescence confocal laser scanning microscope located at the Cross Cancer Institute (University of Alberta).

Each image was collected within the linear range of fluorescence intensity based on the imaging software. Image overlays represent samples acquired with the use of the sequential mode for double-label collection. Final image manipulations were done in Adobe (Mountain View, CA) Photoshop 5.0. To ensure optimal comparisons, images of cells of similar size (10–20 μm diameter) and brightness were captured with the use of similar pinhole and laser inten-

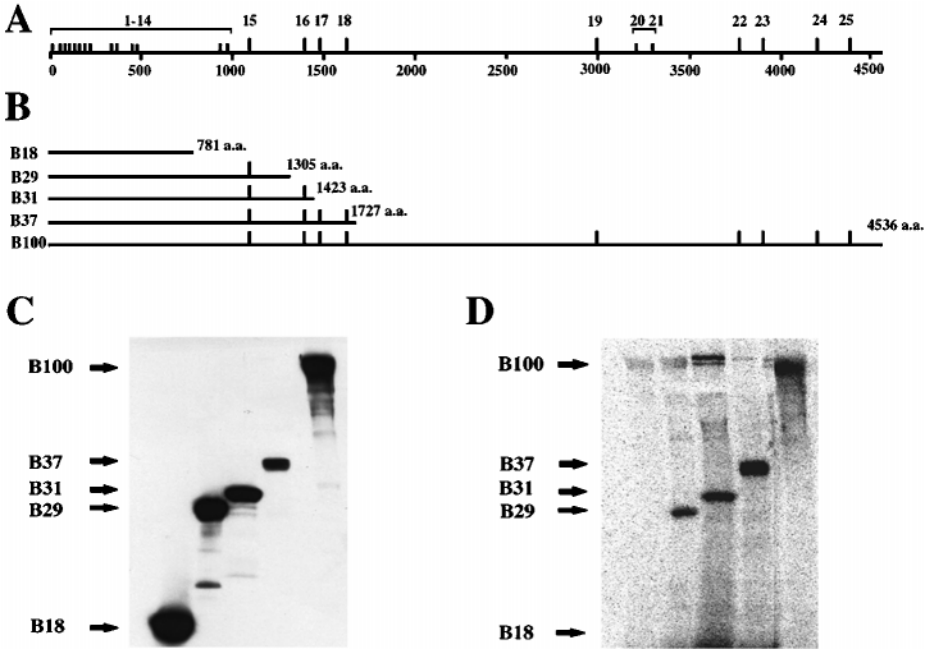

Figure 1. Incorporation of [^{125}I]iodopalmitate into secreted apoBs: apoB-29, apoB-31, apoB-37, and apoB-100 incorporate [^{125}I]iodopalmitate, whereas apoB-18 does not. Distribution of cysteine residues in apoB-100 (A) and various apoB truncated constructs (B). Free cysteine residues are represented by long ticks, and short ticks represent cysteine residues found in disulfide linkages. (C) Western blot analysis of various secreted [^{125}I]iodopalmitate-labeled apoB constructs with the use of the 1D1 anti-human apoB mouse mAb demonstrates the presence of the various apoBs on the PVDF membrane. (D) Incorporation of [^{125}I]iodopalmitate into corresponding secreted apoB proteins visualized by autoradiography of the PVDF membrane. Exposure time was 3 d with the use of Molecular Dynamics phosphorimager cassettes.

sity. No cross-reaction or bleed through was detected with the combinations of primary and secondary antibodies listed above.

RESULTS

Identification of Palmitoylated Cysteine Residue(s) in apoB

To begin the investigation of the role of palmitoylation of apoB, we needed to identify which of the nine cysteines of apoB that is (are) not involved in intramolecular disulfide bond formation (Yang *et al.*, 1990) is (are) palmitoylated. Maps describing the positions of these cysteine residues in various apoBs are shown in Figure 1, A and B. Therefore, we used a deletion analysis approach combined with metabolic labeling of truncated human apoBs (apoB-18, apoB-29, apoB-31, apoB-37) secreted from McArdle-RH7777 cells to narrow the region of palmitoylation. Then, we used site-directed mutagenesis to eliminate a putative palmitoylation site. Incorporation of [^{125}I]iodopalmitate (Berthiaume *et al.*, 1995) into the various secreted apoBs is depicted in Figures 1 and 2.

In Figure 1, C and D, various apoBs are shown to be secreted from McArdle-RH7777 or HepG2 cells, and our metabolic labeling study shows that human apoB-29, apoB-31, apoB-37, and apoB-100 secreted from cells incorporate [^{125}I]iodopalmitate, whereas apoB-18 does not. Because apoB-29 contains a single free cysteine residue and incorporates [^{125}I]iodopalmitate, this suggests that Cys-1085 of apoB-29 appears to be a palmitate acceptor (Figure 1D). Also noteworthy, radiolabeling signals resulting from apparent covalent incorporation of [^{125}I]iodopalmitate into apoB proteins are progressively more intense in apoBs larger than apoB-29 (Figure 1D). These results suggest that other cysteine residues in apoB-31, apoB-37, and apoB-100 also are potentially palmitoylated. Consistent with this possibility, for apoB-29, apoB-31, and apoB-37, the intensity of the signal is proportional to the number of free cysteine residues present in those truncated apoBs (Figure 1, B and D). In this study, different McArdle-RH7777 cell lines stably expressing various apoBs were used and exhibit various ratios of endogenous apoB to human apoB. This accounts for variations in the amounts of endogenous apoB-100 present on PVDF membranes, as detected by autoradiography and shown in Figure 1D.

We also validated our methodology to ensure that the autoradiographic signal obtained from our metabolic labeling approach was not the result of nonspecific binding of indirectly radiolabeled glycerolipids to apoBs. In a parallel experiment, we metabolically labeled cells with 60 μCi of [^{3}H]glycerol (instead of [^{125}I]iodopalmitate) and processed samples as described in MATERIALS AND METHODS. Bands corresponding to apoB were excised from the PVDF membrane and counted by scintillation. No [^{3}H]radioactivity could be detected in those samples (data not shown). This result suggests that our methodology, which uses a pulse-chase labeling approach, organic extraction of secreted apoBs followed by SDS-PAGE analysis, and Western blotting before autoradiography, removed all detectable traces of glycerolipids specifically or nonspecifically bound to apoBs.

ApoB Cysteine Residue 1085 Is Palmitoylated via a Thioester Bond

The chemical nature of the bond linking the [^{125}I]iodopalmitate analogue and apoB-29 was investigated by treatment with 1 M neutral hydroxylamine, which cleaves thioester bonds but leaves ester (carboxyl ester or oxyester) bonds intact (Schlesinger, 1993). Figure 2A shows the autoradiogram corresponding to two radiolabeled bands containing

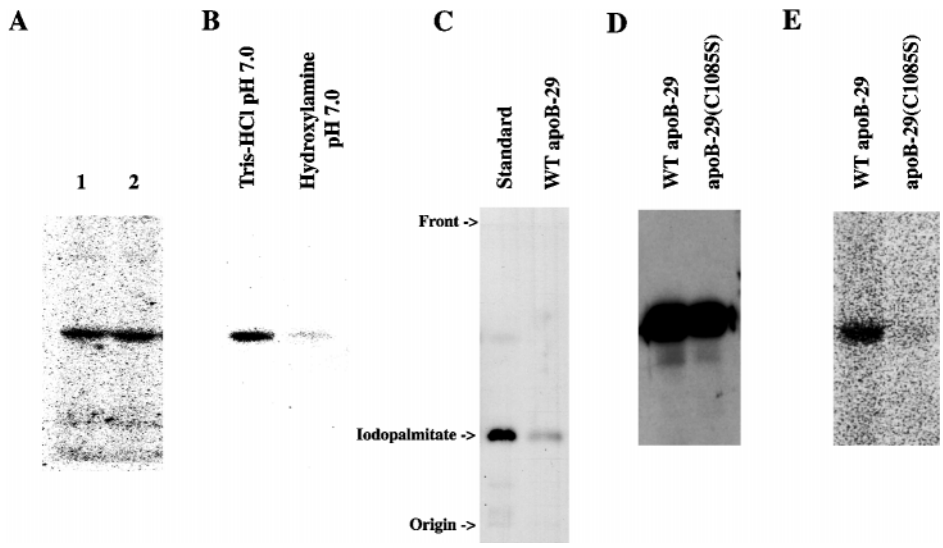

Figure 2. ApoB-29 is palmitoylated on cysteine residue 1085 via a hydroxylamine-sensitive thioester bond. (A) Autoradiogram of [^{125}I]iodopalmitate-labeled secreted WT apoB-29 starting material (lanes 1 and 2) blotted onto a PVDF membrane before hydrolytic treatment. (B) Autoradiogram of a PVDF membrane after a 72-h treatment with either 1 M Tris-HCl, pH 7.0, or 1.0 M hydroxylamine-HCl, pH 7.0. (C) TLC analysis of [^{125}I]iodopalmitate standard and hydrolyzed radiolabel extracted from [^{125}I]iodopalmitate-labeled apoB-29. Exposure time was 14 d on film with an intensifying screen. (D) Western blot analysis of WT apoB-29 and Cys1085Ser apoB-29 secreted from corresponding [^{125}I]iodopalmitate-labeled McArdle-RH7777 stable cell lines. (E) Autoradiogram of WT apoB-29 and Cys1085Ser apoB-29 secreted from corresponding [^{125}I]iodopalmitate-labeled McArdle-RH7777 stable cell lines. Exposure time was 3 d with the use of Molecular Dynamics phosphorimager cassettes.

apoB-29 on PVDF membranes before hydrolysis with neutral hydroxylamine or Tris-HCl, pH 7.0, as a control. As shown in Figure 2B, neutral hydroxylamine treatment resulted in a loss of radioactivity from apoB-29, compared with the control treatment with neutral Tris buffer. These results confirm that apoB-29 radiolabel was incorporated into apoB-29 through a thioester bond. Hydroxylamine treatment is typically performed in gel slices (Schlesinger, 1993) or even in solution (Schroeder *et al.*, 1996). In the present case, hydroxylamine hydrolysis was performed on apoB-29 bound to PVDF membranes because hydroxylamine treatment caused aggregation of apoBs tested in solution (our unpublished observation) and thus potentially in gel as well. To ensure that an equivalent amount of apoB-29 remained bound to the PVDF membrane, the membrane was stained with Coomassie. Hydroxylamine treatment did not alter the amount of apoB-29 present on the membrane (data not shown).

To further confirm the chemical nature of the labeled moiety bound to apoB-29, we hydrolyzed a polyacrylamide gel band containing radiolabeled apoB-29 with 0.5 M KOH at 4°C for 16 h and extracted the acidified (pH 5.0) hydrolysate with chloroform. TLC analysis of this hydrolysate revealed that the radioactive signal hydrolyzed from apoB-29 comigrated and corresponded to [^{125}I]iodopalmitate used as a standard (Figure 2C). To control for the possibility that our hydrolytic treatment might have cleaved off fatty acid moieties linked to glycerolipids via carboxyl ester linkages, we incubated tritiated triolein in 0.5 M KOH at 4°C for 16 h, acidified it (pH 5.0), and extracted the mixture with chloroform. The organic phase extract was then analyzed by TLC with appropriate standards. Under these conditions, no [^{3}H]oleate was released from the glycerol backbone of the tritiated triolein (data not shown).

To test whether the only free cysteine residue of apoB-29, Cys-1085, was palmitoylated, we substituted a serine residue for this cysteine residue by site-directed mutagenesis. McArdle-RH7777 cell lines stably expressing similar levels of WT and Cys1085Ser mutant apoB-29s were then metabolically labeled with [^{125}I]iodopalmitate, as described in MATERIALS AND METHODS. Incorporation of the [^{125}I]iodopalmitate analogue into both apoB-29s was evaluated after organic extraction, SDS-PAGE, Western blotting (Figure 2D), and autoradiography (Figure 2E). The results indicate that the Cys1085Ser mutant of apoB-29 was not a substrate for palmitoylation.

Palmitoylation Is Critical for the Assembly of Neutral Lipid Core into apoB-29 Lipoprotein Particle

To determine if palmitoylation plays a role in lipoprotein particle assembly, we compared the size and buoyancy of WT and Cys1085Ser apoB-29–containing lipoproteins. We first measured the relative electrophoretic mobilities of palmitoylated and nonpalmitoylated forms of apoB-29 with the use of nondenaturing gradient gel electrophoresis. Second, we analyzed the lipoproteins' densities by isopycnic gradient centrifugation. In four independent determinations, particles containing nonpalmitoylated apoB-29 formed electrophoretically more mobile particles on native gels. Typical results are shown in Figure 3A. Although the difference in electrophoretic mobility was slight, it was reproducible. Our density gradient analyses also demonstrated that nonpalmitoylated apoB-29 lipoproteins formed significantly denser particles. Typical data obtained from an experiment that was repeated seven times are shown in Figure 3B. Figure 3C shows the corresponding graphical representation of relative amounts of apoB-29s calculated as a percentage of the peak value analyzed in fractions 1–14 of each gradient. The maximal distribution of the particles containing mutant apoB-29 in the KBr gradient was up to fraction 7 ± 2 (n = 6), whereas that of the WT apoB-29 was up to fraction 12 ± 2 (n = 7). The average density corre-

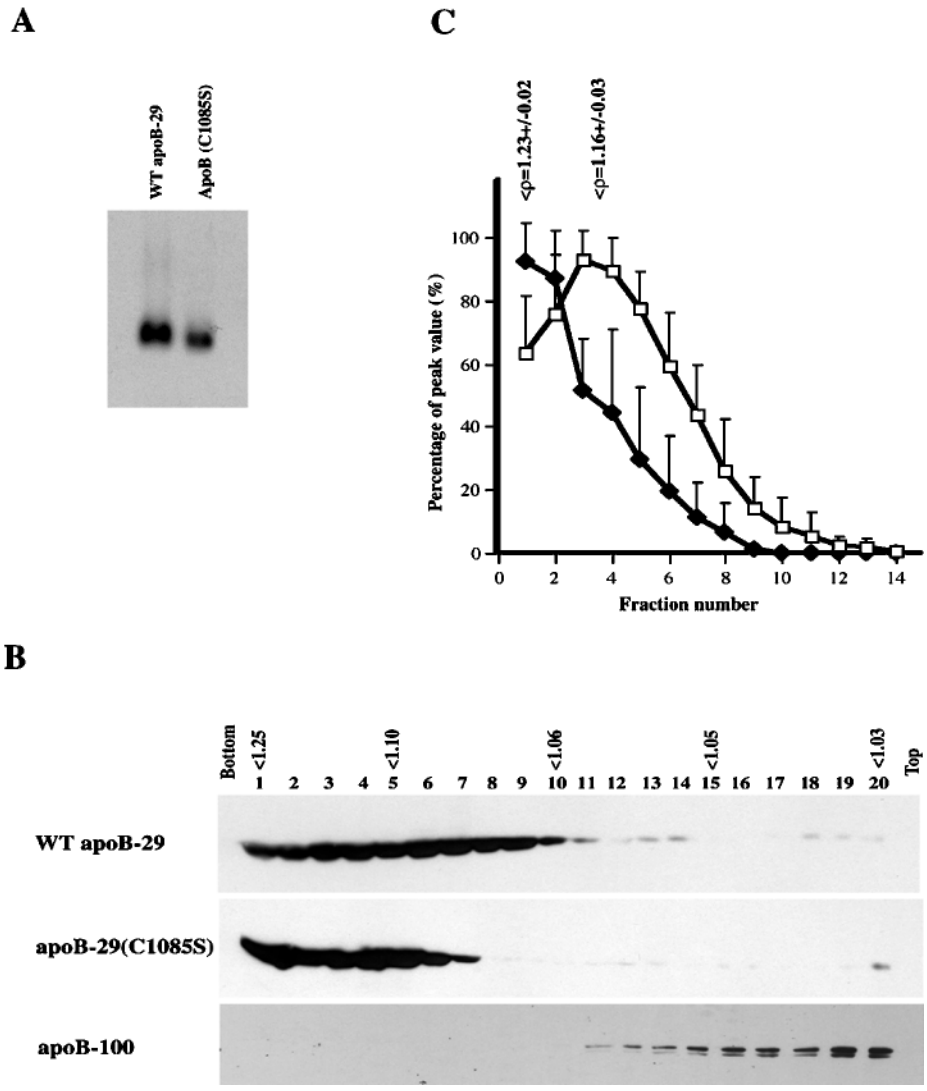

Figure 3. Palmitoylation of apoB-29 is required for secretion of larger and lower-density lipoprotein particles. (A) ApoB-29 particles secreted from McArdle-RH7777 cells were separated on 3–10% nondenaturing gradient gel electrophoresis and analyzed by Western blot analysis. Typical gel migration patterns of WT palmitoylated apoB-29 and nonpalmitoylated Cys1085Ser apoB-29 are shown. (B) Isopycnic density gradient analyses of lipoprotein particles secreted from McArdle-RH7777 cells. Fractions collected from the bottom to the top of the gradient were numbered 1–20. Densities (grams per milliliter) of fractions 1, 5, 10, 15, and 20 are shown above the corresponding fraction numbers. Typical distributions of Cys1085Ser mutant apoB-29, WT apoB-29, and endogenous rat apoB-100 lipoproteins throughout the KBr gradient as revealed by Western blot analysis are shown. (C) Graphical representation of the average relative distribution of WT (□) and Cys1085Ser mutant (♦) apoB-29s in the KBr gradient as a function of fraction number. Relative amounts of apoB-29s were calculated as a percentage of the peak value in fractions 1–14 of each gradient. Average peak densities (grams per milliliter) of particles containing WT (n = 7) or Cys1085Ser mutant (n = 6) apoB-29s are shown above the corresponding curves.

sponding to the tube containing the most apoB-29 (peak), as determined by Western blot analysis, was 1.23 ± 0.02 (n = 6) for the particles containing Cys1085Ser mutant apoB-29, whereas it was significantly lower for particles containing WT apoB-29 (1.16 ± 0.03; n = 7). The peak density reached by lipoprotein particles containing WT apoB-29 in our experiments was similar to that reported by McLeod *et al.* (1996) for particles containing apoB-29 (density of the peak fraction [ρ_{peak}] = 1.12) and for particles containing apoB-28 (ρ_{peak} = 1.17) (Yao *et al.*, 1991). Our average density for WT apoB-29–containing particles is also similar to those reported by Spring *et al.* (1992) for particles containing either apoB-26 (ρ_{peak} = 1.163) or apoB-33 (ρ_{peak} = 1.143). In our experiments, great care was taken to ensure that identical amounts and volumes of WT and mutant apoB-29s were loaded on each gradient to avoid potential differences in spread of the protein through the gradient or position of the peak fraction. The fact that mutant apoB-29 lipoprotein particles formed smaller and denser particles than the WT apoB-29 was consistent with the possibility that fewer lipids were present in the mutant lipoprotein particle. These results were confirmed in two other independent clones for each McArdle-RH7777 cell line stably expressing WT or mutant human apoB-29s (data not shown).

The next experiments compared the lipid content of lipoprotein particles containing the WT and Cys1085Ser mutant apoB-29s. To ensure minimal lipid contribution from endogenous apoB-48 or apoB-100 lipoproteins, secreted lipoproteins were separated on an isopycnic KBr gradient and TLC analysis was performed on the lipid content of fractions containing apoB-29 proteins but not apoB-100, as judged by Western blot analysis of corresponding aliquots (Figure 3B, fractions 1–7). Although particles containing endogenous apoB-100 were effectively separated from those containing apoB-29s, particles containing endogenous apoB-48 were not (data not shown). The particles containing endogenous apoB-48 were typically found in fractions 5–9 (ρ_{peak} = 1.10–1.06) when detected. Indeed, in five independent determinations, levels of secreted apoB-48 were at least 100 times lower than levels of either apoB-29 (n = 3) or were unde-

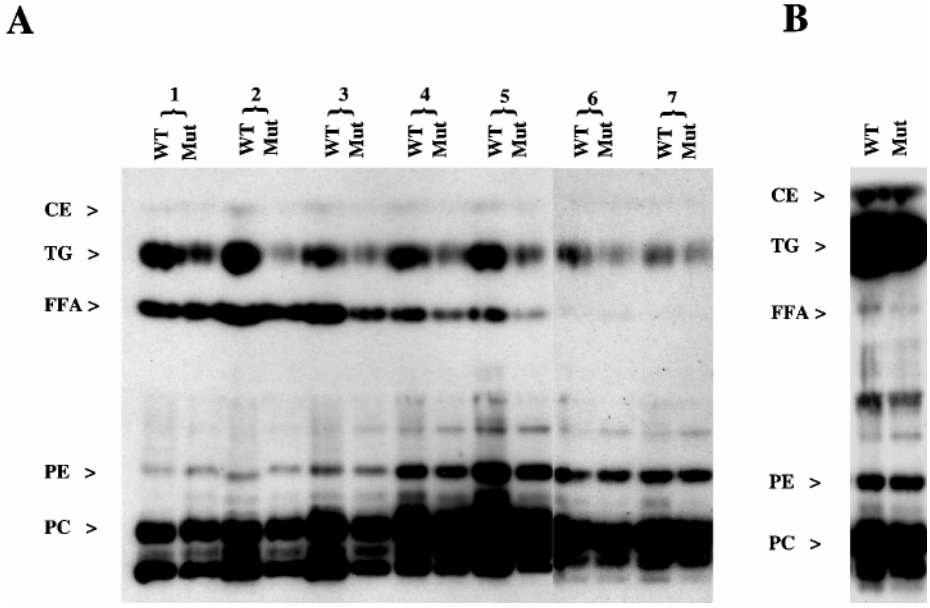

Figure 4. Palmitoylation of apoB-29 is required for assembly of neutral lipids into the hydrophobic core of the lipoprotein particle. (A) TLC analysis of lipid content corresponding to individual density gradient fractions 1–7 (depicted in Figure 3) is shown for WT apoB-29 (WT) or nonpalmitoylated mutant apoB-29 (Mut). (B) An aliquot of the lipid extract corresponding to one-tenth of pooled gradient fractions 11–20 isolated from McArdle-RH7777 cells secreting either WT apoB-29 (WT) or Cys1085Ser mutant apoB-29 (Mut). Positions of lipid standards are indicated on the left for CE, TG, free fatty acid (FFA), phosphatidylethanolamine (PE), and phosphatidylcholine (PC). The TLC plate was exposed to film for 16 h.

tectable (n = 2), as judged by Western blot analysis or Coomassie staining of PVDF membranes (data not shown). As such, the amounts of apoB-48 present in fractions 5–9 were so small that we believe the vast majority of lipids isolated from fractions 1–7 belong to particles containing apoB-29s and not to particles containing endogenous apoB-48. The low levels of apoB-48 in the clones of McArdle-RH7777 cells we obtained from Dr. Zemin Yao have also been reported previously (Hussain *et al.*, 1995).

The lipid content in particles containing nonpalmitoylated Cys1085Ser apoB-29 was significantly different from that in particles containing WT apoB-29 (Figure 4). When the ratios of neutral lipids in WT and mutant particles were analyzed by TLC and quantified by phosphorimager analysis, the relative concentrations of nonpolar lipids were three to four times lower in lipoprotein particles containing apoB-29(Cys1085Ser) compared with those in particles containing WT apoB-29. The mutant particles reproducibly contained 36 ± 8% CE and 26 ± 12% TG of the WT lipoprotein particles in three different experiments. Typical results are shown in Figure 4. In contrast, levels of polar lipids isolated from WT or mutant apoB-29 lipoprotein particles were almost identical (Figure 4). These results are consistent with a structural role for protein palmitoylation in the assembly of the nonpolar hydrophobic core in the apoB lipoprotein. We also determined if expression of various apoB-29s affected the secretion and composition of lipids from endogenous apoB particles from McArdle-RH7777 cells expressing either WT or Cys1085Ser mutant apoB-29s by analyzing an aliquot of pooled fractions 11–20. In Figure 4B, we show that lipid composition and content were not altered significantly in endogenous rat apoB lipoproteins secreted from McArdle-RH7777 cells expressing either WT or Cys1085Ser mutant apoB-29. Furthermore, levels of expression of endogenous apoBs were similar in both cell lines (data not shown). As such, stable expression of either apoB-29 did not have any apparent dominant effects on the cellular machinery responsible for assembling lipids into lipoproteins in McArdle-RH7777 cells. The presence of free fatty acids in fractions 1–5 of the KBr density gradient was attributed to the content of BSA in these fractions (Yao *et al.*, 1991).

Palmitoylation of apoB-29 Is Involved in Intracellular Sorting

To explain the differences in lipid content between WT and mutant apoB-29 lipoprotein particles, we tested whether the lack of palmitoylation could influence the intracellular localization of Cys1085Ser apoB-29 and thus prevent interaction with the neutral lipid–loading machinery. Immunocytochemistry was used to localize either human apoB-29 or endogenous apoB-100 within McArdle-RH7777 cells (Figure 5A). Images obtained from the confocal laser scanning microscope showed that the apparent majority of WT human apoB-29 and endogenous rat apoB-100 was localized in rather large spherical vesicular structures distributed throughout the cytosol (Figure 5A, a, c, d, and f). In addition, both apoB-29s and apoB-100 showed diffuse fluorescence staining throughout the cytosol. The intensity of this apparent cytosolic fluorescence varied from cell to cell. In contrast, nonpalmitoylated apoB-29 was concentrated in a dense perinuclear area similar to that of the Golgi compartment (Figure 5A, b and e). We then tested whether the large vesicular structures containing WT apoB-29 or endogenous apoB-100 would represent the same intracellular structures. Our indirect double immunofluorescence studies showed significant overlap between the fluorescent signals corresponding to WT apoB-29 and those of endogenous rat apoB-100 (Figure 5B). The colocalization was restricted to the large vesicular structures distributed throughout the cytosol (Figure 5B). Typically, the fluorescent signals corresponding to large vesicles containing WT apoB-29 were encompassed within those corresponding to large vesicles containing endogenous apoB-100. This observation is con-

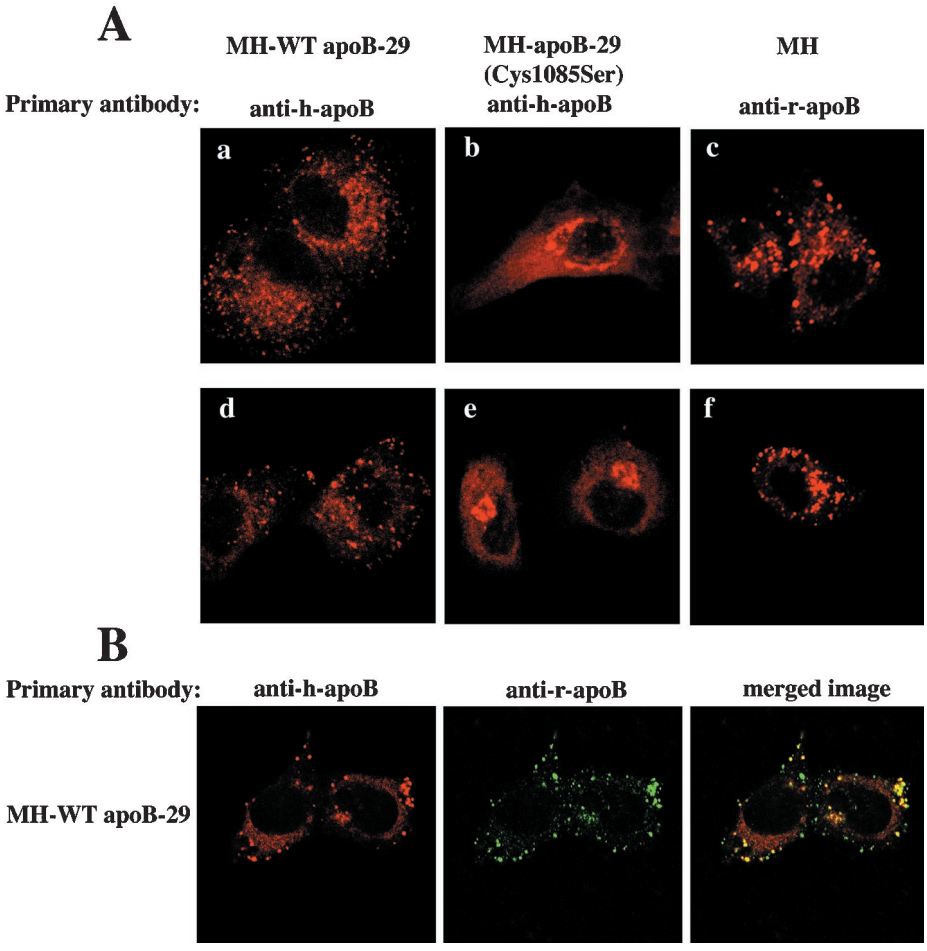

Figure 5. Palmitoylation of apoB-29 confers localization to large vesicular structures containing endogenous apoB-100 in McArdle-RH7777 cells. Confocal microscope images of the intracellular distribution of various apoBs obtained by indirect immunofluorescence. (A) Typical localization of WT human apoB-29 (a and d), nonpalmitoylated Cys1085Ser human apoB-29 (b and e), and endogenous rat apoB-100 (c and f) in different McArdle-RH7777 cells visualized with the use of appropriate secondary antibody conjugated to TR. (B) Double-labeling immunofluorescence was carried out to detect WT human apoB-29 and endogenous rat apoB-100 in McArdle-RH7777 cells stably expressing WT apoB-29 (MH-WT apoB-29). WT apoB-29 or endogenous apoB-100 was visualized with the use of a TR-conjugated secondary antibody (red) in the left panel or an FITC-conjugated secondary antibody (green) in the middle panel, respectively. The merged image is shown in the right panel.

sistent with the possibility that other protein-sorting determinants might lie in the C-terminal 71% of apoB-100.

Based on the similarity in size and distribution of these vesicles and the significant extent of colocalization between WT apoB-29 and endogenous rat apoB (Figure 5, A and B), we conclude that the presence of a single palmitoylated cysteine residue on apoB-29 is a key signaling determinant that is responsible for proper intracellular localization of apoB-29 lipoprotein. Cells expressing nonpalmitoylated Cys1085Ser apoB-29 exhibited some colocalization between mutant apoB-29 and endogenous rat apoB. The extent of this colocalization was restricted to the dense perinuclear area enriched in mutant apoB-29 (results not shown).

Most WT apoB-29 Is Found in a Subcompartment of the ER, Whereas Nonpalmitoylated apoB-29 Is Found Mostly in the Golgi Apparatus

To identify the cellular compartments corresponding to the localization of both apoB-29s, indirect double immunofluorescence colocalization studies were carried out between apoB-29s and known organelle markers of the secretory pathway. Although WT apoB-29 colocalized very significantly with the large vesicular structures containing PDI, an ER organelle marker (Ferrari and Söling, 1999), nonpalmitoylated Cys1085Ser apoB-29 and apoB-18 only partially colocalized with PDI (Figure 6). The extent of the partial colocalization of Cys1085Ser mutant apoB-29 and apoB-18 with PDI was mostly restricted to a dense juxta/perinuclear area. In McArdle-RH7777 cells, the distribution of the fluorescence corresponding to the ER marker PDI was more punctate than reticular (Figure 6), although the shape/structure of the ER appeared normal upon fluorescence staining of the cells with the use of FITC-conjugated concanavalin A (our unpublished results). Cell-specific distribution of PDI was also noticeable (Figure 6, middle panels), especially in cells apparently expressing larger amounts of WT apoB-29. Thus, these results are consistent with the proposition that the large vesicular structures containing WT apoB-29 and/or endogenous apoB represent a distinct subcompartment of the ER. As such, protein palmitoylation appears to be required to localize apoB-29 to these ER subcompartments.

Although the overlap between signals corresponding to large vesicular structures containing WT apoB-29 (or endogenous apoB-100; results not shown) and PDI was predominant, it was rarely total (Figure 6). This finding could be

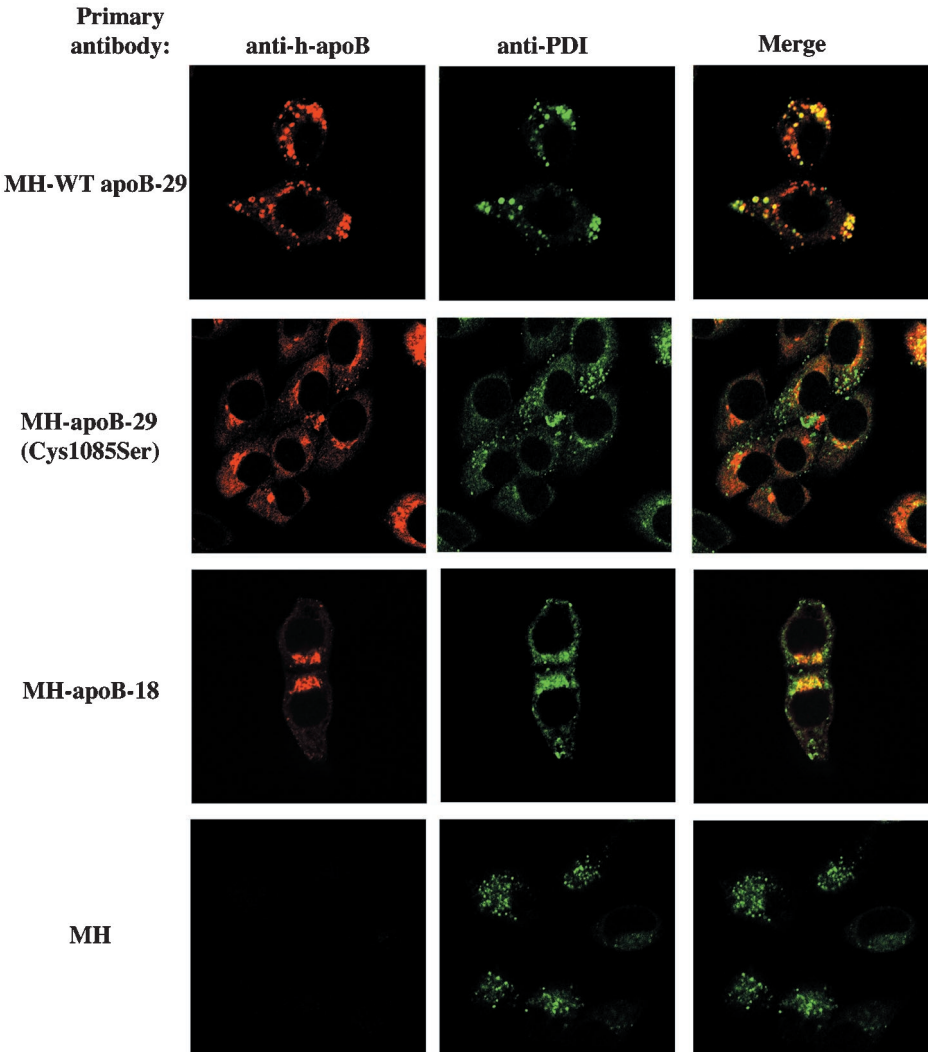

Figure 6. Palmitoylation of apoB-29 is required for localization to a subcompartment of the ER. Colocalization studies of various apoBs with the ER marker protein PDI. Various human apoBs were detected with the 1D1 mouse mAb and are shown in red with the use of anti-mouse IgG-TR–conjugated secondary antibody (left panels). PDI was detected with the use of the rabbit polyclonal anti-PDI antibody and is shown in green with the use of anti-rabbit IgG-FITC–conjugated secondary antibody (middle panels). The merged images are shown in the right panels. Staining of McArdle-RH7777 cells stably expressing WT apoB-29, Cys1085Ser apoB-29, or apoB-18 and nontransfected McArdle-RH7777 cells is shown and identified as follows: MH-WT apoB-29, MH-apoB-29(Cys1085Ser), MH-apoB-18, and MH, respectively.

explained by the fact that some of these large vesicular structures colocalized with the *trans*-Golgi network organelle marker TGN38 (our unpublished results). This latter observation is consistent with the results of Hamilton *et al.* (1991), who showed that apoB-VLDL particles were found in dilated ends of the *trans*-Golgi by electron microscopy of intact Golgi fractions isolated from rat livers.

In the absence of palmitoylation, apoB-29(Cys1085Ser) seemed to be concentrated in a compartment similar to the Golgi apparatus. To test whether this was the case, a double immunofluorescence study was performed between various apoBs and a known marker of the medial Golgi compartment, α-mannosidase II (Velasco *et al.*, 1993). Distinct from nonpalmitoylated forms of apoB, staining corresponding to large vesicular structures containing WT apoB-29 or endogenous apoB-100 showed only partial colocalization with that of α-mannosidase II (Figure 7). The extent of this partial colocalization was restricted to a dense juxta/perinuclear area. In McArdle-RH7777 cell lines stably expressing mutant apoB-29, the localization of the medial Golgi marker α-mannosidase II completely overlapped with the dense perinuclear staining of apoB-29(Cys1085Ser) and apoB-18 (our unpublished observations). These results suggest that nonpalmitoylated apoB-29 and apoB-18 showed an apparent increased concentration in the Golgi apparatus. Under steady-state conditions, it appears that palmitoylated and nonpalmitoylated forms of apoB accumulate in different cellular compartments. This difference is consistent with a change in the rate-limiting step of secretion of these apoBs.

In our study, McArdle-RH7777 cell lines stably expressing either WT or Cys1085Ser apoB-29s were matched for their ability to secrete similar levels of both apoB-29s. Similar intracellular distributions were found with the use of immunofluorescence in five different McArdle-RH7777 cell lines stably expressing WT or mutant apoB-29s (our unpublished results). In those experiments, McArdle-RH7777 cell lines stably expressing mutant apoB-29s were obtained with the use of five independently sequenced Cys1085Ser apoB-29 cDNAs. Thus, we believe that the observed variations in neutral lipid assembly and/or subcellular localization of

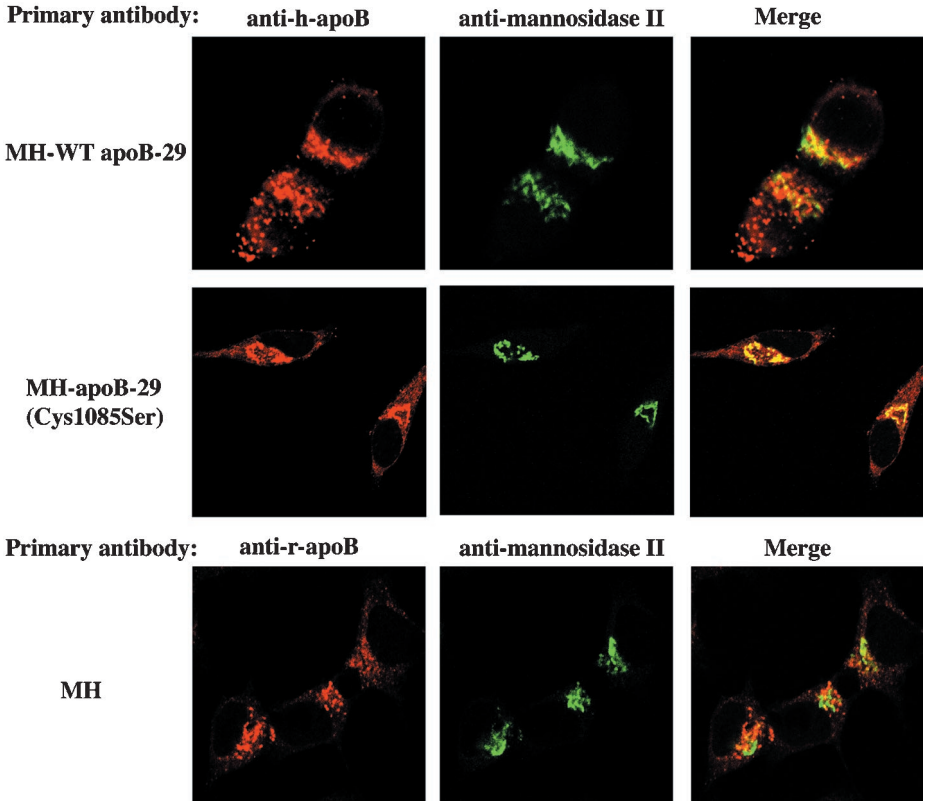

Figure 7. Nonpalmitoylated apoB-29 is concentrated in the Golgi compartment. Colocalization of various apoBs with the Golgi marker protein α-mannosidase II. Various apoBs are visualized in red (left panels) with the use of the 1D1 anti-human (anti-h) apoB or the SY anti-rat (anti-r) apoB primary antibody with appropriate TR-conjugated secondary antibody. α-Mannosidase II staining was detected with the use of rabbit polyclonal anti-α-mannosidase II and is shown in the middle panels in green with the use of appropriate FITC-conjugated secondary antibody. The merged images are shown in the right panels. Abbreviations for the various McArdle-RH7777 cells are as described for Figure 6.

Cys1085Ser apoB-29 are representative and are not caused by deleterious insertion of the expression vector in the genome of McArdle-RH7777 cells or by the presence of a spontaneous secondary mutation resulting in nonspecific alteration of the secretory pathway.

DISCUSSION

ApoB Is Palmitoylated on Cysteine Residue 1085 via a Thioester Bond

The biosynthesis and maturation of apoB-containing lipoprotein particles is a complex multistep process that has been shown to involve cotranslational addition of lipids, several chaperonins, including calreticulin and Bip (Linnik and Herscovitz, 1998), microsomal lipid transfer protein (Wu *et al.*, 1996; Wang *et al.*, 1997; Rustaeus *et al.*, 1998), and a series of posttranslational covalent modifications, including protein fatty acylation (Hoeg *et al.*, 1988; Huang *et al.*, 1988). Here, a fatty acid in the form of a [^{125}I]iodopalmitate analogue was shown to be bound covalently to apoB via a hydroxylamine-sensitive thioester bond. These data confirmed those previously reported by Hoeg *et al.* (1988) and Huang *et al.* (1988). With the use of a combination of metabolic labeling and site-directed mutagenesis, a palmitoylation site was localized to cysteine residue 1085 in human apoB-29. Furthermore, the metabolic labeling results show an increased autoradiographic signal intensity in apoB-31 and apoB-37 compared with apoB-29. This finding suggests that there may be more than one palmitoylation site on apoB. This increase appeared proportional to the number of free cysteine residues present in apoB-31 (two) and apoB-37 (four). Consistent with this possibility, the amino acids surrounding the first three free cysteine residues of human apoB are very similar (LSC^{1085}D, LSC^{1395}D, and LSC^{1478}Q). This amino acid sequence could potentially represent a recognition motif for a protein fatty acyl transferase (PAT) that could palmitoylate apoB. The amino acids surrounding the fourth free cysteine residue are somewhat less related but still are relatively similar to those surrounding the first three (LKC^{1635}S). The sequence context of the remaining five free cysteine residues present in human apoB did not show any apparent similarity with that of the first four. Unfortunately, only the human apoB cDNA sequence is available in the area of Cys-1085 (Segrest *et al.*, 1998). As such, we cannot assess the level of conservation of that cysteine residue among apoBs from various species, nor can we extrapolate our findings.

Palmitoylation of apoB Is Required for Proper Assembly of the Neutral Lipid Core of the Lipoprotein Particle

Studying the role of multiple potential palmitoylation sites in apoB-100 might have complicated the interpretation of results because these palmitoylation sites might have different functions in apoB-100. Fortunately, apoB-29, a truncated lipoprotein that can form functional buoyant lipoprotein particles, offers the unique opportunity to study the role of a

single palmitoylation site in the structure and function of a lipoprotein. The elimination of the single palmitoylation site in apoB-29 resulted in secretion of smaller and denser lipoprotein particles than those containing palmitoylated apoB-29, as judged by native PAGE and isopycnic gradient analyses. Differences in size and density could be explained by the fact that nonpalmitoylated apoB-29 mutant particles contained on average five times less TG and no detectable CE compared with lipoprotein particles containing WT apoB-29; thus, they were less buoyant. Although the lack of palmitoylation of apoB-29 had a striking deleterious effect on the assembly of TG and CE in the neutral lipid core, it did not alter the levels of the polar phospholipids that are components of the amphiphatic shell of the lipoprotein particle. As such, palmitoylation of apoB plays a specific role in the assembly of the neutral lipids (CE and TG) into the hydrophobic core of the lipoprotein particle. Thus, palmitoylation may be involved in the second step of the lipoprotein particle assembly, whereby a lipid droplet rich in CE and TG originating from the smooth ER would fuse to the lipid-poor nascent apoB lipoprotein particle originating from the rough ER (Alexander *et al.*, 1976).

Palmitoylation Is Required for Localization of apoB-29 to Large Vesicular Structures Corresponding to a Subcompartment of the ER

Double-labeling immunofluorescence techniques were used to localize various apoBs within the cell. Using these techniques, we saw profound differences in subcellular localization patterns between the nonpalmitoylated apoB-29 and palmitoylated WT apoB-29 in McArdle-RH7777 cells. Whereas Cys1085Ser mutant apoB-29 was concentrated in the Golgi apparatus, the vast majority of WT apoB-29, like endogenous apoB-100, was located in punctate or large vesicular structures containing the ER marker PDI. As such, these large vesicular structures could represent subcompartments of the rough ER, as described by Mitchell *et al.* (1998), who showed that apoB-100 was present in punctate structures corresponding to extensions of the rough ER. Also, elegant work with the electron microscope by Claude (1970) and Alexander *et al.* (1976) showed that nascent apoB-VLDL particles were present at smooth-surfaced terminal ends of the rough ER cisternae and, in some vesicles, in close proximity to the rough ER or interposed between rough ER cisternae and the Golgi apparatus. These latter structures were then postulated to represent specialized smooth-surfaced tubular elements that could transport nascent apoB-VLDL to the Golgi apparatus. Our results are thus consistent with a role for palmitoylation in the localization of apoB-29 to large spherical structures that may represent extensions or subcompartments of the ER. Whether the spherical/punctate form of the large vesicular structures containing WT apoB-29 and apoB-100 can be attributed to distinct vesicles or a cross-section of tubular elements of the ER is not known at present.

Pre-Golgi elements described by Presley *et al.* (1997) were often larger than 1.5 μm and were similar in size and distribution to our large vesicular structures containing WT apoB-29 or endogenous apoB-100. Thus, our large vesicular structures may represent pre-Golgi elements similar to those involved in the transport of the viral glycoprotein VSV-G (Presley *et al.*,1997). Also, VSV-G has been found in smaller vesicular carriers (40–80 nm) and vesicular-tubular clusters responsible for transporting the protein from the ER to the Golgi apparatus (Balch *et al.*, 1994). Whether our large vesicular structures represent pre-Golgi elements such as those described by Presley *et al.* (1997), vesicular carriers, or vesicular-tubular clusters (Balch *et al.*, 1994) is not known.

The fact that, under steady-state conditions, the majority of WT and Cys1085Ser mutant apoBs do not accumulate in the same subcellular compartments definitely suggests a role for palmitoylation of apoB-29 in the routing of apoB through the secretory pathway. These data are thus consistent with the palmitoylation-dependent retention/sequestration of lipid-poor apoB particles in large vesicular structures (potentially a specialized ER compartment) awaiting the addition of neutral lipids to occur. Once the neutral lipids are added, the lipoprotein particle could be transported to the Golgi apparatus, further concentrated, and secreted by exocytosis (Claude, 1970; Alexander *et al.*, 1976). In the absence of this potential palmitoylation-dependent transport signal, nonpalmitoylated Cys1085Ser apoB-29 would not be routed properly and could be transported by default through the secretory pathway. In that case, transport by default would preclude the addition of neutral lipids in the second-step part of the lipoprotein particle assembly process and nonpalmitoylated apoB-29 would be secreted as part of smaller, denser, lipid-poor lipoprotein particles. Alternatively, or in addition to the subcellular localization signal provided by palmitoylation, palmitoylation may facilitate the fusion between the nascent lipoprotein particle and the lipid droplet originating from the smooth ER in a specialized extension/subcompartment of the ER.

As with any mutagenesis protocol, the risk of generating an inappropriately folded mutant always exists. Although the cysteine-to-serine substitution in mutant apoB-29 results in the net substitution of a sulfur atom for an oxygen atom and thus represents an extremely conservative change, some local structural alterations could occur in addition to the prevention of palmitoylation. The proposed flexible structure of apoB (Segrest *et al.*, 1998) and the fact that apoBs with significant deletions can still bind lipids (Yao *et al.*, 1991; Spring *et al.*, 1992) and form functional lipoprotein particles suggest that the impact of a free cysteine-to-serine substitution on the apoB structure should be minimal. Nonetheless, functions specific to Cys-1085 in the structural context in which it is present in apoB may also account for some of the differences in localization independent of, or in addition to, its role as a palmitate acceptor. By analogy, when the *O*-glycosylation site of erythropoietin (EPO) was abolished by mutagenesis (Ser126Gly mutation), mutant EPO was not secreted (Dubé *et al.*, 1988). However, when secretion of EPO was analyzed in an *O*-glycosylation conditional mutant cell line, EPO was secreted at the nonpermissive temperature (Wasley *et al.*, 1991). This suggested that the mutation of a single residue that was glycosylated had two consequences: 1) prevention of glycosylation; and 2) some disruption in the folding or structure of the protein so that it was retained in the ER. Unlike the Ser126Gly EPO mutant, however, our Cys1085Ser apoB-29 mutant appears to be secreted to a similar extent as WT apoB-29 in several cell lines. Furthermore, in our case, WT apoB-29 is the protein that accumulates in the ER during the secretion process. Clearly, the

Cys1085Ser mutation leads to a loss of function (i.e., proper routing and assembly), whether from the absence of palmitoylation or from the net substitution of the sulfur atom for an oxygen atom. Structural differences attributable to the absence of palmitoylation may occur and could potentially account in part for the differential routing of mutant apoB and its accumulation in the Golgi instead of the ER extensions.

Palmitoylation as a Novel Molecular Sensor for Assembly, Sorting, and Secretion of apoB Lipoprotein Particles

Under anabolic conditions, a proportion of metabolic energy is stored in the form of lipids. One of these lipids, palmitate (C16:0), is a precursor of several other lipids, including oleate (C18:1), CE, TG, and phospholipids. Before it is converted to other fatty acid species or transferred to cholesterol or glycerol moieties, palmitate must be activated to palmitoyl-CoA. Interestingly, palmitoyl-CoA is also the palmitoyl donor in the reaction catalyzed by the PAT enzymes that transfer palmitate or various long-chain fatty acids onto a variety of proteins (Schlesinger, 1993). Under conditions in which levels of palmitoyl-CoA would be greater than the K_m of the putative PAT that palmitoylates apoB and of various enzymes involved in neutral lipid synthesis (e.g., acyl-CoA: cholesterol acyltransferase and glycerol-3-phosphate acyltransferase), palmitoyl-CoA could stimulate in a concerted manner the synthesis of neutral lipids (CE and TG), the palmitoylation of apoB, and the secretion of lipid-rich apoB lipoprotein particles. Thus, palmitoylation of apoB could represent the molecular sensor mechanism that links the availability of newly synthesized neutral lipids to the engagement of apoB lipoprotein for secretion.

Palmitoylation of apoB is required for proper assembly of the TG- and CE-rich hydrophobic core of the lipoprotein particle and mediates its effect by allowing apoB to be localized to a subcompartment of the ER where neutral lipids have been proposed to be assembled into the lipid-poor nascent lipoprotein particle. Because apoB, TG, and CE concentrations in the blood are major risk factors for atherosclerosis, their reduction is associated with a reduction in mortality from coronary heart disease. By understanding the molecular mechanisms by which palmitoylation of apoB occurs and can act as a potential transport signal for the secretion of TG- and CE-rich lipoprotein particles, it should become possible to alter this process in a beneficial manner.

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