Overexpression of the Endoplasmic Reticulum 60 Protein ER-60 Downregulates ApoB100 Secretion by Inducing Its Intracellular Degradation via a Nonproteasomal Pathway: Evidence for an ER-60-Mediated and *p*CMB-Sensitive Intracellular Degradative Pathway†

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ABSTRACT: Co- and posttranslational regulation of apolipoprotein B (apoB) has been postulated to involve degradation by both proteasomal and nonproteasomal pathways; however, nonproteasomal mechanisms of apoB degradation are currently unknown. We have previously demonstrated an intracellular association of newly synthesized apoB with endoplasmic reticulum (ER)-60, an ER-localized protein, possessing both proteolytic and chaperone activities. In the present paper, adenoviral expression vectors containing rat ER-60 cDNA were used to achieve dose- and time-dependent overexpression of ER-60 to investigate its role in apoB100 turnover. Overexpressed ER-60 accumulated in the microsomal lumen of HepG2 cells and was associated with apoB100 in dense lipoprotein particles. Overexpression of ER-60 in HepG2 cells significantly reduced both intracellular and secreted apoB100, with no effect on the secretion of a control protein, albumin. Similar results were obtained in McA-RH7777 rat hepatoma cells. ER-60-stimulated apoB100 degradation and inhibition of apoB100 secretion were sensitive to the protease inhibitor, *p*-chloromercuribenzoate (*p*CMB), in a dose-dependent manner but were unaffected by the proteasomal or lysosomal protease inhibitors, *N*-acetyl-leucinyl-leucinyl-nor-leucinal, E64, and leupeptin. Interestingly, enhanced expression of ER-60 induced apoB100 fragmentation in permeabilized HepG2 cells and resulted in detection of a unique 50 kDa degradation intermediate, a process that could be inhibited by *p*CMB. Intracellular stability and secretion of apoB100 in primary hamster hepatocytes were also found to be sensitive to *p*CMB. When taken together, the data suggest an important role for ER-60 in promoting apoB100 degradation via a *p*CMB-sensitive process in the ER. ER-60 may act directly as a protease or may be involved indirectly as a chaperone/protein factor targeting apoB100 to this nonproteasomal and *p*CMB-sensitive degradative pathway.

Apolipoprotein B (apoB100)[1] is the major protein component of plasma very low-density lipoprotein (VLDL) and low-density lipoprotein (LDL). Increased plasma levels of apoB and its associated lipoproteins are regarded as important risk factors in the development of human atherosclerosis. Hepatic apoB100 secretion is metabolically regulated (*1*−*3*), and acute regulation appears to be posttranscriptional in nature (*4*−*13*). A significant proportion of newly synthesized apoB is rapidly degraded in rat hepatocytes (*14*−*16*), rabbit hepatocytes (*17*), hamster hepatocytes (*18*), and hepatoma cell lines such as HepG2 (*19*−*22*). Available evidence clearly shows the involvement of the proteasome in the intracellular degradation of apoB100 in HepG2 cells. This evidence includes the sensitivity of apoB degradation to various proteasome inhibitors (*23*−*29*) and the detection of ubiquitinated apoB (*25*−*29*). Chen et al. detected ubiquitin−apoB associated with the Sec61 complex and showed that factors including calnexin, which alter translocation, can affect apoB ubiquitination and degradation (*26*). Newly synthesized apoB appears to be exposed to the cytosol and targeted to the proteasome in a cotranslational manner based on evidence

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[1] Abbreviations: apoB, apolipoprotein B; ALLN, *N*-acetyl-leucinyl-leucinyl-nor-leucinal; *p*CMB, *p*-chloromercuribenzoate; CSK, cytoskeletal; ER, endoplasmic reticulum; HDL, high-density lipoprotein; LDL, low-density lipoprotein; moi, multiplicity of infection; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; VLDL, very low-density lipoprotein.

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from both intact (*20*, *29*) and permeabilized cells (*25*, *30*). It has also been suggested that, during most of its association with the endoplasmic reticulum (ER), apoB is close to or within the translocon and is accessible to both the ubiquitin−proteasome and lipoprotein assembly pathways (*28*).

There is also compelling evidence that apoB may be degraded posttranslationally by a proteasome-independent pathway(s). Data from several laboratories, including recent kinetic modeling (*12*), support the involvement of multiple proteolytic pathways in apoB degradation. Wu et al. (*22*) proposed a two-site model for the degradation of apoB in HepG2 cells suggesting that, after the initial rapid degradation process, apoB that is fully translocated into the ER lumen can still undergo degradation via a second proteolytic system that is *N*-acetyl-leucinyl-leucinyl-nor-leucinal (ALLN)-resistant but sensitive to dithiothreitol (DTT). Posttranslational degradation of apoB in HepG2 cells was initially thought to occur in the ER or a closely associated compartment (*31*−*35*), although studies in rat hepatocytes also suggested that degradation of apoB may occur in post-ER compartments (*36*, *37*). There is evidence that apoB degradation induced by insulin or fish oils (*38*, *39*) is unaffected by proteasomal inhibitors (*20*). Similar results have been reported with dexamethasone treatment (*37*). More recently, work by Gillian-Daniel et al. (*40*) showed that overexpression of a mutant recombinant form of the LDL receptor caused enhanced degradation of apoB within the ER, implicating LDL receptor in modulating intracellular apoB stability.

Permeabilized HepG2 cells have been used extensively to investigate posttranslational degradation of apoB (*25*, *30*, *32*, *41*). In permeabilized cells, apoB degradation occurs by a temperature- and pH-dependent and ALLN-sensitive mechanism in an ER-related compartment (*31*, *32*) resulting in the generation of an abundant N-terminal 70 kDa fragment, which can be detected in the lumen of the secretory pathway (*31*, *42*). We previously employed this permeabilized cell system to demonstrate the existence of a nonproteasomal degradative pathway that is responsible for specific fragmentation of apoB and generation of a 70 kDa fragment (*32*). Permeabilized cells that were largely devoid of the cytosolic proteasome continued to degrade apoB generating specific fragments, including a 70 kDa fragment, via a lactacystin-insensitive process (*25*). Further evidence for a second proteolytic pathway came from an earlier finding that newly synthesized apoB can be found associated with an ER-localized protease/chaperone, ER-60, during its transit in the microsomal lumen (*41*). This indirectly suggested a potential role for ER-60 in apoB biogenesis; however, there has been controversy as to whether ER-60 acts as a protease and/or chaperone in modulating apoB turnover or secretion (*43*). ER-60, a protein homologous to phosphoinositide-specific phospholipase (*44*−*48*), was first purified as a protease by Urade et al. from the ER of rat liver (*45*) and found to mediate degradation of ER-resident proteins, such as protein disulfide isomerase, calreticulin, and casein. Proteolytic degradation was inhibited by *p*-chloromercuribenzoate (*p*CMB) (*45*). The ER-60 protease contains two copies of Cys-Gly-His-Cys (CGHC) motifs (*47*). The C-terminal cysteine residue(s) of the CGHC motifs may constitute the active site(s) of ER-60 protease. The function of ER-60 in posttranslational degradation of apoB has been controversial, and it is unknown whether or how this ER-resident protein participates in posttranslational degradation of apoB. Recent studies of apoB100 secretion in a fructose-fed hamster model of insulin resistance have provided further evidence for an association between the ER-60 protein levels and the rate of hepatic apoB100 secretion. An important observation in the fructose-fed hamster was that livers of fructose-fed hamsters expressed a lower level of ER-60 protein concomitant with overproduction of VLDL−apoB100 (*49*, *50*). Interestingly, treatment of fructose-fed hamsters with rosiglitazone, an insulin sensitizer, resulted in normalization of the ER-60 protein mass in the liver (*51*). This was evidence that ER-60 protein levels may be chronically responsive to hepatic insulin signaling. Analysis of the 5′ promoter of the ER-60 gene has revealed putative insulin-responsive elements and one sterol-responsive element that may mediate insulin and/or sterol regulation of the ER-60 gene (unpublished observations).

In the present paper, we attempted to directly investigate the role of ER-60 in apoB100 biogenesis by employing an adenoviral vector to overexpress ER-60 in a number of cultured cell models capable of assembly and secretion of apoB-containing lipoproteins. Data from both intact and permeabilized cell studies appear to further implicate ER-60 in modulation of intracellular stability of apoB100 and its extracellular secretion.

EXPERIMENTAL PROCEDURES

Cell Culture. HEK 293, McA-RH7777, and HepG2 cells were purchased from ATCC (Manassas, VA). HEK293 and McA-RH7777 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, Inc.) containing 10% and 20% fetal calf serum, respectively. HepG2 cells were maintained in α-MEM medium (GibcoBRL) with 10% fetal calf serum (Atlanta Biologicals, Norcross, GA). Media were supplemented with 50 units/mL penicillin and 50 μg/mL streptomycin (Life Technologies, Inc.), and cells were maintained at 37 °C with 5% CO_2. The cells were subcultured 2 days before carrying out the experiments.

Generation of Recombinant Rat ER-60 Adenovirus. The rat ER-60 cDNA (1.5 kb) was cloned as an *Eco*RI/*Xba*I fragment (*47*) into pACCMVpLpA, resulting in the plasmid pACCMV-rER-60. Viral recombination was achieved as described previously (*52*). Briefly, pACCMV-rER-60 (5 μg/100 mm diameter dish) was cotransfected with the plasmid pJM-17 (10 μg/100 mm diameter dish) (*53*) into 70% confluent HEK293 cells. Recombination, as indicated by the cytopathogenic effect, occurred 7−14 days after transduction. The resulting recombinant virus containing the rat ER-60 gene was denoted as AdrER-60 and was replication-defective in cells lacking the E1 region of the adenovirus but was fully infectious. Three clones of recombinant virus were assayed by immunoblotting transducted HEK293 cell lysates with rabbit anti-rat ER-60 antibodies. After two rounds of viral plaque purification, all three clones (AdrER-60#1, AdrER-60#2, and AdrER-60#4) revealed successful integration of the rat ER-60 gene into adenovirus (see Figure 1), and the clone, AdrER-60 #2, was amplified in HEK293 cells for further experiments. The control recombinant adenovirus encoding β-galactosidase (Adβ-gal) (*53*) was amplified and purified the same way as the virus encoding rat ER-60.

Isolation of HepG2 Microsomes. Microsomes were prepared as described previously (*54*). Briefly, HepG2 cells

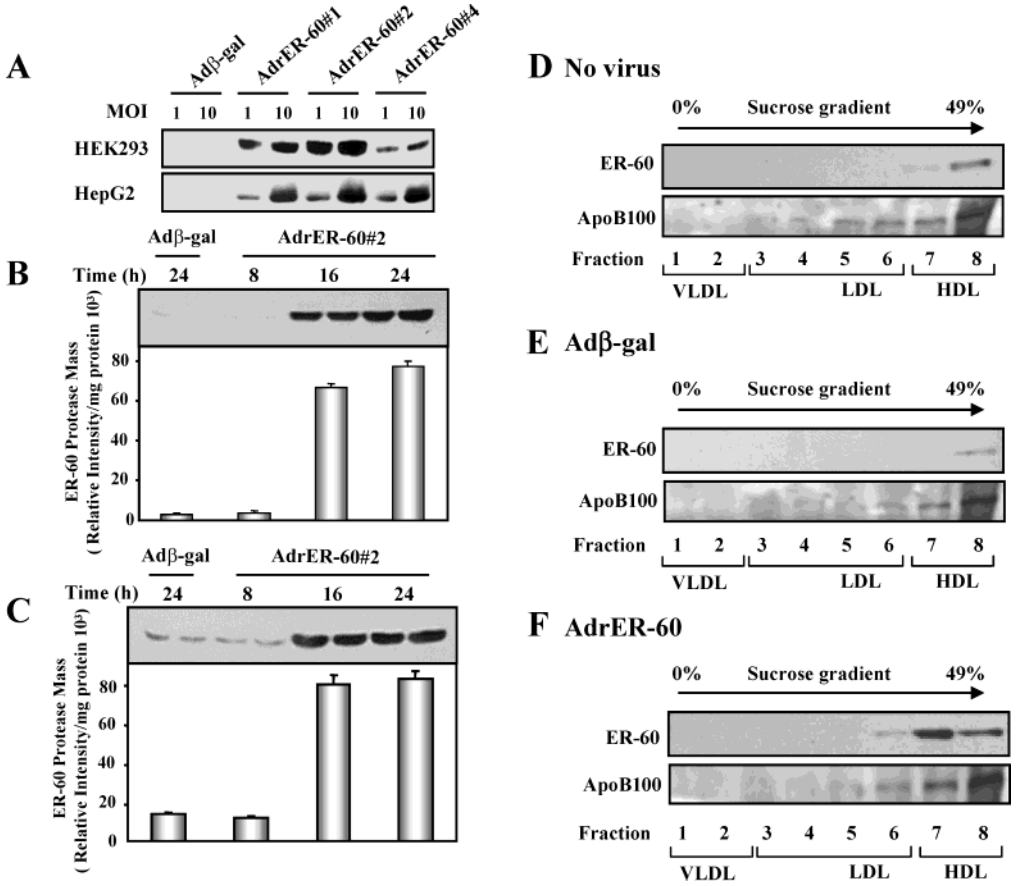

FIGURE 1: Overexpression of rat ER-60 and association with apoB100-lipoprotein particles. (A) HEK293 or HepG2 cells (5×10^5) were transducted with moi of 1 or 10 of rat ER-60 recombinant adenoviruses, AdrER-60#1, AdrER-60#2, and AdrER-60#4. A total of 24 h after transduction, the cells were solubilized and equal amounts of cell protein (20 μg) were resolved by 8% SDS−PAGE. Immunoblotting with rabbit anti-rat ER-60 antiserum was performed to detect the 58 kDa ER-60 protein. Cells were also transducted with adenovirus containing β-galactosidase (Adβ-gal) as a control. Time course (8, 16, or 24 h) of HEK293 (B) and HepG2 (C) cells transducted with AdrER-60#2 at moi of 10. (D−F) Subcellular localization of overexpressed ER-60 and its association with apoB100. Untransducted HepG2 cells (top panel), as well as cells transducted with moi 20 AdrER-60 (lower panel) or Adβ-gal (middle panel) for 48 h. Microsomal lumenal contents were extracted from transducted HepG2 cells and subjected to a discontinuous sucrose gradient, and all fractions were immunoprecipitated with apoB polyclonal antibody conjugated to Affigel 10 beads. Immunoprecipitated proteins were resolved by SDS−PAGE and immunoblotted with either a rabbit polyclonal antibody to ER-60 or a goat polyclonal antibody to apoB. Immunoblots shown are representative of four independent experiments.

(1×10^6), transducted with either AdrER-60 or Adβ-gal, were homogenized with 25 strokes of a Dounce homogenizer in 500 μL of 50 mM sucrose and 3 mM imidazole buffer at pH 7.4, containing 0.1 mM leupeptin, 1 mM PMSF, and 100 KIU/mL Trasylol (aprotinin). After homogenization, 50 μL of a 49% sucrose solution was added and cells were homogenized with 5 additional strokes. The homogenate was centrifuged at 2200*g* for 10 min at 4 °C. The pellet was rinsed with 500 μL of 250 mM sucrose and 3 mM imidazole at pH 7.4 and centrifuged at 2200*g* for an additional 10 min at 4 °C. Supernatants containing microsomes were then pooled and centrifuged at 100000*g* for 60 min at 4 °C.

Extraction of Lumenal Contents and Ultracentrifugation of Lumenal Lipoproteins. Microsomal lumen contents were extracted using the method of Rustaeus et al. (*55*). Briefly, after centrifugation at 100000*g*, the microsomal pellet was solubilized in 1 mL of 100 mM sodium carbonate at pH 11.5, 0.025% deoxycholate, 1.2 M potassium chloride, 0.1 mM leupeptin, 1 mM PMSF, and 100 KIU/mL Trasylol and incubated on ice for 30 min with gentle mixing every 5 min. After the incubation, bovine serum albumin was added to a final concentration of 0.5%. The suspension was centrifuged at 100000*g* for 1 h at 4 °C to separate microsomal membrane from lumen. Lumenal contents were supplemented with additional protease inhibitors and subjected to ultracentrifugation on a discontinuous sucrose gradient (0.5 mL of 49% sucrose, 1.0 mL of 25% sucrose, 0.667 mL of 20% sucrose, 1.06 mL of the sample, 0.63 mL of 5% sucrose, and 0.3 mL of 0% sucrose) at 35 000 rpm (150000*g*) in an SW55Ti rotor (Beckman) for 65 h at 4 °C. Gradients were fractionated into 0.5 mL fractions, and the apoB proteins were immunoprecipitated with an apoB polyclonal antibody (Midland Bioscience Corp.) covalently linked to Affi-Gel 10 gel (Bio-Rad) matrix as suggested by the manufacturer. The immunoprecipitated proteins were resolved on 8% SDS−PAGE, transferred to poly(vinylidene difluoride) (PVDF) membranes, and immunoblotted using a rabbit polyclonal antibody to ER-60.

Transduction of Cell Cultures with Recombinant Adenoviruses. HEK293, McA-RH7777, and HepG2 (5×10^5) cells were seeded on collagen-coated 6-well plates. A total of 4 h after seeding, cells were transducted with AdrER-60 at multiplicity of infection (moi) from 1 to 50. Adβ-gal transduction was used as an adenovirus control, and 1× phosphate buffered saline (PBS) was used as a nontransduction (no virus) control. A total of 2 h after incubation at

37 °C, adenovirus medium was removed and replaced with complete cell medium containing 5% fetal calf serum. The samples were harvested at 44 h after transduction. The expression levels of apoB and rat ER-60 were determined by immunoblot analysis.

Immunoblot Analysis. After transduction with recombinant adenoviruses, the cultured cells were washed twice with 1× PBS and lysed using a solubilizing buffer (1× PBS containing 1% Nonidet P-40, 1% deoxycholate, 5 mM EDTA, 1 mM EGTA, 2 mM PMSF, 0.1 mM leupeptin, 100 KIU/mL Trasylol, and 0.5 μM ALLN), and an equal amount of cell lysates were resolved on 8% SDS−PAGE mini gels. After SDS−PAGE, the protein was transferred electrophoretically for 18 h at 4 °C onto PVDF. The membranes were blocked with a 4% solution of fat-free dry milk powder, incubated with a rabbit anti-rat ER-60 antiserum kindly provided by Drs. M. Kito and R. Urade (Kyoto University), washed, and incubated with a goat anti-rabbit secondary antibody conjugated to horseradish peroxidase. Membranes were then incubated in an enhanced chemiluminescence detection reagent (Amersham Pharmacia Biotech) for 1−5 min and exposed to Kodak Hyperfilm. Films were developed, and quantitative analysis was performed using an imaging densitometer.

Metabolic Labeling of Adenovirus Transducted Cells. A total of 48 h after transduction of HepG2 or McA-RH7777 with the recombinant adenoviruses, the cell cultures were preincubated in methionine/cysteine-free minimum essential medium at 37 °C for 1 h and labeled with 50−100 μCi/mL [^{35}S]methionine/cysteine for 1 h. After the pulse, the medium was harvested for immunoprecipitation of secreted apoB. In pulse−chase experiments, the transducted HepG2 cells were incubated in methionine/cysteine-free MEM in the presence or absence of various amount of inhibitors at 37 °C for 1 h, labeled with 50−100 μCi/mL [^{35}S]methionine/cysteine for 15−30 min, and then chased for 0, 1, or 2 h under the conditions described in the figure captions. The chase media or cell lysates were collected for immunoprecipitation.

Permeabilization of HepG2 Cells Transducted with Recombinant Adenoviruses. A total of 48 h after transduction of HepG2 cultures with recombinant adenoviruses, the cells were incubated with methionine/cysteine-free MEM for 1 h and pulse-labeled for 15 min with 50 μCi/mL [^{35}S]methionine/cysteine. To examine apoB degradation in permeabilized cells, intact cells were chased for 10 min and then washed with cytoskeletal (CSK) buffer [0.3 M sucrose, 0.1 M KCl, 2.5 mM $MgCl_2$, 1 mM sodium-free EDTA, and 10 mM piperazine-1,4-bis(2-ethanesulfonic acid) at pH 6.8]. The cells were incubated for 10 min at room temperature in CSK buffer containing 50 mg/mL digitonin. Permeabilized cells were washed three times in CSK buffer and then incubated in CSK buffer under the conditions described in the figure captions. The cells were harvested in solubilizing buffer, and the cell extracts were subjected to immunoprecipitation.

Immunoprecipitation, SDS−PAGE, and Fluorography. Immunoprecipitation was performed as described previously. Immunoprecipitates were washed 3 times with wash buffer (10 mM Tris-HCl at pH 7.4, 2 mM EDTA, 0.1% SDS, and 1% Triton X-100) and prepared for 5% SDS−PAGE by resuspension and boiling in 30 μL of Laemmli buffer. SDS−PAGE was performed essentially as described (*50*). The gels were fixed and saturated with Amplify (Amersham Pharmacia Biotech) before being dried and exposed to Kodak Hyperfilm at −80 °C for 1−4 days. In some experiments, after SDS−PAGE, the protein was transferred electrophoretically at 4 °C for 18 h onto PVDF membranes using a Bio-Rad Wet Transfer System. The membrane was exposed to Kodak Hyperfilm at room temperature. Films were developed, and quantitative analysis of apoB bands was performed using an imaging densitometer.

RESULTS

Dose- and Time-Dependent Overexpression of ER-60 Protein in Cultured Cells. ER-60 was overexpressed in HEK293 and HepG2 cells using AdrER-60, an adenoviral expression vector carrying a rat ER-60 cDNA. Under basal conditions, low levels of endogenous ER-60 protein were detectable in both cell lines. After transduction of HEK293 or HepG2 with AdrER-60, recombinant ER-60 protein was found to be highly overexpressed in both cell lines. As shown in Figure 1A, the protein mass of ER-60 in both cell lines increased with an increasing dose of adenovirus (moi = 1 or 10) using any of the three different clones (AdrER-60#1, AdrER-60#2, or AdrER-60#4). ER-60 protein levels also increased with an increasing time of transduction (16 or 24 h). At 16 h posttransduction, ER-60 protein level was dramatically increased by 39.4 ± 0.99-fold in HEK293 cells (Figure 1B) and by 7.2 ± 0.47-fold in HepG2 cells (Figure 1C) in comparison to control cells transducted with an adenovirus carrying β-galactosidase (Adβ-gal). Overexpressed ER-60 was undetectable in the culture medium (data not shown), confirming its intracellular localization.

Overexpressed ER-60 Is Localized within the Microsomal Lumen of HepG2 Cells in Association with ApoB100. We had previously reported the intracellular association of ER-60 to newly synthesized apoB100 (*41*). Here, we investigated whether lumenal ER-60 associated with apoB100 was increased in cells transducted with AdrER-60. After extraction of the lumenal contents from microsomes of nontransducted, as well as HepG2 cells transducted with AdrER-60 or Adβ-gal for 48 h, the lumenal lipoprotein-associated apoB100 was immunoprecipitated with an apoB polyclonal antibody linked to Affigel beads, immunoblotted, and probed with a polyclonal antibody to ER-60. Overexpression of ER-60 led to an increased association of ER-60 with apoB100 (15.8 ± 2.9-fold, $p < 0.01$), predominantly in dense high density lipoprotein (HDL)-sized lipoproteins (fractions 7 and 8) of the microsomal lumen (parts D, E, and F of Figure 1). This suggests that overexpressed ER-60 is localized in the microsomal lumen of HepG2 cells and confirms our previous observation that ER-60 is directly associated with apoB100 intralumenally (*41*).

Effect of ER-60 Overexpression on ApoB100 Secretion from HepG2 Cells. To examine whether ER-60 overexpression directly alters apoB100 secretion, HepG2 cells were transducted with ER-60 and the control adenovirus at moi of 50 for 48 h. After 1 h of metabolic labeling with [^{35}S]methionine/cysteine, cells were harvested and tested for expressed ER-60 protein by immunoblotting. ER-60 protein mass (Figure 2A) was increased to 18.8 ± 1.4-fold ($p < 0.01$) in HepG2 cells transducted with AdrER-60 in comparison with control cells transducted with Adβ-gal. To investigate the effect of increased ER-60 expression on

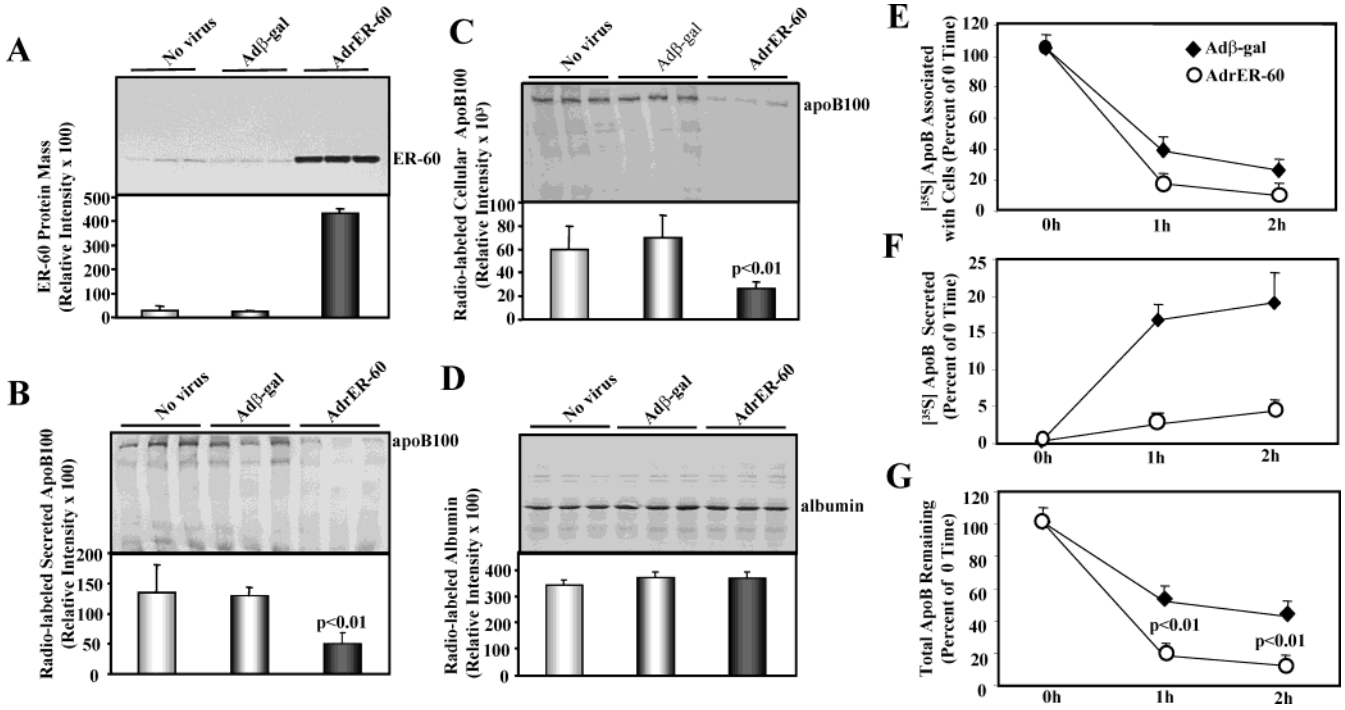

FIGURE 2: Effect of ER-60 overexpression on apoB100 turnover in HepG2 cells. HepG2 cells (5×10^5) were transducted with moi of 50 of rat ER-60 (AdrER-60) or β-galactosidase (Adβ-gal) recombinant adenovirus and nontransducted cells as a control. A total of 48 h after transduction, the cells were pulsed with 100 μCi/mL [^{35}S]methionine/cysteine. After a 1 h pulse, (A) the cells were solubilized and equal amounts of cell lysates (20 μL) were subjected to 8% SDS−PAGE to assess rat ER-60 expression levels. The media (B) and cell lysates (C) were collected and immunoprecipitated with anti-human apoB antiserum. After resolving by 5% SDS−PAGE, the samples were transferred onto a PVDF membrane and then exposed to Kodak Hyperfilm. (D) The apoB100 depleted media were immunoprecipitated with anti-human albumin antiserum and then resolved in 8% SDS−PAGE. HepG2 cells (5×10^5) were transducted with moi 20 AdrER-60 or Adβ-gal. A total of 48 h after transduction, the cells were pulsed with 100 μCi/mL [^{35}S]methionine/cysteine. After a 15 min pulse, the radioactivity was chased at time 0, 1, or 2 h. The cell lysates (E) and medium (F) were collected and immunoprecipitated with anti-human apoB antiserum. After resolving by 5% SDS−PAGE, the samples were transferred onto a PVDF membrane and then exposed to Kodak Hyperfilm. Corresponding bands were scanned, quantified, and (G) presented as total radiolabeled apoB100 (cells and medium). Each blot is a representative of three independent experiments and expressed as mean ± standard deviation (SD).

apoB100 secretion, the medium and cell lysates were collected and immunoprecipitated with an anti-human apoB antiserum. Secreted and cellular apoB100 were significantly reduced by 60.6 ± 14.2% ($p < 0.01$) and 62.4 ± 12.4% ($p < 0.01$), respectively, in cells transducted with AdrER-60 compared with Adβ-gal-transducted controls (parts B and C of Figure 2). To determine the specificity of the effect on apoB100 secretion, the same apoB-depleted cell medium was subjected to a second immunoprecipitation with anti-human albumin antiserum. As shown in Figure 2D, no change was observed in secreted albumin in HepG2 cells transducted with AdrER-60 in comparison to those transducted with Adβ-gal. The total apoB100 mass as assessed by Western blotting did not significantly change with overexpression of ER-60 or β-gal (data not shown), suggesting that the effect of ER-60 on apoB100 degradation was more detectable on the newly synthesized apoB100 pool.

To investigate apoB100 turnover in HepG2 cells overexpressing ER-60, a series of pulse−chase experiments were carried out. HepG2 cells transducted with either AdrER-60 or Adβ-gal were pulse-labeled for 15 min and chased for 0, 1, and 2 h. The distribution of full-length apoB100 in the cells, secreted into the media, and the total apoB100 are shown in parts E, F, and G of Figure 2, respectively. Only 17.6 ± 2.6% ($p < 0.01$) and 11.7 ± 2.1% ($p < 0.01$) of the total apoB100 were recovered in cells transducted with AdrER-60 after a 1 and 2 h chase, respectively, compared with 50.8 ± 3.9% and 41.5 ± 1.4% of the total apoB100 in the cells transducted with Adβ-gal (Figure 2G). The data appear to suggest a considerable reduction in intracellular apoB100 stability in cells overexpressing ER-60.

Overexpression of ER-60 Downregulates ApoB100 Secretion in McA-RH7777 Cells. A second cell model, McA-RH7777 rat hepatoma cells, commonly used for studies of apoB biogenesis, was also employed to confirm our observation in HepG2 cells. Overexpression of ER-60 was achieved in a time- and dose-dependent manner in McA-RH7777 cells after transduction with AdrER-60 (parts A and B of Figure 3). To investigate the effect of an increased ER-60 expression on apoB100 secretion, McA-RH777 cells were transducted with ER-60 and the Adβ-gal at moi of 20 for 48 h. After 1 h of metabolic labeling with [^{35}S]methionine/cysteine, cell lysates and media were collected and immunoprecipitated with an anti-rat apoB antiserum. Cellular and secreted apoB100 were significantly reduced by 64.0 ± 9.0% ($p < 0.01$) and 63.8 ± 7.5% ($p < 0.01$), respectively, in cells transducted with AdrER-60 compared with Adβ-gal-transducted controls (parts C and D of Figure 3).

ER-60-Induced Downregulation of ApoB100 Secretion in Intact HepG2 Cells Is Sensitive to the Protease Inhibitor, pCMB. To investigate the mechanism of ER-60-stimulated apoB100 degradation, the apoB100 turnover was monitored in ER-60-overexpressing HepG2 cells treated with a series of protease inhibitors. Among the inhibitors tested, a protease inhibitor, *p*CMB, previously shown to inhibit ER-60 protease activity was found to be effective. A pulse experiment was

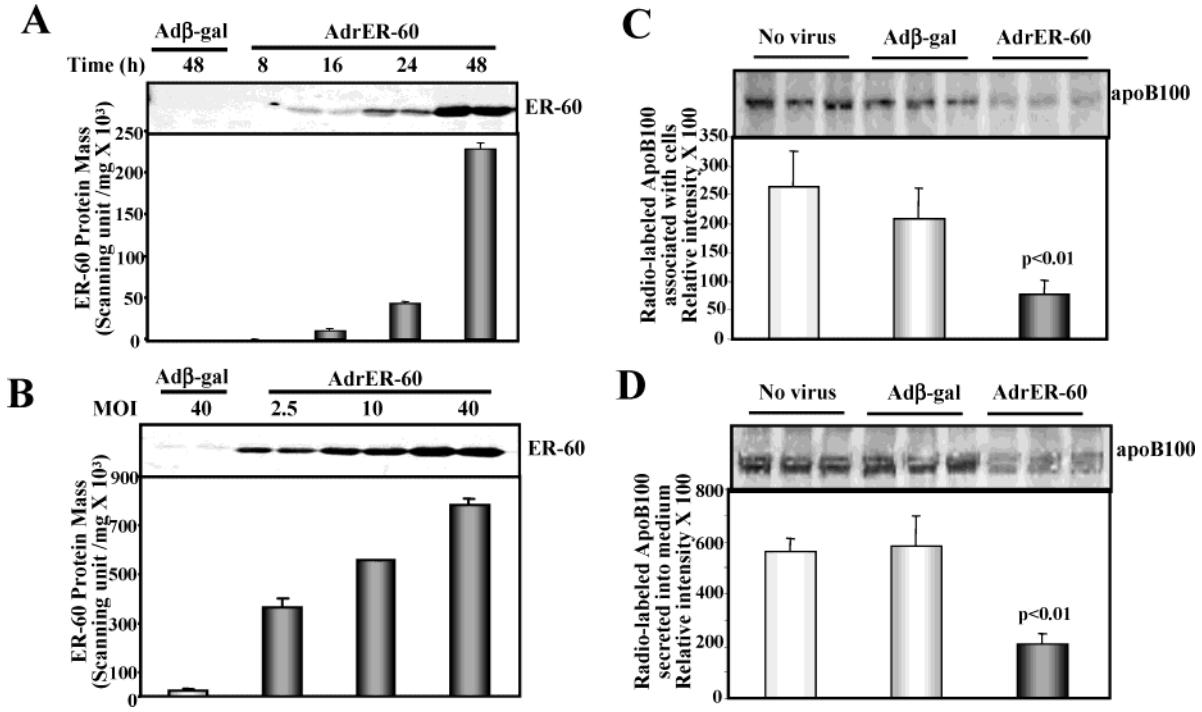

FIGURE 3: Effect of ER-60 overexpression on apoB100 secretion in McA-RH7777 cells. (A) McA-RH7777 cells (5×10^5) were transducted with moi 10 rat ER-60 recombinant adenoviruses and the control Adβgal. At 8, 16, 24, and 48 h after transduction, the cells were solubilized and equal amounts of cell protein (20 μg) were resolved by 8% SDS−PAGE. Immunoblotting with rabbit anti-rat ER-60 antiserum was performed to detect the 58 kDa ER-60 protein. (B) Similar experiments as in A (above) were performed following transduction with different doses of adenovirus (moi 2.5, 10, and 40 AdrER-60) for 48 h. (C and D) After a 48 h transduction of McA-RH7777 cells with AdrER-60 and Adβ-gal at moi of 20, the cells were pulsed with 100 μCi/mL [^{35}S]methionine/cysteine for 1 h. The radiolabeled cellular (C) or secreted (D) apoB100 were immunoprecipitated with anti-rat apoB antibody and then resolved by SDS−PAGE. Each panel is a representative of three independent experiments and expressed as mean ± SD.

first carried out to examine whether *p*CMB could enhance accumulation and stability of newly synthesized apoB100. HepG2 cells transducted with either AdrER-60 or Adβ-gal were metabolically labeled with [^{35}S]methionine/cysteine for various time periods in the presence or absence of *p*CMB. As shown in Figure 4A, there was no significant change in the rate of radiolabeled apoB100 accumulated in the presence or absence of *p*CMB at 5 and 15 min pulse times. However, at a 30 min pulse, the addition of *p*CMB significantly increased radiolabeled apoB100 accumulated in HepG2 cells transducted with AdrER-60 [$(13.4 \pm 1.4) \times 10^3$ counts per minute (CPM) versus $(6.9 \pm 1.6) \times 10^3$ CPM, $p < 0.05$] but not in cells transducted with the control Adβ-gal. Because the effect of *p*CMB was not observed at early pulse times and only became apparent at 30 min, the data appear to suggest no significant effect on the synthesis of apoB100. At 30 min, a significant portion of the newly synthesized apoB100, subject to intracellular degradation and increased apoB100 accummulation in the presence of *p*CMB, is likely to reflect an inhibition of degradation rather than an effect on apoB100 synthesis. Nevertheless, a slight effect on apoB100 synthesis cannot be ruled out.

Next, we examined the effect of *p*CMB on ER-60-induced apoB degradation using pulse−chase labeling experiments. The cells were metabolically labeled with [^{35}S]methionine/cysteine for 15 min and then chased for 0, 1, and 2 h in the absence or presence of an increasing *p*CMB concentration. As shown in Figure 4B, apoB100 secretion remained unchanged in the presence of 5 or 25 μM *p*CMB after a 2 h chase period in no virus control cells (open bars). ApoB100 secretion was slightly, but not significantly, increased in the presence of 25 μM *p*CMB in Adβ-gal-transducted HepG2 cells (gray bars). In contrast, apoB100 secretion, which was reduced in AdrER-60-transducted HepG2 cells, was significantly increased in the presence of 25 μM *p*CMB. ApoB100 secretion was increased by 2.0 ± 0.13-fold ($p < 0.01$) or 2.9 ± 0.13-fold ($p < 0.01$) in AdrER-60-transducted cells in the presence of 5 or 25 μM *p*CMB, respectively, compared with untreated cells (Figure 4B). Thus, ER-60-stimulated apoB100 degradation was blocked by *p*CMB in a dose-dependent manner. Similar results were seen for the total apoB100 (parts C−E of Figure 4). The data suggest that ER-60 overexpression stimulates an intracellular degradation pathway that is sensitive to *p*CMB inhibition.

An experiment was also carried out to determine whether *p*CMB affects the density distribution of apoB-containing lipoprotein particles secreted by HepG2 cells overexpressing ER-60 protein. After transduction of HepG2 cells with AdrER-60, the cells were metabolically labeled with [^{35}S]methionine/cysteine for 1 h and chased for 2 h in the presence or absence of 25 μM *p*CMB. As shown in Figure 4F, density gradient ultracentrifugation of conditioned medium from control and *p*CMB-treated cells showed a slight shift in particle density, with *p*CMB-treated cells secreting slightly larger HDL-size particles (detected in the 7th fraction) and a 2-fold higher level of LDL-dense particles (based on the amount of radiolabeled apoB (CPM) detected in the 5th and 6th fractions).

Stability and Secretion of Hamster ApoB100 Are Sensitive to pCMB in Intact Primary Hamster Hepatocytes. To further examine the physiological relevance of the *p*CMB-sensitive degradative pathway, we repeated the above experiments in

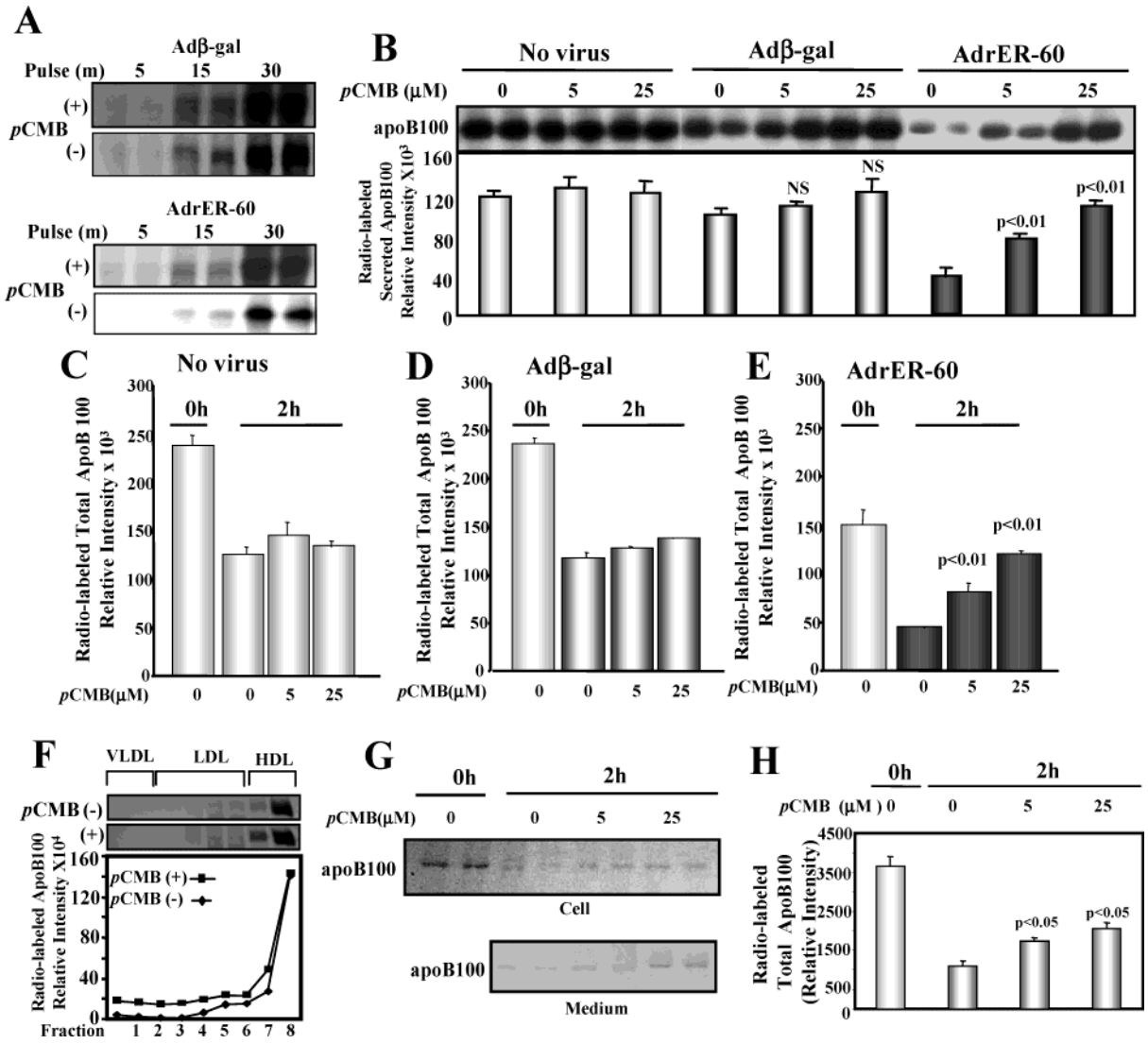

FIGURE 4: Effect of *p*CMB on apoB100 degradation in intact HepG2 cells overexpressing ER-60 and primary hamster hepatocytes. (A) A total of 48 h after transduction of HepG2 cells with the recombinant adenoviruses moi 20 AdrER-60 and Adβ-gal, the cell cultures were preincubated in methionine/cysteine-free MEM in the absence or presence of 25 μM *p*CMB and the cells were pulsed with 50 μCi/mL [^{35}S]methionine/cysteine for 5, 15, or 30 min; the radiolabeled apoB100 was immunoprecipitated with anti-human apoB antibody and then resolved by SDS−PAGE. (B−E) After a 48 h transduction of HepG2 cells with AdrER-60 and Adβ-gal at moi of 20, the cells were pulsed with 50 μCi/mL [^{35}S]methionine/cysteine for 15 min and chased for 2 h in the presence of 0, 5, or 25 μM *p*-CMB. ApoB100 secreted in the media is shown in panel B; the total cellular and secreted apoB are shown in panels C−E. (F) After a 48 h transduction of HepG2 cells with AdrER-60 at moi of 20, the cells were pulsed with 50 μCi/mL [^{35}S]methionine/cysteine for 1 h and chased for 2 h in the absence or presence of 25 μM *p*CMB. The chase media were subjected to sucrose gradient centrifugation, and all fractions were immunoprecipitated with antihuman apoB polyclonal antibody and resolved by SDS−PAGE. Panels G and H show the effects of *p*CMB on apoB100 in primary hepatocytes. Primary hepatocytes (1.5×10^6) isolated from chow-fed hamsters were labeled with 100 μCi/mL [^{35}S]methionine/cysteine for 1 h and then chased for 2 h in the presence of 0, 5, or 25 μM *p*CMB. Cell associated apoB100 and apoB100 secreted into the media are presented in panel G. The total radiolabeled apoB100 (cell plus media) is presented in panel H.

primary hamster hepatocytes isolated from chow-fed Syrian golden hamsters. Pulse−chase experiments were performed in the presence and absence of *p*CMB. *p*CMB (0, 5, and 25 μM) appeared to decrease apoB100 degradation in a dose-dependent manner with significant increases in apoB100 recovery in both cells and media (Figure 4G) as well as in the total apoB100 (Figure 4H) after a 2 h chase.

ER-60-Induced Downregulation of ApoB100 Secretion in Intact HepG2 Cells Is Insensitive to Proteasomal or Lysosomal Inhibitors. To assess the involvement of other proteolytic pathways, the effects of other proteasomal and lysosomal inhibitors on apoB100 stability and secretion were examined. A pulse−chase experiment was carried out in HepG2 cells transducted with AdrER-60 or the control Adβ-gal in the absence or presence of ALLN (5 μg/mL), a commonly used proteasomal inhibitor, which also inhibits other calpains. A total of 48 h after transduction of HepG2 cells with moi 20 AdrER-60 or Adβ-gal, the cells were pulsed with [^{35}S]methionine/cysteine for 30 min. At a 0 h chase time (Figure 5A), in the absence of ALLN, only $69.5 \pm 2.8\%$ ($p < 0.01$) of apoB100 remained in the cells transducted with AdrER-60 compared with the control Adβ-gal, suggesting apoB100 degradation during the pulse following overexpression of ER-60. The apoB100 level did not significantly change when transducted with AdrER-60 in the presence of ALLN compared with cells transducted with AdER-60 in the absence of ALLN. In contrast, higher intracellular levels of apoB100 ($142 \pm 12.6\%$, $p < 0.05$)

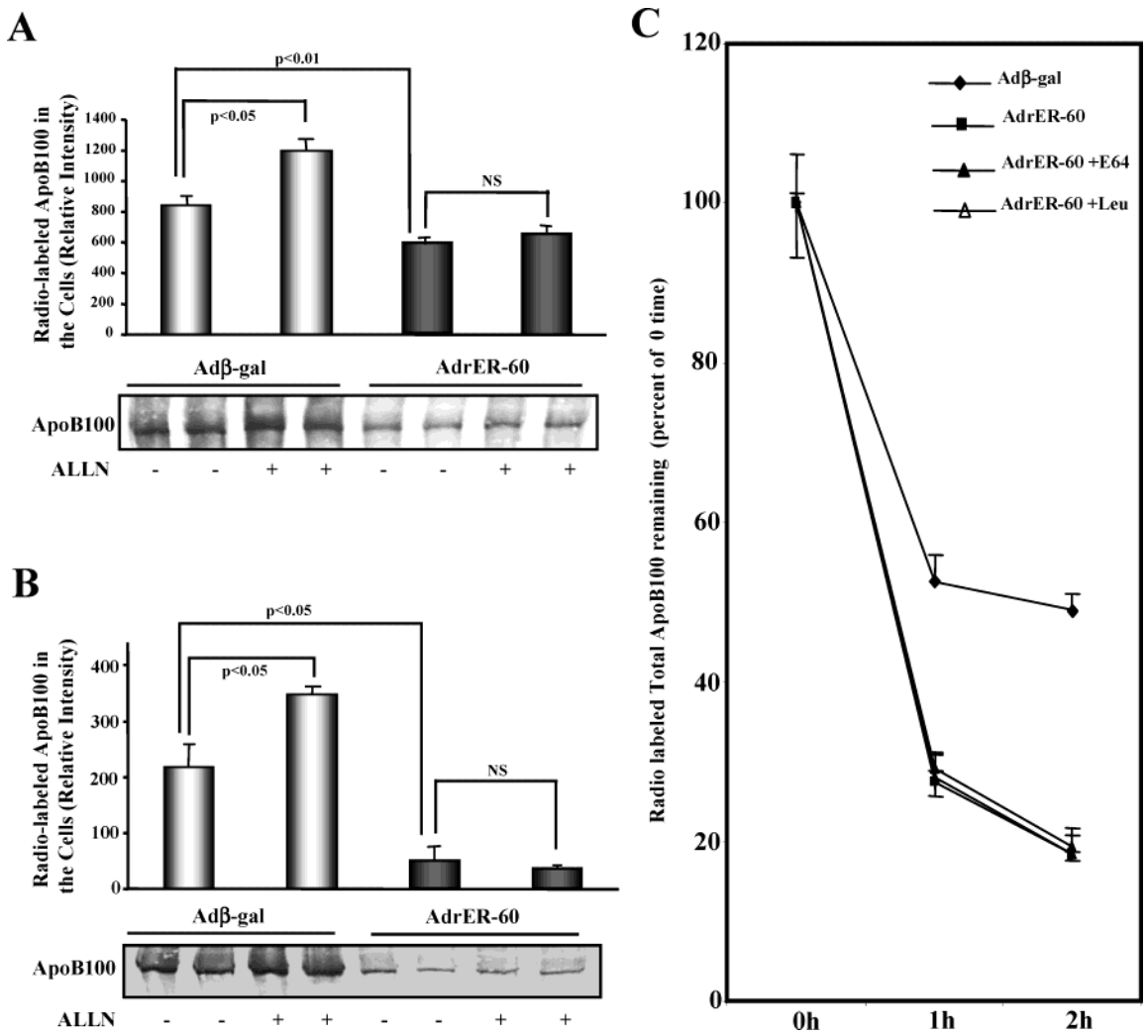

FIGURE 5: Effect of proteasomal and lysosomal inhibitors on apoB100 degradation in intact HepG2 cells overexpressing ER-60. A total of 48 h after transduction of HepG2 cells with the recombinant adenoviruses, AdER-60, Adβ-gal, or no virus control, the cells were preincubated with 5 μg/mL ALLN (panels A and B) or 25 μg/mL E64 or leupeptin (panel C) at 37 °C for 1 h and pulsed with 100 μCi/mL [^{35}S]methionine/cysteine for 15−30 min. The radioactivity was chased for 0, 1, and 2 h. Chase media and cell lysates were collected for immunoprecipitation with anti-human apoB antiserum. Corresponding bands were scanned, quantified, and expressed as mean ± SD. Panel A is the cell-associated apoB at 0 time chase (±ALLN); panel B is the cell-associated apoB at 2 h chase (±ALLN); and panel C is the total apoB remaining in the presence or absence of E64 or leupeptin.

could be immunoprecipitated from the cells transducted with the control Adβ-gal in the presence of ALLN in comparison with cellular apoB100 immunoprecipitated in the absence of ALLN. At the 2 h chase time point (Figure 5B), less apoB100 (23.04 ± 11.52%, $p < 0.05$) remained in the cell when transducted with AdrER-60 in the absence of ALLN in comparison with that of the control adenovirus, Adβ-gal. The apoB100 level was unchanged with ALLN indicating that ALLN could not protect apoB100 from degradation in the cells overexpressing ER-60. By contrast, much higher amounts of apoB100 (159.9 ± 6.4%, $p < 0.05$) could be recovered from cells transducted with the control adenovirus, Adβ-gal, in the presence of ALLN. Similar results were observed in the pulse−chase experiments in the presence of higher concentrations of ALLN (25 μg/mL) (data not shown). Overall, these data suggest that, although ALLN can normally protect apoB100 from degradation in HepG2 cells, this protection could be blocked by overexpression of ER-60.

Two lysosomal protease inhibitors, E64 and leupeptin, were also used to determine whether apoB100 was protected from degradation in HepG2 cells overexpressing ER-60. Pulse−chase experiments were carried out in the absence or presence of E64 (25 μg/mL) or leupeptin (25 μg/mL). Neither E64 nor leupeptin could protect apoB100 from the degradation stimulated by ER-60 overexpression (Figure 5C). These data argue against the stimulation of the lysosomal degradative system by ER-60 overexpression.

Overexpression of ER-60 Protein Induced ApoB100 Fragmentation and Generation of Unique Intermediates in Permeabilized HepG2 Cells. Next, we directly investigated the effect of ER-60 overexpression in permeabilized HepG2 cells, an in vitro model of proteasome-independent apoB degradation (*25*). HepG2 cells were transducted with moi 50 AdrER-60 and subjected to a brief pulse and chase, and the cells were then permeabilized with digitonin. As shown in Figure 6A (upper panel), apoB100 turnover was significantly higher in permeabilized HepG2 cells transducted with ER-60 following a 1 and 2 h chase. To confirm specificity of the effect on apoB, stability of albumin, a control protein was also monitored and was found to be unaffected (lower panel of Figure 6A). In cells transducted with ER-60,

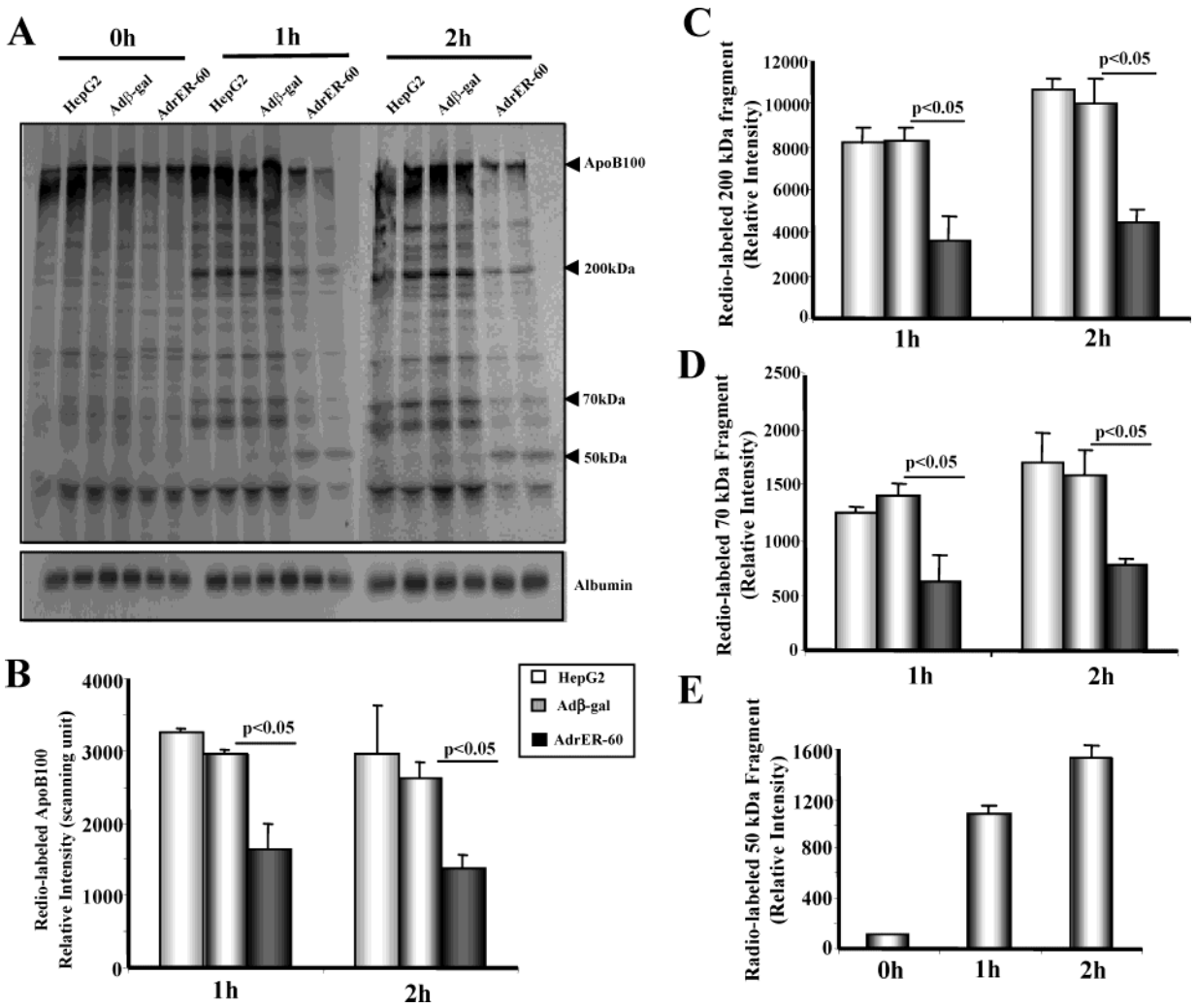

FIGURE 6: Overexpression of ER-60-induced apoB100 degradation and generation of unique fragments in permeabilized HepG2 cells. HepG2 cells (5×10^5) were transducted with moi 50 AdER-60 or Adβ-gal. A total of 48 h after transduction, the cells were pulsed with 50 μCi/mL [^{35}S]methionine/cysteine for 15 min and then chased for 10 min. Cells were permeabilized with CSK buffer containing 50 μg/mL digitonin for 10 min. At 0, 1, or 2 h after incubation in CSK buffer, cell lysates were immunoprecipitated with anti-human apoB (A, upper panel) followed by a second immunoprecipitation using anti-human albumin antiserum (A, lower panel). The samples were subjected to SDS−PAGE and fluorography. Corresponding apoB bands were scanned, quantified, and expressed as mean ± SD. Graphs are shown as intact apoB100 (B) and apoB fragments including 200 kDa (C), 70 kDa (D), and 50 kDa (E) in permeabilized HepG2 cells overexpressing ER-60.

apoB100 was more rapidly degraded with 55.0 ± 11.9% ($p < 0.05$) of apoB100 remaining following a 1 h chase and 52.0 ± 7.4% ($p < 0.05$) following a 2 h chase (Figure 6B). In addition to the intact apoB100, there appeared to be a significant increase in the turnover of a number of apoB100 degradation intermediates. A 200 kDa fragment of apoB was found to be reduced to 43.7 ± 13.9% ($p < 0.05$) at a 1 h chase and 43.0 ± 5.9% ($p < 0.05$) at a 2 h chase (Figure 6C). The 70 kDa fragment of apoB was also significantly reduced to 44.4 ± 15.9% ($p < 0.05$) at a 1 h chase and 48.4 ± 3.0% ($p < 0.05$) at a 2 h chase (Figure 6D) following transduction with ER-60 compared with the control cells treated with Adβ-gal. Interestingly, an abundant 50 kDa fragment of apoB was observed in cells transducted with AdrER-60. Although the 50 kDa fragment was also observed in the control, Adβ-gal-transducted cells, its abundance was significantly enhanced by 8.7 ± 0.7-fold and 12.4 ± 0.8-fold at a 1 and 2 h chase, respectively, in permeabilized HepG2 cells overexpressing ER-60 (Figure 6E).

ER-60-Induced Generation of the 50 kDa ApoB Fragment in Permeabilized HepG2 Cells Is Sensitive to pCMB. We further investigated the effect of *p*CMB on the appearance of unique apoB fragments. After transduction with AdrER-60, similar experiments were performed using permeabilized conditions as in Figure 6 but in the presence or absence of *p*CMB. As depicted in Figure 7, the generation of the unique 50 kDa fragment of apoB100 (observed with ER-60 overexpression) was significantly reduced in the presence of *p*CMB (Figure 7A). There was an almost 80% decrease in the intensity of the 50 kDa fragment relative to apoB100 in the presence of *p*CMB (Figure 7B). Direct comparison of the intensity of the 50 kDa fragment revealed a 4-fold reduction in the level of the fragment generated in the presence of *p*CMB (Figure 7C). In contrast, cells transducted with Adβ-gal showed a very low level of the 50 kDa fragment at 2 h in the absence of *p*CMB, while the fragment appeared to become undetectable in the presence of the inhibitor.

DISCUSSION

Several lines of evidence have consistently suggested the involvement of protease systems other than the cytosolic

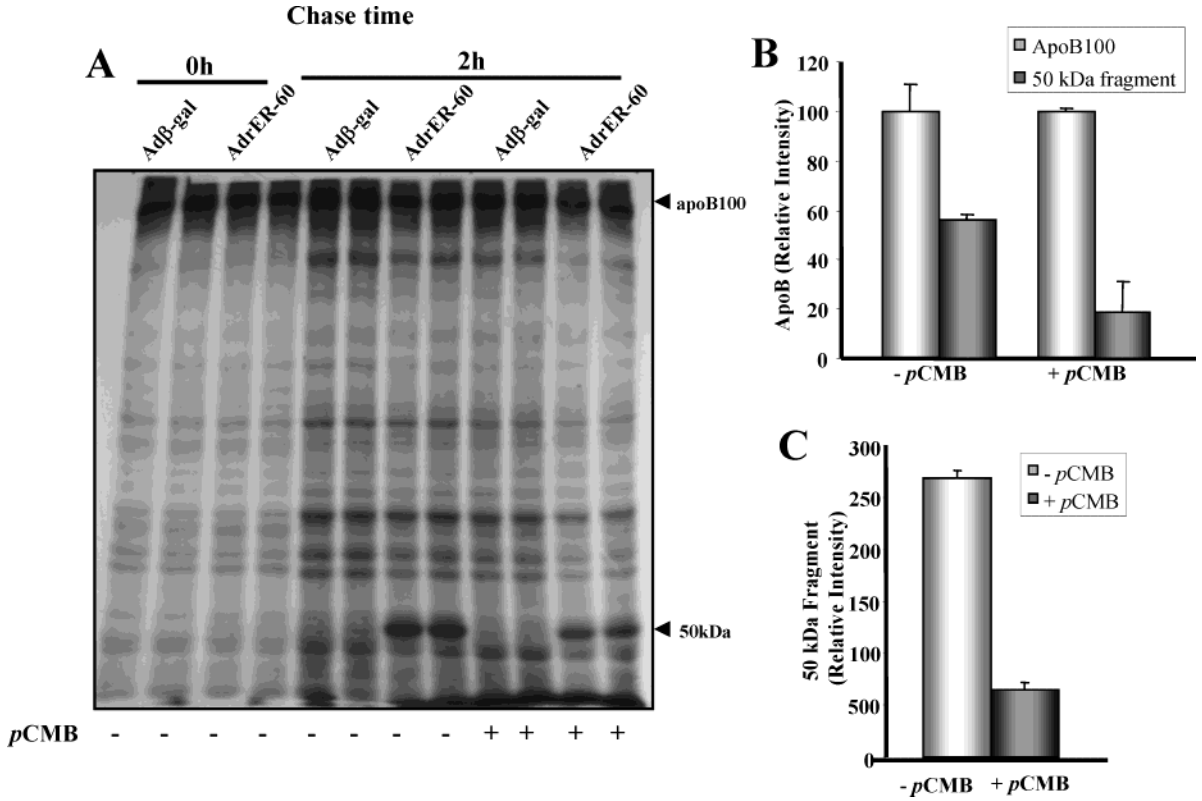

FIGURE 7: Generation of the 50 kDa fragment in permeabilized HepG2 cells is sensitive to inhibition by *p*CMB. HepG2 cells (5×10^5) were transducted with moi 20 AdER-60 or Adβ-gal. A total of 48 h after transduction, the cells were pulsed with 50 μCi/mL [^{35}S]methionine/cysteine for 15 min and then chased for 10 min. Cells were permeabilized with CSK buffer for 10 min. At 0 or 2 h following incubation in CSK buffer, cell lysates were immunoprecipitated with anti-human apoB polyclonal antibody. *p*CMB was first added to the indicated samples during the chase and was present during all subsequent steps. The samples were subjected to SDS−PAGE and fluorography (A). Corresponding apoB bands were scanned for the intensity of the 50 kDa fragment (expressed as a percent of the apoB100 band) in the presence and absence of *p*CMB (B). Direct comparison of the intensity of the 50 kDa fragment in the presence or absence of *p*CMB (C).

proteasome in apoB degradation, particularly at the post-translational level (*12*, *41*, *43*, *56*). Nonproteasomal degradation of apoB in the secretory pathway may play an important role in the turnover of lumenal and lipoprotein-associated apoB pools and may act as an alternative pathway to proteasomal degradation (*43*). It has been suggested that even following translocation into the ER lumen, apoB can potentially undergo further degradation by a lumenal DTT-sensitive degradative system suggesting the existence of an ER-localized proteolytic system distinct from the ubiquitin−proteasome system (*32*). Studies in our laboratory have also provided evidence for nonproteasomal degradation of apoB. ApoB was found to be specifically fragmented in the ER generating an abundant 70 kDa fragment in a regulated manner (*32*). Interestingly, permeabilized cells that were devoid of functional proteasomes continued to degrade apoB generating a 70 kDa fragment (*25*). Coimmunoprecipitation and DSP (dithiobissuccinimidyl propionate) mediated cross-linking studies in permeabilized HepG2 cells also revealed an association of apoB with an ER-localized protease/chaperone, ER-60 (*41*). These observations combined with previous data showing cysteine protease activity of ER-60 (*47*, *48*) implicated this protein in intralumenal degradation of apoB in the ER.

To delineate mechanisms responsible for the apoB non-proteasomal degradative pathway, here, we have further investigated the role of ER-60 by adenoviral-mediated overexpression in HepG2 cells and assessed the impact on apoB degradation. We hypothesized that the ER-60 may be potentially involved in posttranslational fragmentation of apoB, and increased abundance of this protein in cultured cells would result in increased posttranslational fragmentation of apoB and generation of intermediary fragments. To achieve overexpression of ER-60 protease in the cultured cells, we transducted HepG2 cells with rat ER-60 cDNA using an adenoviral vector and achieved successful overexpression in a dose- and time-dependent manner in HEK293, McA-RH7777, and HepG2. ER-60 was found to accumulate in the microsomal lumen of HepG2 cells after transduction of AdrER-60 and directly associate with dense, HDL-size apoB100-containing lipoproteins. This evidence extends our previous observation of an association between apoB and ER-60 (*41*) and demonstrates association of ER-60 with a poorly lipidated fraction of apoB-containing lipoprotein particles that have been previously suggested to be secretion-incompetent and destined for intracellular degradation (*6*, *42*).

Overexpression of ER-60 was found to directly enhance apoB100 turnover and downregulate apoB100 secretion in HepG2 cells. Importantly, however, the ER-60-induced degradation was specific to apoB100 because the secretion of albumin was unaffected. A pulse−chase experiment provided evidence that downregulation of apoB100 by ER-60 directly induces additional apoB100 degradation. Similar observations were made in primary hepatocytes isolated from fructose-fed hamsters, an animal model in which there is considerable overproduction of VLDL−apoB100 by the liver. ER-60 overexpression in primary hepatocytes signifi-

cantly reduced cell-associated apoB100, suggesting that it can block fructose-induced apoB100 overproduction (W.Q. and K.A., unpublished observations). Thus, overexpression studies in these different cell culture models support the notion that hepatic apoB100 secretion may be regulated by ER-60 acting as either a protease or a chaperone/protein factor targeting apoB100 to intracellular degradation. These data also support our recent observation that ER-60 levels are downregulated in a fructose-fed hamster model of insulin resistance, concomitant with enhanced assembly and secretion of apoB-containing lipoproteins (*50*). Thus, physiological suppression of ER-60 expression mediated by insulin resistance could potentially contribute to a VLDL overproduction state and metabolic dyslipidemia. There is no direct evidence currently for insulin-mediated regulation of ER-60 expression. However, treatment of fructose-fed hamsters with a peroxisome proliferator-activated receptor γ agonist and insulin sensitizer, rosiglitazone, normalized the high hepatic levels of ER-60 (*51*), suggesting that cellular levels of ER-60 protein are responsive to changes in hepatic insulin sensitivity. It is interesting to note that several factors such as inhibition of phosphatidylinositol 3-kinase activity are able to inhibit the degradation of apoB induced by insulin (*57*). Whether insulin exerts its effect on apoB via changes in the ER-60 protein is currently unknown and requires further investigation.

Interestingly, ER-60 overexpression induced intracellular apoB100 degradation in a process that appears to be independent of both proteasomal and lysosomal degradative pathways based on experiments with ALLN, E64, and leupeptin. None of these inhibitors could significantly protect radiolabeled apoB100 from degradation in cells overexpressing ER-60. However, *p*CMB, a thiol protease inhibitor previously shown to inhibit ER-60-mediated protein degradation (*45*), could protect downregulation of apoB100 secretion induced by ER-60 in a dose-dependent manner. Hence, these data appear to suggest that ER-60-induced apoB100 degradation is independent of the proteasome and lysosomal pathways and implicates a *p*CMB-sensitive/ER-60-stimulated pathway in apoB100 turnover. It further suggests the potential involvement of multiple proteolytic systems in the intracellular itinerary of newly synthesized apoB100 molecules posttranslationally as suggested previously (*58*). However, it should be emphasized that direct evidence for a proteolytic function of ER-60 directly acting on the apoB100 molecule is still lacking and that the ER-60-mediated stimulation of apoB100 degradation may be indirect and could involve activation of a yet unknown protease or proteolytic system in the secretory pathway.

An intriguing observation was that HDL-size apoB100 turnover in HepG2 cells transducted with AdrER-60 appeared to be largely insensitive to *p*CMB, bringing into question the physiological importance of the *p*CMB-sensitive/ER-60-mediated proteolytic pathway. It should be noted, however, that the vast majority of apoB100 degradation in intact HepG2 cells is known to be proteasomal in nature and that inhibition of a small pool of apoB100 degraded by this second pathway may not be readily detectable in this cell line. Support for this notion comes from experiments in primary hamster hepatocytes, which showed a significant inhibition of hamster apoB100 degradation by *p*CMB, suggesting that this second pathway may play a more important role in primary hepatocyte systems. There appears to be a higher recovery of apoB in the presence of *p*CMB in cells overexpressing ER-60 compared to that observed in control untransducted cells. This may suggest that overexpression of ER-60 can potentially redirect some of the apoB pool that is destined to other degradative pathways toward the *p*CMB-sensitive degradative mechanism. Thus, in cells overexpressing ER-60, a greater proportion of newly synthesized apoB may be degraded via a *p*CMB-sensitive mechanism. ER-60 may potentially act as a chaperone that targets apoB to this latter pathway.

Interestingly, inhibition of ER-60-induced degradation by *p*CMB appeared to induce secretion of slightly larger and more buoyant apoB-containing lipoprotein particles. These data appear to suggest that inhibition of the ER-60-induced degradation may promote lipidation of newly synthesized apoB particles in the secretory pathway and lead to secretion of more buoyant lipoprotein particles. This effect will need to be further confirmed however in other hepatocyte cell systems capable of normal assembly and secretion of larger VLDL-sized particles.

Experiments in permeabilized HepG2 cells overexpressing ER-60 appeared to shed further light on the mechanisms mediating apoB100 degradation. Our previous work indicated that permeabilized cells lack proteasomal activity because of a major loss of both functional and structural 20S proteasomal subunits following permeabilization of these cells (*25*). Thus, intracellular fragmentation of apoB100 and generation of interemediates such as the 70 kDa fragment were found to be lactacystin insensitive and largely independent of the proteasomal system. Intriguingly, ER-60 overexpression appeared to significantly reduce not only the intact apoB100 but also the 200 and 70 kDa fragments. In addition, a 50 kDa fragment was generated in cells overexpressing ER-60. This fragment may represent a new degradation intermediate or may be a product of further degradation of the 200 and/or 70 kDa fragments. However, the fact that both intact as well as degradation intermediates of apoB100 were reduced with ER-60 overexpression clearly implicates this protein in posttranslational turnover of apoB. The generation of the 50 kDa fragment in cells overexpressing ER-60 was found to be sensitive to inhibition by *p*CMB, supporting the notion that this fragment is the product of an ER-60-mediated posttranslational apoB degradative pathway. Interestingly, the 50 kDa fragment generated in cells overexpressing ER-60 is strikingly similar to a 50 kDa fragment found previously after permeabilization of primary hamster hepatocytes (*18*). Importantly, the generation of the 50 kDa fragment was specific to ER-60 overexpression because overexpression of a control ER chaperone protein, BiP, did not induce the generation of this fragment in permeabilized HepG2 cells (W.Q. and K.A., unpublished observation). These observations further support the notion that the 50 kDa fragment is the product of a physiologically relevant system rather than a result of nonspecific degradation of apoB because of the overexpression of ER-60.

In summary, the current paper provides further direct evidence for the involvement of ER-60 in intracellular itinerary of apoB100 and modulation of its extracellular secretion. ER-60 appears to be a critical factor in a nonproteasomal, ER-associated degradative mechanism that controls the intracellular stability of apoB100 during its

transit in the secretory pathway. We hypothesize that this pathway may be involved in acute regulation of apoB secretion and may mediate the inhibitory effect of factors such as omega-3 fatty acids on apoB secretion (*58*). Whether ER-60 functions as a protease responsible for direct fragmentation of apoB100 or as a chaperone/protein factor involved in the targeting of apoB100 to a degradative machinery is currently unknown and will require further investigation.

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