Ubiquitin-Proteasome Pathway Mediates Intracellular Degradation of Apolipoprotein B†

S. Jim Yeung, San Hwan Chen, and Lawrence Chan*

Departments of Medicine and Cell Biology, Baylor College of Medicine, Houston, Texas 77030

Received July 29, 1996⊗

ABSTRACT: Newly synthesized apolipoprotein B (apoB) is degraded by a proteolytic process in the pre-Golgi compartment that can be inhibited by *N*-acetyl-L-leucinyl-L-leucinyl-L-norleucinal (ALLN) but not by several other protease inhibitors. We have tested the hypothesis that the ubiquitin-proteasome pathway is involved in the intracellular degradation of apoB in liver cells. We found that inhibitors of proteasomes blocked the degradation of apoB in cultured human hepatoma (HepG2) cells. Protein degradation by proteasomes is ATP-dependent, and ATP depletion by dinitrophenol and 2-deoxyglucose also inhibited apoB degradation in these cells. Furthermore, the intracellular human apoB isolated by immunoprecipitation was shown to react specifically with anti-ubiquitin antibody by immunoblotting. This result was corroborated by sequential immunoprecipitation of [^{35}S]methionine-labeled proteins by anti-human apoB and anti-ubiquitin antisera. In contrast, secreted apoB was not ubiquitinated. The amount of intracellular ubiquitinated apoB was increased by the proteasome inhibitors, ALLN and carbobenzoxyl-leucinyl-leucinyl-norvalinal-H (MG115). Our findings suggest that the ubiquitin-proteasome pathway is one mechanism for the intracellular degradation of apoB.

Elevated apolipoprotein B (apoB)[1] is a major risk factor for atherosclerosis (Chan, 1992). The plasma level of apoB is a balance between secretion of apoB-containing particles and their removal from the circulation. The rate of secretion of apoB-100-containing particles by liver cells seems to be primarily determined by the rate of intracellular degradation of newly synthesized apoB. The level of intracellular apoB mRNA is quite stable and generally does not change when the secretion of apoB-containing particles is changed by metabolic perturbations (Pullinger *et al*., 1989; Dashti *et al*., 1989). Up to approximately one-half of *de novo* synthesized apoB is degraded. Therefore, it is important to understand this degradative mechanism that regulates the amount of apoB secreted by liver cells before potential targets for therapeutic intervention can be identified.

The proteolytic process involved in the degradation of *de novo* synthesized apoB has been elusive although previous work has defined its location in a pre-Golgi compartment and exclued the lysosomal pathway (Furukawa *et al*., 1992). *N*-Acetyl-L-leucinyl-L-leucinyl-L-norleucinal (ALLN) inhibits the degradation of nascent apoB (Thrift *et al*., 1992; Sakata *et al*., 1993; Adeli, 1994), but a number of commonly used protease inhibitors do not. Recent preliminary evidence suggests that other proteolytic pathways can degrade apoB in addition to the ALLN-sensitive process (Ginsberg *et al*., 1995).

A major proteolytic enzyme activity in the cell is in the proteasomes. Intracellular protein degradation by the ubiquitin-proteasome pathway has been documented for a number of proteins. This protein degradation system is involved in the turnover of regulatory proteins and elimination of abnormal proteins such as proteins that are misfolded or "unused" proteins that would otherwise be part of a larger multicomponent complex (Jentsch & Schlenker, 1995; Ciehanover, 1994; Peters, 1994). Oleate facilitates secretion of apoB and decreases intracellular degradation (Dixon *et al*., 1991). Misfolding in apoB or increased "unused" apoB trapped in the membrane of ER due to nonavailability of fatty acids makes the apoB susceptible to degradation (Ginsberg, 1995). We hypothesize that the proteasome is a degradative mechanism of *de novo* synthesized apoB. In this paper, we present evidence that the intracellular degradation of apoB in HepG2 cells is mediated at least in part by the ubiquitin-proteasome pathway.

MATERIALS AND METHODS

Polyclonal anti-ubiquitin rabbit antibodies were from Chemicon and Sigma. Monoclonal anti-ubiquitin mouse antibody and polyclonal anti-human apoB goat antibodies were from Chemicon. Rabbit polyclonal anti-human low-density lipoprotein (that contained apoB-100 as its only apolipoprotein) antiserum was a generous gift of Dr. W. A. Bradley (University of Alabama in Birmingham). ALLN was from Bachem (King of Prussia, PA). Anti-goat IgG peroxidase conjugate (affinity purified from swine) and anti-rabbit IgG peroxidase conjugate (affinity purified from goat) were from Boehringer Mannheim Biochemicals (Indianapolis, IN). MG115 (carbobenzoxyl-leucinyl-leucinyl-norvalinal-H, also known as Z-LLnV) was a generous gift of Myogenics, Inc. (Cambridge, MA).

† This work was supported by National Institutes of Health Grant HL16512 to L.C. and Fellowship GM17159 to S.J.Y.

* Address correspondence to this author at Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030. Phone (713) 798-4478. FAX: (713) 798-8764.

⊗ Abstract published in *Advance ACS Abstracts,* October 1, 1996.

[1] Abbreviations: apoB, apolipoprotein B; ALLN, *N*-acetyl-L-leucinyl-L-leucinyl-L-norleucinal; EDTA, ethylenediaminetetraacetic acid; ER, endoplasmic reticulum; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; α-MEM, α-modified Eagle's minimum essential medium; MG115, carbobenzoxyl-leucinyl-leucinyl-norvalinal-H; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; PVDF, polyvinylidene difluoride; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; Ub, ubiquitin.

S0006-2960(96)01877-6 CCC: $12.00

Cell Culture. Monolayer cultures of HepG2 cells (ATCC HB8065, American Type Culture Collection) were maintained at 37 °C in an atmosphere with 5% CO_2 and in α-modified Eagle's minimum essential medium (α-MEM) supplemented with 100 units of penicillin-G/mL, 10 μg of streptomycin sulfate/mL, 2 mM glutamine, and 10% (v/v) fetal bovine serum. Depending on the experiment, cells were grown in six-well plates (well diameter, 35 mm) or 175 cm^2 dishes and were used at about 90% confluency.

Cell Labeling and Pulse−Chase Experiments. Cultured cells were pre-incubated for 15 min with MEM without serum and methionine, and then they were incubated for a specified time period with up to 0.5 mCi of L-[^{35}S]-methionine/mL (1000 Ci/mmol) dissolved in MEM without serum and methionine. After labeling, the cells were either lysed immediately after two washes with ice-cold PBS or they were washed with PBS (150 mM NaCl, 50 mM sodium phosphate, pH 7.4) at 37 °C and "chased" by addition of MEM containing "cold" L-methionine (15 mg/L) and no L-[^{35}S]methionine for a specified time period before lysis. Unless stated otherwise, lysis was performed in a buffer containing 0.5% sodium deoxycholate, 0.5% Triton X-100, 5 mM EDTA, 150 mM NaCl, 62.5 mM sucrose, 1 mM PMSF and 50 mM Tris, pH 7.4 with the help of a sterile cell-scraper. Insoluble material in the cell lysate was removed by centrifugation at maximum speed at 4 °C in a microcentrifuge.

Protein concentrations in cell lysates were measured using Bio-Rad DC protein assay. Both protein concentration and radioactivity per mg of protein in the cell lysates were monitored to ensure comparability of the samples.

Immunoprecipitation (Single and Sequential). Single immunoprecipitation was performed on cells lysed with the lysis buffer described above. Antiserum was added to the precleared lysate and incubated on ice for 1 h. Then 25 μL of Gamma-Bind (Protein G-Sepharose, Pharmacia) per mL of lysate was added and incubated for another hour with intermittent inversion of tube to resuspend the Sepharose. After being washed 3 times with the lysis buffer, the protein G-Sepharose was boiled for 5 min in SDS sample buffer (4% SDS, 20% glycerol, 2% 2-mercaptoethanol, 0.001% bromphenol blue, 125 mM Tris, pH 6.8) and the immunoprecipitates were analyzed by SDS−PAGE or liquid scintillation counting. For each cell lysate sample in experiments using liquid scintillation counting, background and nonspecific radioactivity was measured by performing the immunoprecipitation procedure without the antiserum on an aliqout of the same cell lysate, and this was subtracted from the radioactivity in the immunoprecipitate.

Sequential immunoprecipitation was performed using the method of Ou *et al*. (1993) with minor modifications. Cells were lysed in 2% sodium cholate in HEPES-buffered saline (50 mM HEPES, pH 7.5, 200 mM NaCl, 1 mM PMSF). For both the first and second immunoprecipitation, the mixture was incubated on ice for 1 h after addition and antiserum; protein G-Sepharose was then added, and the mixture was incubated for another hour with intermittent inversion of tube to resuspend the Sepharose. For the first immunoprecipitation, the protein G-Sepharose was washed twice with 2% sodium cholate in HEPES-buffered saline. 1% SDS in HEPES-buffered saline (0.1 mL) was then added to the protein G-Sepharose beads, and the mixture was heated at 90 °C for 3 min. After centrifugation, the supernatant was diluted to 1 mL with 1% Triton X-100 in HEPES-buffered saline, which was then used for the second immunoprecipitation. After washing twice with 1% Triton X-100 in HEPES-buffered saline, the protein G-Sepharose from the second immunoprecipitation was boiled for 5 min in SDS sample buffer.

SDS−PAGE. Electrophoresis was performed at room temperature on precast 4−15% gradient polyacrylamide gels or 4% polyacrylamide gels at a current of 20 mA/gel. Prestained protein markers were used for molecular weight calibration. Gels were fixed with 10% acetic acid and 10% methanol and then dried on filter paper. For [^{35}S]methionine labeling experiments, the dried gels were exposed to Kodak X-Omat AR5 films at −80 °C for up to 3 weeks.

Western Blot. Electrophoretic transfer of proteins from an SDS−PAGE gel onto a PVDF membrane or supported nitrocellulose membrane was performed in Tris−glycine−methanol buffer (20 mM Tris, 150 mM glycine, 20% methanol, pH 8.0) at 25 V at 4 °C overnight. After blotting, antibody incubation and visualization were performed with an enhanced chemiluminescence kit (Amersham Life Science). The blocking solution used was 5% nonfat dry milk in PBS. Washing was performed with 0.1% Tween-20 in PBS. The chemiluminescence image was recorded on Kodak Biomax MR films. To reprobe the same membrane with a different antibody, it was incubated at 50 °C for 30 min with occasional agitation in stripping buffer containing 100 mM 2-mercaptoethanol, 2% SDS, 50 mM Tris, pH 6.8, and washed with 0.1% Tween-20 in PBS before it was reblocked with 5% nonfat dry milk in PBS and used for immunodetection with another primary antibody.

RESULTS

Inhibition of ApoB Degradation by Proteasome Inhibitors

MG115 and ALLN are inhibitors of proteasomes (Rock *et al*., 1994). To see if intracellular apoB degradation was inhibited by these inhibitors, we examined the effects of MG115 and ALLN on the rate of degradation of newly synthesized apoB. HepG2 cells cultured in six-well plates were labeled with L-[^{35}S]methionine for 30 min and chased for different lengths of time (20, 45, 80, 150 min) afterward in the presence or absence of ALLN (0.1 mM) or MG115 (85 μM) in serum-free α-MEM containing "cold" L-methionine (15 mg/L). The concentrations of the inhibitors were chosen according to published data (Rock *et al*., 1994). [^{35}S]Methionine-labeled apoB was purified by immunoprecipitation, and the radioactivity in each immunoprecipitate was quantitated by liquid scintillation spectrophotometry. The background radioactivity as measured by control immunoprecipitation without antiserum was subtracted, and the radioactivity in immunoprecipitated apoB was corrected for slight variations in protein concentration in the cell lysates. In the absence of inhibitors, about 50% of newly synthesized apoB present at 20 min is degraded at 80 min (Figure 1A). ALLN inhibited the degradation of apoB ($n = 3$, Student's t-test, $p < 0.05$) when compared to the control at 80 min. MG115 inhibited the degradation of apoB ($n = 3$, Student's t-test, $p < 0.05$) when compared to the control at 80 and 150 min. The amount of labeled apoB secreted into the chase medium was also monitored. ALLN and MG115 did not affect the secretion of labeled apoB (data not shown), and

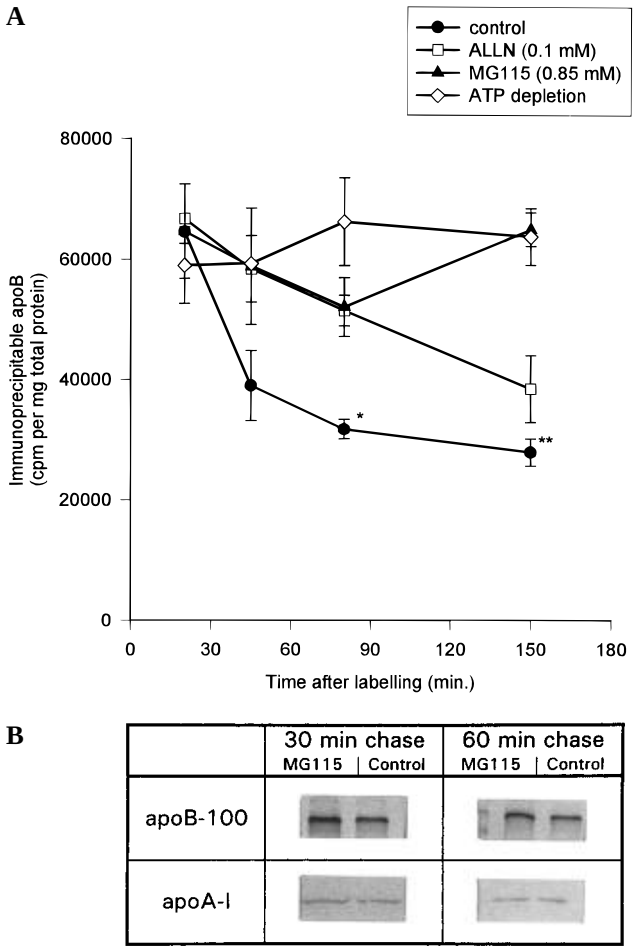

FIGURE 1: (A) Effects of ATP depletion, MG115 and ALLN on the intracellular degradation of apoB. HepG2 cells were labeled with L-[S^{35}]methionine for 30 min followed by "chase" with "cold" L-methionine in the presence or absence of inhibitors for various periods. The amount of intracellular apoB was measured by immunoprecipitation with anti-human apoB. Background and nonspecific radioactivity were subtracted, and the radioactivity was corrected for variations in protein concentration in the cell lysates. The error bars in the graph indicate standard errors. (*) All three treatments were significantly different from control ($p < 0.05$). (**) MG115-treated cells and ATP-depleted cells were significantly different from control ($p < 0.05$). (B) MG115 inhibited degradation of intracellular apoB. Autoradiographic images shown are SDS−PAGE from immunoprecipitates of HepG2 cells labeled with L-[S^{35}]-methionine followed by "chase" with "cold" L-methionine in the presence or absence of MG115 for 30 or 60 min. MG115-treated cells contained more labeled apoB than the control cells at both times. In contrast, the amount of labeled apoA-I was not changed by MG115.

the maximum secreted labeled apoB was about 10% of the maximum labeled apoB in the cell lysate.

The effect of MG115 on intracellular apolipoprotein degradation was specific for apoB (Figure 1B). HepG2 cells were cultured in six-well plates and labeled with L-[^{35}S]-methionine for 60 min. After being washed twice with PBS, the cells were chased with unlabeled methionine in serum-free α-MEM with and without MG115 (0.1 mM) for 30 and 60 min. We performed immunoprecipitation of the radiolabeled protein in aliquots of the cell lysate using anti-human apoB antibody and, as a control, anti-human apoA-I antibody and analyzed the immunoprecipitated protein by SDS−PAGE and autoradiography. The autoradiographic image was analysed by integrating the optical density over the area of each band (Visage, Bio-Image, Ann Arbor, MI). In cells chased in the presence of MG115 for 30 min, the integrated optical density of the apoB-100 band was 1.9-fold that in the control; at 60 min, it was 1.4-fold that in the corresponding control. MG115 did not have any effect on radiolabeled apoA-I at the two time points examined (integrated optical density ratios of MG115 over control were 1.10 and 1.03 at 30 and 60 min, respectively).

Inhibition of ApoB Degradation by ATP Depletion

Both ubiquitination and degradation of ubiquitinated proteins by the proteasomes are ATP-dependent. If apoB degradation is mediated by the ubiquitin-proteasome pathway, depletion of ATP by uncoupling oxidative phosphorylation using dinitrophenol and inhibiting carbohydrate metabolism using 2-deoxyglucose would be expected to inhibit the degradation of apoB. HepG2 cells were labeled with L-[^{35}S]methionine for 30 min and then chased in α-MEM [containing unlabeled L-methionine (15 mg/L) but no glucose or fetal bovine serum] in the presence or absence of dinitrophenol 0.5 mM and 2-deoxyglucose 15 mM for 20, 45, 80, and 150 min, and immunochemical quantitation of radiolabeled apoB was performed as before. ATP depletion inhibited the degradation of apoB ($n = 3$, Student's t-test $p < 0.05$) compared to control at 80 and 150 min (Figure 1A). The amount of labeled apoB secreted into the chase medium was also monitored. ATP depletion inhibited secretion of labeled apoB compared to control (data not shown). Since the maximum secreted labeled apoB was about 10% of the maximum labeled apoB in the cell lysate, inhibition of apoB secretion by ATP depletion could not have accounted for the difference seen in the intracellular apoB between the ATP-depleted cells and the control. Therefore, intracellular apoB degradation is at least partly ATP-dependent and its inhibition by ATP depletion and by proteasome inhibitors supports the conclusion that proteasomes are involved in this degradative process.

Intracellular but Not Secreted ApoB is Ubiquitinated

To investigate whether intracellular apoB was ubiquitinated, we tested for the presence of ubiquitin immunoreactivity in immunoreactive apoB. We immunoprecipitated apoB from HepG2 cell lysates using a polyclonal goat anti-human apoB antiserum and fractionated the immunoprecipitate on SDS−PAGE gels. Western blotting was performed on these gels using a rabbit polyclonal anti-ubiquitin antiserum and immunoreactive ubiquitin was visualized by chemiluminescence using anti-rabbit IgG peroxidase conjugate for detection (Figure 2A). It is clear that ubiquitin immunoreactivity was present in the immunoprecipitated apoB and the relative amount of immunoreactivity is proportional to the amount of anti-apoB antiserum in the initial immunoprecipitation.

The specificity of the anti-ubiquitin immunoreactivity was demonstrated by the following controls (Figure 2B). The ubiquitin immunoreactivity was absent when either the anti-apoB antiserum (lane 1) or the cell lysate (lane 2) was omitted in the immunoprecipitation or when the anti-apoB antiserum was replaced by preimmune goat serum (lane 3). It was also absent when excess ubiquitin was present during incubation with anti-ubiquitin antiserum (bottom panel), indicating the specificity of the antiserum.

The major anti-ubiquitin immunoreactivity was a broad band with maximal intensity at a higher apparent molecular

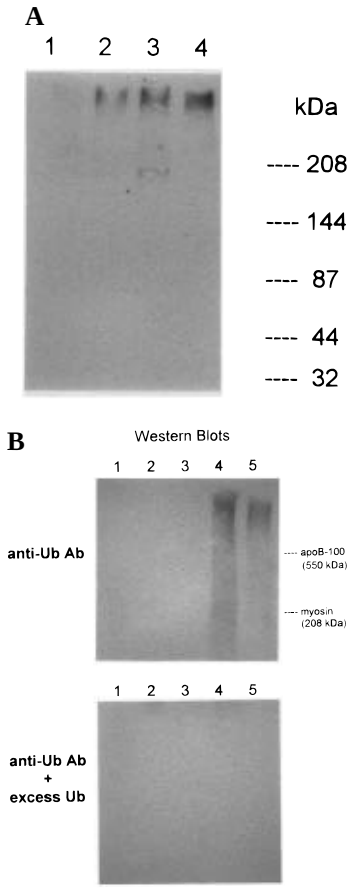

FIGURE 2: (A) Ubiquitin immunoreactivity in anti-apoB immunoprecipitates. Samples loaded in lanes 1−4 of the 4−15% gradient polyacrylamide gel were immunoprecipitates with 0.5, 1, 2.5, and 5 μL, respectively, of anti-apoB antiserum per mL of HepG2 cell lysate. The anti-ubiquitin immunoreactivity increased as the amount of immunoprecipitated apoB increased. (B) Specificity of ubiquitin immunoreactivity. The upper panel is an anti-ubiquitin Western blot of immunoprecipitated apoB fractionated on 4% SDS−PAGE. The lower panel is similar except that the antibody incubation was performed in the presence of excess ubiquitin. Upper and lower panels: lane 1, no anti-apoB antiserum added; lane 2, immunoprecipitation on lysis buffer instead of cell lysate; lane 3, anti-apoB antibody replaced by preimmune goat serum; lanes 4, 20 μL of anti-apoB antiserum; lane 5, 7 μL of anti-apoB serum. The anti-ubiquitin immunoreactivity was competitively blocked by excess ubiquitin, and it required the presence of HepG2 cell lysate and anti-apoB antibody in the immunoprecipitation.

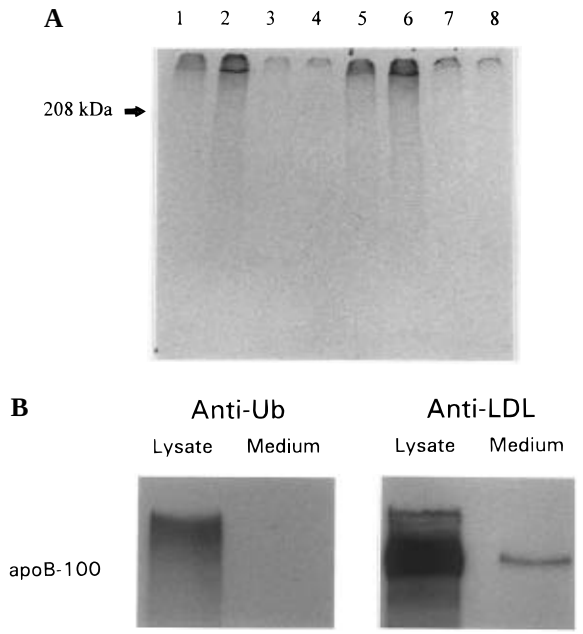

FIGURE 3: (A) An autoradiograph of L-[S^{35}]methionine-labeled proteins sequentially immunoprecipitated by anti-ubiquitin and anti-apoB. The proteins were fractionated on a 4−15% gradient gel. Lanes 1 and 2: 10 and 20 μL, respectively, of sequential (anti-apoB, then anti-ubiquitin) immunoprecipitate. Lanes 3 and 4: 10 and 20 μL, respectively, of sequential (anti-apoB, then no antibody) immunoprecipitate. Lanes 5 and 6: 10 and 20 μl, respectively, of sequential (anti-ubiquitin, then anti-apoB) immunoprecipitate. Lanes 7 and 8: 10 and 20 μL, respectively, of sequential (anti-ubiquitin, then no antibody) immunoprecipitate. Radiolabeled ubiquitinated apoB was seen in lanes 1, 2, 5, and 6. (B) Secreted apoB is not ubiquitinated. Immunoprecipitation with goat anti-human apoB anti-serum was performed using HepG2 cell lysate and incubation medium. In the left panel, a Western blot using rabbit anti-ubiquitin antiserum was shown. No anti-ubiquitin immunoreactivity was detected in the anti-apoB immunoprecipitate from the medium. In the right panel, a Western blot using rabbit anti-LDL antiserum was shown. A sharp band of apoB was detected in the medium.

weight than apoB-100 in both the 4−15% gradient gel (Figure 2A) and the 4% gel (Figure 2B). This was an expected finding because ubiquitin has a molecular weight of 8.5 kDa and the process of polyubiquitination involved multiple conjugation sites on the substrate molecule and ubiquitin polymers of variable lengths. Therefore, the polyubiquitinated apoB in the high molecular weight range (>700 kDa) contains large numbers (>100) of ubiquitin units per apoB molecule and thus exhibits much stronger immunoreactivity toward anti-ubiquitin antiserum than anti-apoB antiserum.

We also used an alternative method to demonstrate the presence of ubiquitinated apoB by sequentially immunoprecipitating radiolabeled protein with anti-apoB and anti-ubiquitin antibodies. Cultured HepG2 cells were incubated in L-[^{35}S]methionine (67 μCi/mL) in α-MEM for 2 h. After being washed with ice cold PBS, the cells were lysed with 2% cholate in HEPES-buffered saline with PMSF, and sequential immunoprecipitation was performed with pre-cleared lysates. The proteins from the second immunoprecipitation were analyzed by SDS−PAGE, and the radiolabeled proteins were visualized by autoradiography (Figure 3A). Radiolabeled protein products mainly of the size of apoB-100 or larger were detected in the double immunoprecipitate (lanes 1 and 2). Similar results were obtained when the order of the two antibodies added was reversed (lanes 5 and 6). The omission of the second antibody led to absence of radiolabeled material (lanes 3, 4, 7, and 8).

Ubiquitinated proteins are destined for degradation and are not secreted. Therefore, only unubiquitinated proteins would be expected to be secreted into the medium. We tested the culture medium for the presence of ubiquitinated apoB. HepG2 cells were incubated for 1 h with α-MEM with fetal bovine serum. At the end of incubation, immunoprecipitation with anti-apoB antibody was performed on the culture medium. The immunoprecipitate was analyzed by SDS−PAGE and Western blotting (Figure 3B). There was no detectable ubiquitin immunoreactivity associated with the immunoreactive apoB secreted into the medium (left panel). In contrast, intracellular ubiquitinated apoB immunoreactivity was clearly detected in the cell lysate. Again, we note that, as in Figure 2, the ubiquitin immunoreactivity associated with apoB migrates as a broad band with its highest intensity substantially higher than unubiquitinated apoB (Figure 3B, left panel). Immunoreactive apoB was

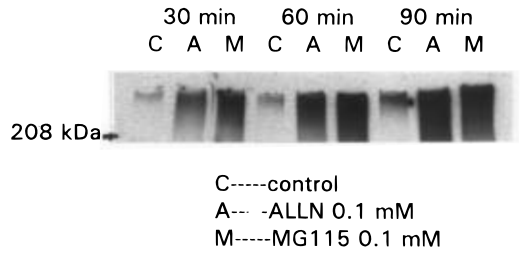

FIGURE 4: Proteasome inhibitors increased ubiquitinated apoB. HepG2 cells were incubated with 0−1 mM ALLN (A) 0.1 mM MG115 (M), or control medium (C) for 30, 60, or 90 min. The immunoprecipitated apoB was fractionated on a 4−15% gradient gel, and Western blot analysis was performed using anti-ubiquitin antiserum. Treatment with the proteasome inhibitors led to accumulation of ubiquitinated apoB.

detected in both cell lysate and the medium (Figure 3B, right panel). Therefore, only intracellular but not secreted apoB is ubiquitinated.

Inhibitors of Proteasomes Increase Intracellular Ubiquitinated ApoB

Involvement of the ubiquitin-proteasome pathway in the degradation of apoB would predict that inhibitors of proteasomes cause an accumulation of intracellular ubiquitinated apoB. We examined the time course study of the effects of ALLN and MG115 on the amount of intracellular ubiquitinated apoB. HepG2 cells were incubated in α-MEM (without fetal bovine serum) in the presence or absence of MG115 (0.1 mM) or ALLN (0.1 mM) for 30, 60, and 90 min. After incubation, the cells were lysed with lysis buffer and immunoprecipitation was performed with anti-apoB antibody. The immunoprecipitates were analyzed by SDS−PAGE and Western blot using anti-ubiquitin antibody (Figure 4). The amount of anti-ubiquitin immunoreactivity in apoB was increased in the presence of ALLN and MG115 compared with controls at each of the three time points examine, with amounts of anti-ubiquitin−positive immunoreactive apoB increasing over time. Therefore, these proteasome inhibitors led to the intracellular accumulation of ubiquitinated apoB.

DISCUSSION

Protein degradation by proteasomes has been reviewed recently (Jentsch & Schlenker, 1995; Ciehanover, 1994; Peters, 1994). Characteristics of protein degradation by proteasomes include ATP dependence, requirement of ubiquitin conjugation with few exceptions, and inhibition by specific peptide aldehydes such as MG115 and ALLN. An involvement of the ubiquitin-proteasome pathway in the intracellular degradation of apoB predicts the following: (i) apoB degradation is ATP-dependent, (ii) the intracellular apoB destined for this pathway is ubiquitinated whereas secreted apoB is not, and (iii) proteasome inhibitors will inhibit apoB degradation as well as lead to the accumulation of ubiquitinated apoB. We have examined these criteria and conclude that the intracellular degradation of apoB in HepG2 cells is at least in part mediated by the ubiquitin-proteasome pathway.

The ubiquitin-proteasome pathway is ATP-dependent (Jentsch & Schlenker, 1995; Ciehanover, 1994; Peters, 1994). In intact HepG2 cells, we found that intracellular apoB degradation is ATP-dependent; the depletion of ATP by dinitrophenol and 2-deoxyglucose inhibited the degradation of intracellular apoB. Our data corroborate and extend the findings by Adeli (1994) that ATP supplementation enhances, but cyanide plus fluoride treatment inhibits, the degradation of apoB in permeabilized HepG2 cells.

A major piece of evidence for the involvement of the ubiquitin-proteasome pathway in apoB degradation is the presence of ubiquitinated apoB in HepG2 cells. We have demonstrated the presence of ubiquitinated apoB by two different methods: (i) immunoprecipitation followed by Western blotting and (ii) sequential immunoprecipitation of radiolabeled proteins. The binding specificity of the anti-ubiquitin antibodies was confirmed by the various negative controls and complete competition by excess ubiquitin. The absence of any detectable ubiquitination of secreted apoB indicates that the intracellular ubiquitinated apoB is destined for degradation and is never secreted by the cells. The distribution of immunoreactivity to ubiquitin and apoB is different because near the molecular weight of apoB-100, few ubiquitin molecules are conjugated to an apoB molecule, whereas in the high molecular weight range, hundreds of ubiquitin molecules are conjugated to an apoB molecule. The electrophoretic appearance of ubiquitinated apoB in the form of a smear is precisely what one would predict based on the nature of ubiquitination (i.e., multiple conjugation sites with polyubiquitin of variable length) and the huge size of apoB. This characteristic appearance further supports the conclusion that intracellular apoB is ubiquitinated.

ALLN inhibits different types of proteolytic enzyme activities, including cathepsin B, cathepsin L, and calpains (Rock *et al*., 1994; Sasaki *et al*., 1990). Cathepsin B and cathepsin L are lysosomal enzymes; various laboratories have demonstrated that the degradation of apoB is extralysosomal (Adeli, 1994; Borchardt & Davis, 1987; Sato *et al*., 1990). Furthermore, depletion of intracellular calcium does not affect apoB degradation (Adeli, 1994), suggesting that calpains are not the major enzymes involved. Recently, ALLN is found to inhibit proteasomes (Rock *et al*., 1994; Lowe *et al*., 1995). MG115 is a more potent proteasome inhibitor than ALLN but is much less potent than the latter against cathepsin B and calpain. The higher degree of inhibition of apoB degradation exhibited by MG115 compared to ALLN (Figure 1) is consistent with an involvement of proteasomes in the intracellular degradation of apoB.

Our observation that MG115 and ALLN led to the accumulation of ubiquitinated apoB further strengthens the case for the inhibition of proteasomes as the mechanism by which these compounds inhibit apoB degradation. On the basis of our data, we conclude that the ubiquitin-proteasome pathway is at least one mechanism by which intracellular apoB is degraded.

More than 90% of the apoB associated with the rough and smooth ER fractions is membrane-bound, whereas approximately 67% and 85% of the apoB is in the lumen of the cis-enriched and trans-enriched Golgi fractions, respectively (Cartwright & Higgins, 1992). The known components of the ubiquitin-proteasome pathway are associated with the cytosol. One may raise the question of how apoB comes into contact with components of the ubiquitin-proteasome pathway. Although most secretory proteins are synthesized on ribosomes in association with the cytosolic surface of the rough ER and are rapidly translocated into the ER lumen (Ng & Walter, 1994), the majority of newly

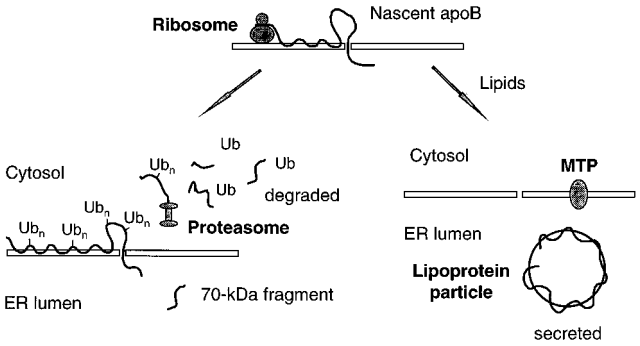

FIGURE 5: Model of apoB biogenesis and intracellular degradation in HepG2 cells. Ub, ubiquitin; MTP, microsomal triglycercide transfer protein; ER, endoplasmic reticulum. Translocation of nascent apoB across the ER membrane requires lipids and MTP. Incomplete translocation leaves the apoB accessible to ubiquitination and proteolysis by the proteasome pathway.

synthesized apoB remains membrane bound (Boren *et al*., 1990). Newly synthesized apoB is partially translocated with about 70 kDa of its N-terminus in the ER lumen and the remaining major portion exposed on the cytosolic side of the ER membrane (Furukawa *et al*., 1992; Davis *et al*., 1989, 1990), which would be accessible to the components of the ubiquitin-proteasome pathway. At least another membrane-spanning protein, the cystic fibrosis transmembrane conductance regulator, has been found to be degraded by the ubiquitin-proteasome pathway (Ward *et al*., 1995; Jensen *et al*., 1995).

On the basis of our results and other investigators' data, we present a model for the regulation of apoB degradation and secretion (Figure 5). ApoB is synthesized and partially translocated in the ER membrane. Adequate supply of lipid is required for efficient formation of lipoprotein particles. Microsomal triglyceride transfer protein (MTP) seems to be also involved in the lipidation of newly synthesized apoB (Gregg & Wetterau, 1994; Wu *et al*., 1996). Once apoB-containing particles mature in the ER lumen, the apoB becomes resistant to degradation and is destined for secretion. ApoB translocation is retarded when lipid supply is inadequate, and these partially translocated apoBs exposed on the cytosolic side of the ER membrane are susceptible to ubiquitination and subsequent degradation by proteasomes.

REFERENCES

Adeli, K. (1994) *J. Biol. Chem. 269*, 9166−9175.

Borchardt, R. A., & Davis, R. A. (1987) *J. Biol. Chem. 262*, 16394−16402.

Boren, J.; Wettesten, M., Sjoberg, A., Thorlin, T., Bondjers, G., Wiklund, O., & Olofsson, S. O. (1990) *J. Biol. Chem. 265*, 10556−10564.

Cartwright, I. J., & Higgins, J. A. (1992) *Biochem. J. 285*, 153−159.

Chan, L. (1992) *J. Biol. Chem. 267*, 25621−25624.

Ciehanover, A. (1994) *Cell 79*, 13−21.

Dashti, N., Williams, D. L., & Alaupovic, P. (1989) *J. Lipid Res. 30*, 1365−1373.

Davis, R. A., Prewett, A. B., Chan, D. C. F., Thompson, J. J., Borchardt, R. A., & Gallaher, W. R. (1989) *J. Lipid Res. 30*, 1185−1196.

Davis, R. A., Thrift, R. N., Wu, C. C., & Howell, K. E. (1990) *J. Biol. Chem. 265*, 10005−10011.

Dixon, J. L., Furukawa, S., & Ginsberg, H. N. (1991) *J. Biol. Chem. 266*, 5080−5086.

Furukawa, S., Sakata, N., Ginsberg, H. N., & Dixon, J. L. (1992), *J. Biol. Chem. 267*, 22630−22638.

Gregg, R. E., & Wetterau, J. R. (1994) *Cur. Opin. Lipidol. 5*, 81−86.

Ginsberg, H. N. (1995) *Cur. Opin. Lipidol. 6*, 275−280.

Ginsberg, H. N., Sakata, N., Lele, K. M., Zhou, M., & Wu, X. (1995) *Circulation 92*, I-228.

Jensen, T. J., Loo, M. A., Find, S., Williams, D. B., Goldberg, A. L., & Riordan, J. R. (1995) *Cell 83*, 129−135.

Jentsch, S., & Schlenker, S. (1995) *Cell 82*, 881−884.

Lowe, J., Stock, D., Jap, B., Zwickl, P., Baumeister, W., & Huber, R. (1995) *Science 268*, 533−539.

Ng, T. T. W., & Walter, P. (1994) *Curr. Opin. Cell Biol. 6*, 510−516.

Ou, W.-J., Cameron, P. H., Thomas, D. Y., & Bergeron, J. J. M. (1993) *Nature 364*, 771−776.

Peters, J. (1994) *Trends Biochem. Sci. 19*, 377−382.

Pullinger, C. R., North, J. D., Teng, B. B., Vicent, A., Rifici, A. E., de Brito, R., & Scott, J. (1989) *J. Lipid Res. 30*, 1065−1077.

Rock, K. L., Gramm, C., Rothstein, L., Clark, K., Stein, R., Dick, L., Hwang, D., & Goldberg, A. L. (1994) *Cell 78*, 761−771.

Sakata, N., Wu, X., Dixon, J. L., & Ginsberg, H. N. (1993), *J. Biol. Chem. 268*, 22967−22970.

Sasaki, T., Kishi, M., Saito, M., Tanaka, T., Higuchi, N., Kominami, E., Katunuma, N., & Murachi, T. (1990) *J. Enzyme Inhib. 3*, 195−201.

Sato, R., Imanaka, T., Takatsuki, A., & Takano, T. (1990) *J. Biol. Chem. 265*, 11880−11884.

Thrift, R. N., Drisko, J., Dueland, S. Trawick, J. D., & Davis, R. A. (1992), *Proc. Natl. Acad. Sci. U.S.A. 89*, 9161−9165.

Ward, C. L., Omura, S., & Kopito, R. R. (1995) *Cell 83*, 121−127.

Wu, X., Zhou, M., Huang, L.-S., Wetterau, J., & Ginsberg, H. N. (1996), *J. Biol. Chem. 271*, 10277−10281.

BI9618777