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Variation in the Human ApoB Signal Peptide Modulates ApoB17 Translocation

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The functional effects of the common 27- or 24-amino-acid (aa) variants in the human apoB signal peptide (SP) on intracellular and secreted apoB17 were investigated *in vitro.* Only in the presence of oleate was a significant difference in intracellular and secreted SP27-B17 compared to SP24-B17 observed ($P = 0.01$ and $P < 0.0007$, respectively), although in the presence or absence of oleate mRNA levels from the two constructs were similar. After fractionation, oleate treatment enhanced microsomal SP27-B17 by 150% ($P < 0.0005$) with a modest but significant effect on SP24-B17 (32% $P = 0.007$). Oleate stimulated SP24-B17 accumulation in the nonmicrosomal fraction. The data suggest that the presence of oleate leads to inefficient translocation of the 24-amino-acid signal peptide, possibly resulting in increased retrograde translocation into the cytoplasm and reduced intracellular and secreted levels compared to the "wildtype" 27 aa SP. This implies a direct role of the SP variants in the regulation of apoB intracellular metabolism.

Key Words: **apolipoprotein B; signal peptide variation.**

Apolipoprotein (apo) B plays a critical role in lipid metabolism as the major structural component of triglyceride (TG)-rich lipoprotein particles (TGRL), synthesized in the liver and intestine, and as the ligand for the cell surface low density lipoprotein receptor (LDL-R) required for removal of apoB-containing lipoproteins from the circulation. Thus perturbations in either apoB synthesis or catabolism could lead to raised levels of plasma TG and/or cholesterol, both of which are risk factors for coronary artery disease (CAD) (1, 2). Both environmental and genetic factors influence levels of apoB and apoB-containing lipoproteins. In humans, environmental modification such as dietary change (3, 4) or lipid lowering drugs (5, 6) can alter the production rate of apoB from hepatocytes or enterocytes. Path analysis and twin studies have shown that the heritability of apoB is high (7–9) and variation in the *APOB* gene has been shown in part to influence plasma cholesterol (10) and TG (11) levels and risk of CAD (12). Much of the research into the role of variation in *APOB* has been concerned with the effect on receptor binding and catabolism (13), however genetic variation that affects synthesis and secretion of apoB itself, could have a profound effect on the production of apoB-containing particles.

ApoB, as a secretory protein, is synthesized initially in precursor form with an N-terminal signal peptide (SP) sequence that directs its insertion into, and translocation across, the endoplasmic reticulum (ER). The SP is removed by signal peptidase through an endoproteolytic cleavage reaction on the luminal side of the ER membrane (14). Factors such as insulin or substrate levels that cause changes in apoB secretion do not alter the level of apoB mRNA (15), and thus the regulation of apoB secretion appears to be primarily posttranslational, with a key step being the translocation of newly synthesized apoB into the inner leaflet of the ER where the nascent lipoprotein particles are assembled. However, compared to secreted proteins such as albumin, this process is inefficient and slow (16, 17) with only a fraction of the synthesized apoB finally assembled into very low density lipoprotein particles (VLDL). Thus the majority of apoB does not get utilized and is degraded, at least in part, via the ubiquitin-proteasome pathway (18). This degradation is thought to occur primarily on the cytosolic side of the ER (19, 20) and factors that

Abbreviations used: apoB, apolipoprotein B; LDL-R, LDL-receptor; VLDL, very low density lipoproteins; TG, triglycerides; TGRL, triglyceride-rich lipoprotein, CAD, coronary artery disease; ER, endoplasmic reticulum, SP, signal peptide; aa, amino acid, RT-PCR, reverse transcriptase polymerase chain reaction; MTP, microsomal triglyceride transfer protein; PDI, protein disulfide isomerase; McA-RH7777, McArdle-7777 rat hepatocyte cell line.

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affect the translocation, such as the hydrophobicity of the signal peptide, may influence the amount of nascent apoB that is degraded.

SPs are positively charged at the N-terminus and have a hydrophobic core, which appears to be essential to direct the nascent protein to the ER membrane and thus for efficient translocation (21). Several studies have characterized the effect of mutations within the hydrophobic core which decrease the efficiency of translocation (22–24). Human apoB is unusual, but not unique, among human secreted proteins in that there is common variation within the SP (25). The "wildtype" SP is 27aa long (SP27); a commonly occurring variant of 24aa (SP24), the result of deletion of Leu-Ala-Leu residues from the hydrophobic core, occurs at a carrier frequency of about 30% in Caucasian (11, 26, 27), 21% in Blacks (27) and 19% in Chinese (28). Individuals who carry an SP24 allele had lower plasma triglyceride levels (11, 29) and raised plasma glucose levels (26), although there is some controversy in the literature concerning these association studies (13). The apoB signal peptide may itself be functional or it may act as a marker for a functional change elsewhere in the apoB gene.

The functional effect of the apoB SP variants was studied in a heterologous yeast expression system, using the yeast invertase gene as a reporter to follow their effect on the secretion and glycosylation of invertase (30). Compared to the wildtype SP27, SP24 mediated inefficient translocation of the invertase and was secretion defective. Although clear differences in the secretion conferred by the SP variants in these yeast studies were observed, yeast represent a heterologous, non-mammalian system and it is possible that these studies did not fully replicate the role of the SP variants in the normal context of a mammalian cell. Furthermore, sequences C-terminal to the signal peptide cleavage site have been demonstrated to play a role in SP processing and were not studied in the yeast system due to the nature of the reporters used.

In this study we tested the hypothesis that naturally occurring variation in the hydrophobic core of the apoB SP modulates the translocation of truncated apoB17 in mammalian cell lines; a homologous system, rat hepatoma McArdle 7777 (McA-RH7777) cells which synthesize apoB and package lipoproteins due to the presence of microsomal triglyceride transfer protein (MTP) and the heterologous system, green monkey kidney cells, COS1, which do not synthesize apoB but can efficiently secrete truncated apoB species (31).

METHODS

Construction of the truncated apolipoprotein B expression plasmids. The apoB SP27/invertase fusion gene, used in the yeast expression studies (30), was excised in an *Eco*RI/*Hin*dIII fragment from the yeast vector apoBSP/invertase/pS5 and ligated into the *Eco*RI/*Hin*dIII site of pCMV5, a mammalian expression vector which contains the CMV promoter, and which is highly expressed in the liver cells. The pCMV5 apoBSP27/invertase construct was digested with *Nar*I/*Hin*dIII to remove the *suc2* gene. The apoB cDNA was derived from pB53LL, a pCMV construct that contains apoB53 (a kind gift of Dr. Zemin Yao). *Nar*I/*Hin*dIII digest of pB53LL yielded apoB17, excluding its signal peptide. This 2.2-kb apoB17 *Nar*I/*Hin*dIII fragment was inserted to generate the apoBSP27/apoB17 fusion construct. The SP24-B17 was generated from SP27-B17. ApoB SP24 synthetic oligonucleotides (Gibco BRL, Paisley, UK) 5′apoB SP24 AATTCATGGACCCGCCGAGGCCCGCGCTGCTGGCGCTGCCTG-CGCTGCTGCTGCTGGTG and 3′ apoB SP24 CGCCCGCCAGCAG-CAGCAGCAGCAGCGCAGGCAGCCCCAGCAGCGCGAGGGCCT-CGGCGGG, were annealed at 92°C and then allowed to reach room temperature and subsequently phosphorylated with T4 polynucleotide kinase (Gibco BRL, Paisley, UK). These oligonucleotides had *Nar*I and *Eco*RI flanking sites. SP27/B17 was digested with *Eco*RI and *Nar*I to remove the signal peptide and the annealed SP24 oligonucleotides were ligated into the *Nar*I/*Eco*RI site creating SP24-B17. Three different plasmid preparations were used in subsequent experiments.

Cell culture. McA-RH7777 cells (obtained from ATCC) were grown in MEM (Gibco BRL, Paisley, UK) supplemented with 10% fetal bovine serum (v/v), 0.1 mM nonessential amino acids, 1 mM sodium pyruvate and penicillin (100 Units/ml)/streptomycin (100 μg/ml) (Gibco BRL, Paisley, UK). The cells were fed fresh complete medium every 3 days and maintained in a CO_2 incubator.

Transfection of McA-RH7777 cells. Cells were grown for 36–48 h prior to harvesting at 80% confluency. Transfections using Lipofectin were performed according to the manufacturer's instructions (Gibco BRL, Paisley, UK). Briefly, plasmid DNA was mixed with OPTI-MEM I reduced serum medium (Gibco BRL, Paisley, UK). This was gently added to an equal volume of diluted Lipofectin in OPTI-MEM and incubated at room temperature for 15 min, whereupon a further volume of OPTI-MEM was added. This mixture was added in 1-ml aliquots to duplicate wells of McA-RH7777 cells (60% confluent) in 6-well plates. Serum and antibiotics were not present in the OPTI-MEM medium during the incubation and transfection time. After incubation at 37°C for 15 h, the transfected cells were incubated with fresh medium containing serum and antibiotics and allowed to recover for 24 h. Transfection efficiency of 10–30% was routinely obtained, monitored by parallel transfection with a β-galactosidase expression plasmid, pSV-β-galactosidase (β-Gal).

β-Galactosidase assay. Cells were harvested 48–72 h after transfection and enzyme extracts were made by three freeze/thaw cycles. 150 μl cell extract was mixed with 150 μl of assay buffer (120 mM Na_2HPO_4, 80 mM NaH_2PO_4, 2 mM $MgCl_2$, 100 mM mercaptoethanol, 1.33 mg/ml ONPG) and incubated overnight at 37°C. Sodium carbonate (1 M) was added to stop the reaction and distilled water was added to dilute the precipitate. β-Galactosidase activity was measured as A_{420} and used as a measure of transfection efficiency. One aliquot was taken out for the β-Gal assay and the rest was treated for 15 min at 65°C. The values (cpm/h/pmol plasmid) were normalized against β-Gal activity to compensate for variations in transfection efficiency.

Isolation of total RNA and reversed transcription coupled with PCR (RT-PCR). Total RNA was isolated using Trizol (Gibco BRL, Paisley, UK) (36). To perform RT-PCR, the method of Celi *et al.* was followed (32). 1.5 μg of total RNA was incubated at 65°C for 10 min in a reaction mixture (100:1) of 10 mM Tris–HCl (pH 8.3)/50 mM KCl/1.5 mM $MgCl_2$/2 mM, DTT/200 mM, dNTP/80 units RNAsin (Promega UK, Southampton, UK). 100 nM reverse primer A1, 25 units of Avian myeblostosis virus (AMV) reverse transcriptase (Gibco BRL, Paisley, UK) was added to the reaction, followed by incubation at 42°C for 1 h. Two primers specific for human apoB were designed. PCR was carried out using *Taq* DNA polymerase (Gibco BRL, Paisley, UK) under the following conditions: 1 min denaturation at 95°C,

2 min annealing at 50°C, and 3 min extension at 72°C. The forward primer was a 19-mer (a_{19}) corresponding to the target sequence. The reverse primer ($b_{26}c_{19}$) was 45 nucleotides in length in which 26 nucleotides at the 3′ end (segment b_{26}) corresponded to the opposite strand of the target sequence, and 19 nucleotides at the 5′ end (c_{19}) corresponded to the target sequence 60 nucleotides upstream of segment b_{26}, thus allowing for the synthesis of an internal standard. The second PCR was coamplified with varying amounts of cDNA with a fixed quantity of internal standard and a pair of conventional PCR primers a_{19} and c_{19}. Using this approach, two products were generated (234 and 151 bp), 234 bp for the endogenous template, and 151 bp for the internal standard. 5′GATGCGACCCGATTCAAGC (a19) and 3′AGAGTTCTTGGTTTTCTTCAGCGGATGAAGCTGC-AGAGCTGGGGA (b26c19) 151-bp PCR product. 3′AGAGTTCTTG-GTTTTCTTCAGC (c19) 234-bp PCR product.

^{3}H-labeling and treatment of cells. Exponentially growing cells were labeled with [^{3}H]leucine (135 Ci/mmol, catalogue no TRK683; Amersham International, Amersham UK) approximately 24 h post-transfection. The medium was first replaced with 2 ml of serum free and leucine-free MEM for 2 h at 37°C. 150 μCi of [^{3}H]leucine was added to each plate serum free, leucine-free, supplemented with 1.5% fatty acid-free albumin and incubated for 30 min. For oleate treatment, the cells were labeled in medium containing either 1.5% BSA (catalog No. A 6003, essentially fatty acid free, Sigma, Poole, UK) (control) or oleate, 1.5% BSA (oleate), prepared as follows: sodium oleate (Sigma, Poole, UK) was dissolved in 1 ml ethanol and dried to a thin film under nitrogen. Medium containing 1.5% BSA (0.244 mM) was added, and the mixture was stirred until the oleate was completely dissolved. The solution was sterile-filtered and used within 2 days. In all experiments the oleate concentration used was 0.8 mM oleate, 1.5% BSA (molar ratio, 3.6:1). Controls cells were always treated with medium containing 1.5% oleate-free BSA. Medium was collected into a tube containing protease inhibitors (1 mM benzamidine, 5 mM EDTA, 0.86 mM PMSF, 100 Kallikrein-inactivating units/ml aprotinin and 10 mM Hepes, pH 8) and the conditioned medium was centrifuged at 1000*g* for 5 min to remove cell debris. ApoB was then immunoprecipitated as described below. Cells were washed twice with cold PBS and collected in ice-cold lysis buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris, pH 7.4, 0.0625 M sucrose, 0.5% Triton X-100 and 0.5% deoxycholate, 50 μg/ml leupeptin and 50 μg/ml pepstatin A) in addition to the protease inhibitor mixture. The cells were scraped off the dish and a cell extract (volume, 1 ml) was prepared by incubating overnight at 4°C on a rocking platform. The next morning, after centrifugation (12,000*g*, 20min), the cell extract supernatants were taken for apoB immunoprecipitation. All experiments were performed twice in triplicate.

Immunoprecipitation, SDS polyacrylamide gradient gel electrophoresis and fluorography. [^{3}H]Leucine-labeled apoB was immunoprecipitated from the medium and cells. An aliquot of medium or cell homogenate was diluted with the same volume of NET buffer: (150 mM NaCl, 5 mM EDTA, 50 mM Tris, pH 7.4, 0.5% Triton X-100 and 0.1% SDS) and immunoprecipitated with an excess of anti-human apoB polyclonal antibody. The goat anti-human apoB antiserum (Sigma, Poole, UK) immunoprecipitated both human and rat apoB. The mixture was incubated at 4°C for at least 15 h. Protein A-Sepharose CL4B (1.5%) was added to the mixture and incubation was continued for at least another 5 h. Unbound antibody was removed by centrifugation at 1000 rpm for 2–3 min, supernatant was discarded and the protein A-antibody complex was washed 5 times with NET buffer. 50 μl of electrophoresis sample buffer (0.125 M Tris–HCl, pH 6.8, 4% SDS, 20% glycerol and 10% mercaptoethanol) was added and apoB was separated from the beads by boiling for 5 min. [^{3}H]Leucine-labeled proteins were resolved by electrophoresis in 3–10% gradient SDS–PAGE with a 3% stacking gel. 5 μl of [^{14}C]-methylated protein were run on gel as molecular weight standards, gels were fixed in 40% (v/v) methanol, 10% (v/v) acetic acid, impregnated with EN-HANCE (Amersham International, Amersham, UK) for 30 min, dried and exposed to Hyperfilm MP (Amersham International, Amersham, UK) for 3–14 days.

Isolation and fractionation of microsomes. Cell transfection was carried out and microsomes were prepared, according to methods described previously (33). Briefly, 48 h after transfection, cells were labeled with [^{3}H]leucine for 30 min. The cells were scraped in phosphate-buffered saline after washing once with ice-cold phosphate-buffered saline. Cells were pelleted by centrifugation at 500*g* for 5 min. The cells were then homogenized by sonication in 0.25 M sucrose and 5 mM Hepes (pH 7.4) on ice. The postnuclear supernatant was obtained by centrifugation at 10,000*g* for 10 min at 4°C, and subsequently the microsomes and the nonmicrosomal fraction were separated by centrifugation at 100,000*g* for 60 min at 4°C. All experiments were repeated twice in triplicate.

Protein estimation and trichloroacetic acid precipitation. Aliquots were used to determine total protein contents according to Bradford (34) using BSA as the protein standard. Aliquots of cells and media were used to measure incorporation of [^{3}H]leucine into protein by trichloroacetic acid precipitation onto Whatman 1MM paper filter and scintillation counting.

RESULTS

To study the effects of the apoB SP polymorphism on the intracellular and secreted apoB17, rat hepatoma line, McA-RH7777, were transiently transfected with the two constructs, under control of the CMV promoter. To track apoB directed by the apoB signal peptide variants, and to distinguish this apoB from the endogenous rat apoB48 and apoB100, the 27-aa SP (SP27) and 24-aa SP (SP24) coding fragments were ligated in frame to an apoB cDNA construct encoding the N-terminal 17% of apoB100, producing an 87-kDa protein.

Time Course of ApoB17 Synthesis and Secretion

Transiently transfected McA-RH7777 cells were washed with PBS and incubated in labeling medium. To optimize the conditions for the expression of the intracellular and secreted levels of apoB17 fusion proteins, labeling times were increased from 10 min to 30 min, and monitored. Aliquots of medium and cell extracts were withdrawn, and the intracellular and secreted [^{3}H] apoB were measured by immunoprecipitation. A 30-min pulse gave the optimum results where both the intracellular and secreted apoB17 fusion proteins could be clearly followed (data not shown). Thus in all subsequent experiments a 30 min radiolabeling was used. All results were estimated by scanning densitometry and normalized for total proteins and for β-galactosidase levels. When fatty acid-free BSA was used, the amount of intracellular SP27-B17 (set at 100%) was modestly but significantly greater than SP24-B17 (93%, $P < 0.0001$). However, there was no difference in the amount secreted into the medium. These results are presented by a representative autoradiograph and a histogram summarizing the data from the repeat experiments (Fig. 1).

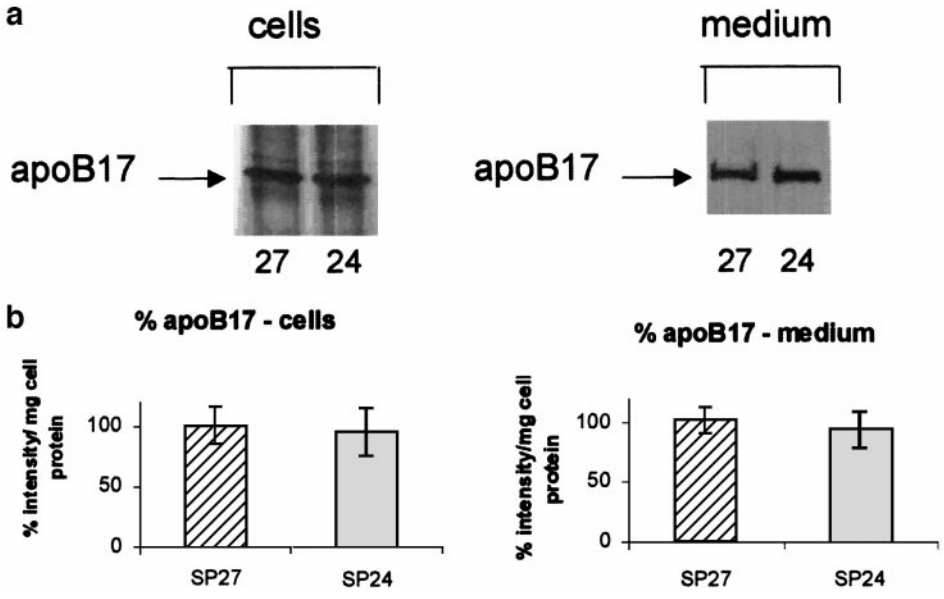

FIG. 1. The effect of the SP variants on intracellular and secreted apoB 17 after a 30-min labeling. SP27-B17 and SP24-B17 transiently transfected McA-RH7777 cells were labeled with [^{3}H] leucine for 30 min. Cells and medium were harvested and subjected to immunoprecipitation with sheep anti-human apoB. Immunoprecipitates were separated by SDS–PAGE and autoradiographed. (a) Representative experiments showing immunoprecipitation from cells and medium and (b) histogram of from the repeat experiments quantified by scanning densitometry of the autoradiographs. Values were normalized for β-gal and total protein content and expressed as a percentage of SP27-B17, which was set at 100%. Results are the mean (SD) from 4 repeat experiments each done in triplicate or more.

Effect of Oleate on ApoB 17 Synthesis

The addition of the fatty acid, oleic acid, as oleate-albumin, into the medium has been reported to rescue apoB from degradation and direct it into the secretory pathway (35, 36), although there is some controversy whether smaller C-terminal truncations of apoB, e.g., B17 are affected by oleate (33, 35). We tested whether the presence of oleate could stimulate intracellular and secreted levels of apoB17, and whether the length of the apoB signal peptide affected this. Transiently transfected McA-RH7777 cells were washed with PBS and preincubated in medium containing either 1.5% BSA (control) or 1.5% BSA plus 0.8 mM oleate for 4 h. The cells were then incubated in labeling medium containing 1.5% BSA or 1.5% BSA plus 0.8 mM oleate for 30 min. Aliquots of medium and cell extracts were withdrawn, and intracellular and secreted [^{3}H] apoB was measured by immunoprecipitation. All results were estimated by scanning densitometry and normalized for total proteins and for β-galactosidase transfection. Control results were similar to the previous experiment. Contrary to previous reports, in the oleate-treated cells both intracellular and secreted levels of SP27-B17 were stimulated, by 76% ($P = 0.002$) and 127% ($P < 0.0001$), respectively. Oleate had non-significant effects on SP24-B17 synthesis (+19%) and secretion (+29%). Once again these results are presented by a representative autoradiograph and a histogram summarizing the data from the repeat experiments (Fig. 2).

Quantification of the Expression of ApoB17 SP Variant mRNA Levels by RT-PCR

The discrepancy between intracellular and secreted levels of apoB17 after oleic acid treatment of McA-RH7777 cells transfected with the SP24-B17 construct, compared to the SP27-B17 construct, could reflect differences in mRNA expression levels. To clarify this, total RNA was isolated from the transfected McA-RH7777 cells with or without oleic acid treatment and a quantitative RT-PCR method was followed to estimate mRNA levels (32). The quantification was achieved by titrating relative amounts of the internal standard and the apoB products. Competitive RT-PCR showed that the ratio of target sequence to internal standard was comparable for the two constructs in the control or in the presence of oleic acid (Fig. 3). Thus the difference in amounts of apoB17 from the two transfections represent differences in apoB17 levels and not mRNA expression.

Localization of SP27-B17 and SP24-B17 in the Microsome and Cytosol of Control and Oleate-Treated Cells

The previous experiment suggested that the intracellular and secreted SP27-B17 was simulated in the presence of oleate, while SP24-B17 was minimally affected by oleate treatment. In an attempt to clarify these differences and explore further the mechanisms by which oleate enhances intracellular levels of SP27-

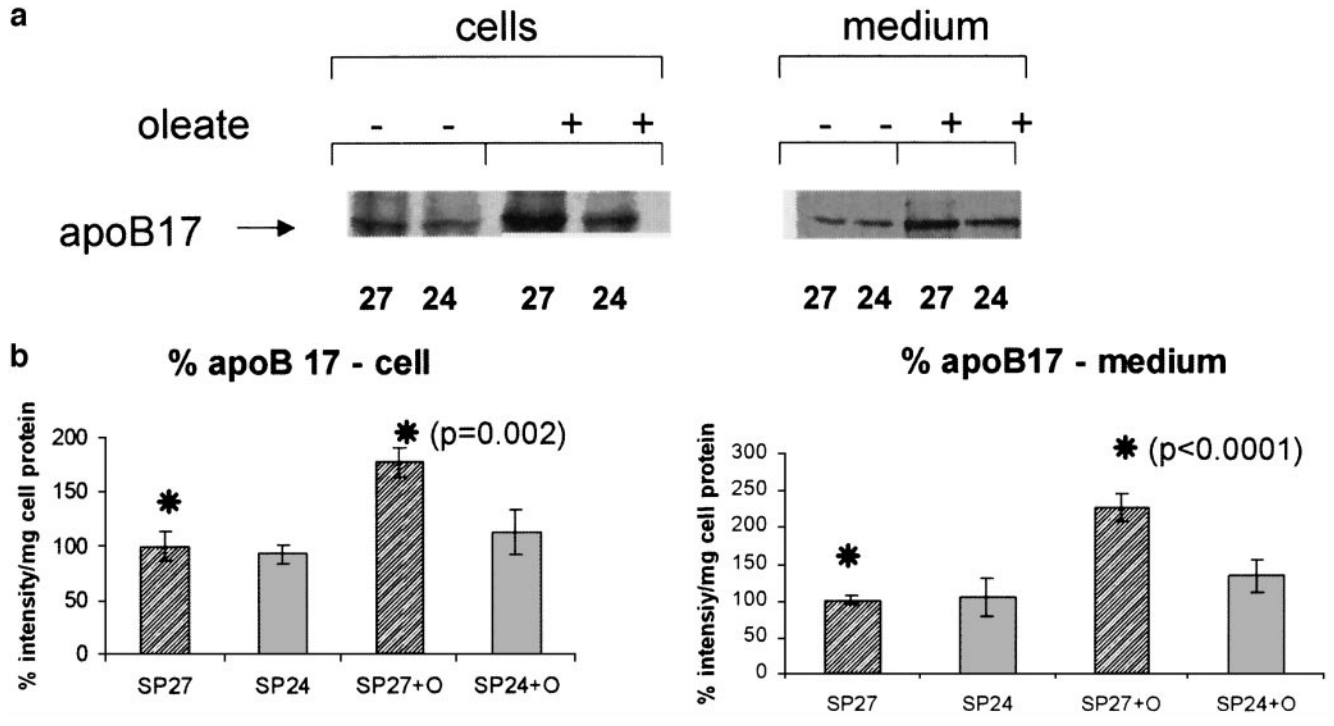

FIG. 2. The effect of the SP variants on intracellular and secreted apoB 17 after a 30-min labeling under control conditions (fatty acid-free BSA) or pretreated in the presence of 0.8 mM oleate. McA-RH7777 cells were transiently transfected with SP27-B17 or SP24-B17 and labeled with [^{3}H]leucine for 30 min. Cells and medium were harvested and subjected to immunoprecipitation with sheep anti-human apoB and separated by SDS–PAGE and autoradiographed. (a) Representative experiments showing immunoprecipitation from cells and medium grown in the absence or presence of oleate. (b) Histogram of repeat experiments quantified by scanning densitometry of the autoradiographs. Values were normalized for β-gal and total protein content and expressed as a percentage of SP27-B17 that was set at 100%. Results are the mean (SD) from 3 repeat experiments, each done in triplicate. The effect of oleate on SP27-B17 was to increase significantly the intracellular ($P = 0.002$) and secreted ($P < 0.0001$) apoB17. There was no significant effect of oleate on intracellular or secreted SP24-B17.

B17, we examined the distribution of apoB17 in the ER and cytosol of control (untreated) or oleate-treated transfected cells. Control and oleate-treated transfected McARH-7777 cells were labeled with [^{3}H]leucine for 30 min and the microsomal and non-microsomal (cytosolic) fractions were isolated by differential centrifugation and analyzed by immunoprecipitation and SDS–PAGE.

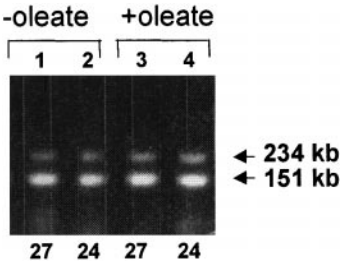

FIG. 3. Expression levels of apoB17 mRNA in McRG-7777 cells assessed by RT-PCR. A constant amount of cDNA prepared by reverse transcription was mixed with increasing amounts of the internal standard. PCR amplification was performed with apoB-specific primers to give a product of 234 and 151 bp from the target and competitor sequences, respectively. The gel shows results when 3.2×10^6 copies (3 μl) of internal standard were mixed with the target.

In the microsomes from control cells, as expected there was no difference in the level of SP27-B17 compared to SP24-B17 ($P = 0.54$). Oleate enhanced SP27-B17 levels by 150% ($P < 0.0005$) and although SP24-B17 levels in the microsomes were also higher than in control cells, this was a much smaller effect of +32% ($P < 0.007$). As a result of this, in microsomes from oleate treated cells the level of SP27-B17 was 95% higher than SP24-B17 ($P < 0.005$) (Figs. 4a and 4b). Thus, while oleate appeared to enhance SP27-B17 translocation, its effect on microsomal SP24-B17 levels was substantially less. This suggests that translocation of SP27-B17 was stimulated by oleate and carried to completion, hence its appearance only in the microsomal fraction. This did not seem to be true for SP24-B17, which only increased slightly in the microsomes after oleate treatment.

In the nonmicrosomal fraction, which includes the cytoplasm, in the control experiment there was a distinct SP24-B17 band present while no SP27-B17 was detected (Fig. 4c). This SP24-B17 band was enhanced in the presence of oleate. These experiments were repeated twice (in triplicate) and the figures are representative of one such experiment.

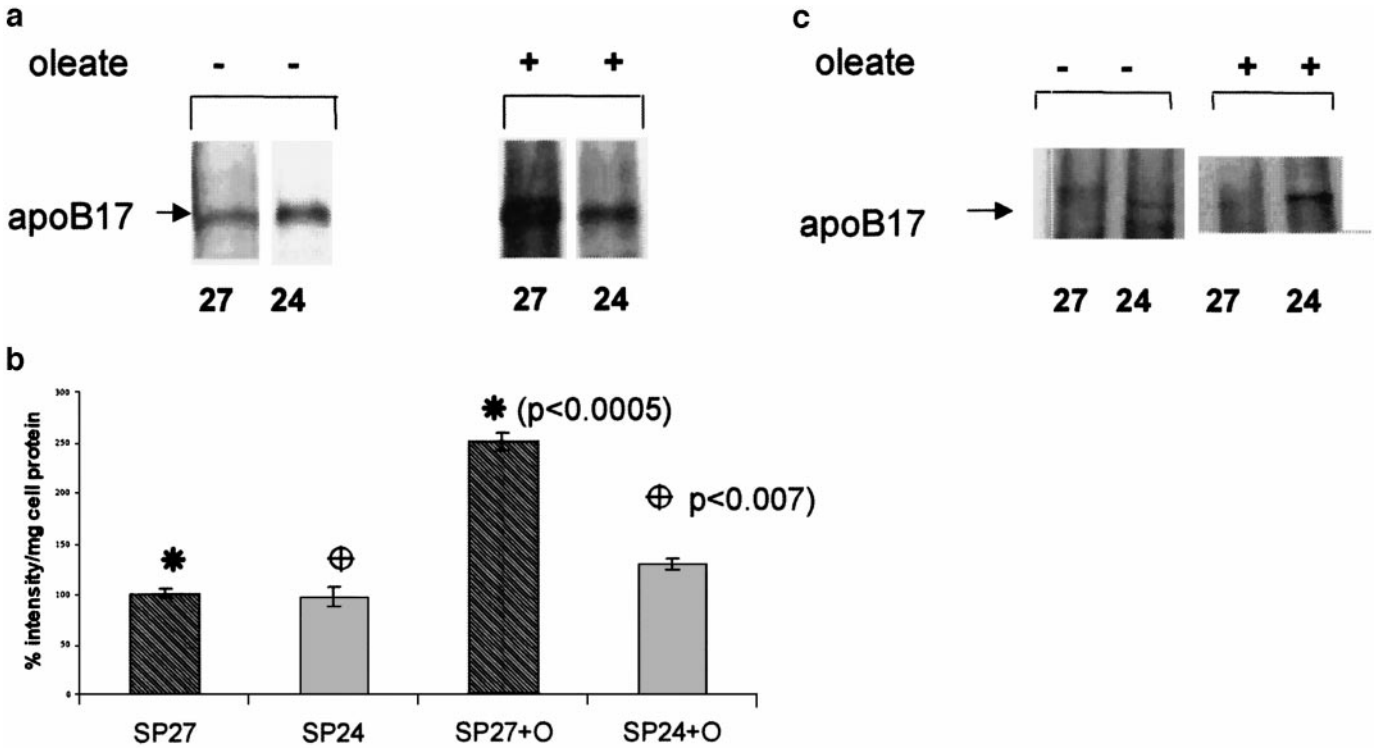

FIG. 4. The effect of the SP variants on the intracellular distribution of apoB17 after a 30-min labeling in the presence or absence of 0.8 mM oleate, followed by cell fractionation. McA-RH7777 cells were transiently transfected with SP27-B17 or SP24-B17 and labeled with [^{3}H]leucine for 30 min. Cells were fractionated as described under Methods and the microsomes and the nonmicrosomal fraction were separated by centrifugation. Immunoprecipitation with sheep anti-human apoB was followed by SDS–PAGE and autoradiographed. (a) A representative experiments showing immunoprecipitation from microsomes from cells grown in absence (control) or presence of oleate (b) Histogram of repeat experiments quantified by scanning densitometry of the autoradiographs. Values were normalized for β-gal and total protein content and expressed as a percentage of SP27-B17 that was set at 100%. Results are the mean (SD) from 2 repeat experiments each done in triplicate. Oleate enhanced SP27-B17 levels by 150% ($P < 0.0005$) and although SP24-B17 levels in the microsomes were also higher than in control cells, this was a much smaller effect of +32% ($P < 0.007$). (c) Representative experiments showing immunoprecipitation from nonmicrosomal fraction from cells grown in absence (control) or presence of oleate.

DISCUSSION

ApoB is constitutively transcribed and translated and readily available for lipoprotein particle formation if lipid is present. This may be a homeostatic process necessary to maintain plasma lipid levels. In the absence of adequate lipid, the fate of surplus apoB is degradation (15), which occurs primarily in the cytoplasmic proteosomes but to a lesser extent in the ER (37). To understand the structure/function relationship of this large 560-kDa hydrophobic protein, several studies have investigated the secretion potential and the ability to form lipoprotein particles of carboxy-terminal truncations of apoB in hepatoma cell lines (35, 38, 39). While apoB truncations with a size of apoB28 and larger are incorporated into lipoprotein particles of increasing density, carboxy-terminal truncations of the size of apoB23 are secreted efficiently from the cells but associate poorly with lipid (40). In the presence of oleate larger apoB constructs showed increased secretion while apoB17 was reported to show only a modest increase.

The consensus from several studies is that the translocation of nascent apoB across the ER membrane is the crucial step in the posttranslational regulation of apoB production (16, 17, 19, 41), with translocation being modulated by several different factors. The specific lipid composition of the ER membrane, particularly the phospholipid composition, has been shown to influence apoB translocation (42, 43). It has been shown that interaction with the chaperon protein HSP70 keeps apoB in a translocational competent state (41), but there is now evidence that HSP70 also promotes apoB degradation by targeting it to the ubiquitin-proteasome pathway (20). A modulator of translocation, however, is likely to be the signal peptide sequence itself because of the importance of the hydrophobic core in determining translocation efficiency (44, 45). Although the translocation channel itself is aqueous (46), there is strong evidence that the hydrophobic core of the signal sequence does play a important role by specifying the targeting route to the ER membrane. and thus alterations to the core would

affect translocation efficiency (44). For apoB, theoretical determination of the signal peptide function, based on the optimum angle of insertion into a simulated hydrophobic/hydrophilic interface (representing the ER membrane), and the hydrophobicity potential of the hydrophobic core, predict that compared to SP27, SP24 would be secretion defective (45, 47).

In transfected McA-RH7777 cells, in the absence of oleate there was no significant difference in the amount of SP27-B17 or SP24-B17 secreted, but intracellular levels of SP27-B17 were higher. However this was not due to differences in the amount of RNA transcribed from the two constructs since this was similar by RT-PCR. Oleate treatment of the transfected cells resulted in enhanced intracellular ($P < 0.002$) and secreted of apoB17 ($P < 0.0001$) when fused to the wildtype 27 amino acid signal peptide but only a minimal, nonsignificant effect on the apoB17 fused to the 24 amino acid SP. In the case of the SP27 fusion protein, this represented a 150% increase of apoB17 in the microsomal compartment ($P < 0.0005$), suggesting augmented translocation. The SP24-B17 protein level also increased in the microsomal fraction when control and oleate treatments were compared, but by only by a modest 32% ($P = 0.007$). The most interesting finding was an 87-kDa band (apoB17) present in the nonmicrosomal fraction, representing the cytoplasm, in the SP24-B17 control cells that was increased after oleate treatment. Our interpretation of these data is based on the hypothesis that the shorter signal peptide, in which Leu-Ala-Leu hydrophobic amino acids are missing, is less efficient at translocation (30). Thus, as translation progresses and apoB17 moves through the translocon, the reduced translocation rate of SP24-B17 may result from inefficient targeting of SP to the ER membrane and thus making contact with the translocon or alternatively it might reflect retrograde translocation. In the presence of oleate, the translation of apoB may be stimulated but the shorter apoB SP translocates less well and results in more apoB17 in the nonmicrosomal fraction.

We did not test the ability of proteosome degradation inhibitors, such as lactacystin and acetyl-leucine, leucine, norleucal (ALLN) to modify the synthesis and secretion of apoB17, since it has been reported that short lengths of apoB, in the order of apoB16 are not ubiquitinated, a prerequisite for proteosomal degradation, and therefore do not undergo proteosomal degradation. The fact that SP24-B17 was present in the cytoplasmic fraction supports this.

Both naturally occurring mutations in signal peptides, and site-directed mutation studies have shed light on the role of SPs in translocation. For example, Gigher-Najjat Type II disease, caused by deficiency of UDP glucuronosyltransferase, is a result of a mutation in the signal peptide that substitutes Leu by Ala thus altering the hydrophobic core, with translocation *in vitro* reduced by >99% (48). Studies in yeast suggest that changes to the hydrophobic residues in the signal peptide, affecting the presentation of the cleavage site at position −1 of the signal peptide to the signal peptidase alter its substrate specificity, as can mutations in the signal peptidase itself (49).

We have previously tested the translocation efficiency of the apoB signal peptide variants in a yeast expression system (30). Compared to SP27, SP24 showed 75% reduced secretion, but relative to the normal invertase SP, the apoB SP isoforms showed a reduction of 40% (SP27) and 75% (SP24). This raised the question as to whether studies using such hybrid-reporter-protein fusions were a true reflection of the functional role of apoB SP variation *in vivo*. Our present findings, in a homologous system with apoB signal peptide in its normal sequence context, demonstrate no difference in translocation of SP27- or SP24-B17 under normal growth conditions, which suggests that the yeast heterologous system did not reflect completely the homologous situation.

These experiments have demonstrated that the naturally occurring variants in the apoB SP play a functional role in apoB17 secretion and that the presence of oleate, in combination with the more hydrophobic signal peptide, SP27, shows greater efficiency of translocation. Our results go someway to explain the discrepancies in the literature concerning the association of the apoB signal peptide variants and lipid levels. While in some reports the SP24 is associated with lower plasma TG levels, in others it is associated with higher levels (reviewed in (13)). The outcome of our present study suggests that the effect of the apoB signal peptide length difference might be exaggerated by the dietary fat intake of the study sample. This hypothesis is supported by the study of Byrne *et al.* (50), who examined the relationship between the free fatty acid response during a fat tolerance test and changes in plasma TGRL levels. In individuals homozygous for the SP27 allele there was a significant positive relationship between maximum FFA concentration and the TGRL ($P = 0.025$). This relationship did not exist in SP24 allele carriers. This suggests that in SP27 homozygous individuals FFAs regulate apoB synthesis and assembly into VLDL, while in SP24 carriers the increased availability of FFA does not influence TGRL synthesis. Riche *et al.* (51), in a study of viscerally obese men, reported that the hepatic secretion of VLDL apoB, was influenced by both apoB SP variation and apoE genotype. In combination with any apoE allele other than E4, SP27 homozygotes had higher hepatic VLDL apoB secretion, suggesting a functional role for the apoB SP with SP27 homozygotes having the greater hepatic production of TGRL.

Thus *in vitro* tracking of apo17 secretion demonstrates in the presence of oleate, a deficiency in the translocation of the 24 aa apoB signal peptide variant.

Future studies will deal with the effects of apoB signal peptide variants on a larger truncated apoB species, which requires lipid for egress from the cell.

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