The Inhibition of Microsomal Triglyceride Transfer Protein Activity in Rat Hepatoma Cells Promotes Proteasomal and Nonproteasomal Degradation of Apoprotein B100†

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ABSTRACT: In the human hepatic cell line, HepG2, apolipoprotein B100 (apoB100) degradation is increased by inhibiting lipid transfer mediated by the microsomal triglyceride transfer protein (MTP) and is predominantly accomplished by the ubiquitin−proteasome pathway. In the current study, we determined whether this degradative pathway was restricted to HepG2 cells or was of more general importance in hepatic apoB100 metabolism. Rat hepatoma McArdle RH7777 cells (McA), compared to HepG2 cells, secrete a large fraction of apoB100 associated with VLDL particles, as does the normal mammalian liver. In McA cells studied under basal conditions, the proteasome inhibitor lactacystin (LAC) increased apoB100 recovery, indicating that the role of the proteasome in apoB100 metabolism is not restricted to HepG2 cells. When apoB100 lipidation was blocked by an inhibitor of MTP (MTPI), recovery of cellular apoB100 was markedly reduced, but LAC was only partially (∼50%) effective in reversing the induced degradation. This partial effectiveness of LAC may have represented either (1) incomplete inhibition by LAC of its preferred target, the chymotrypsin-like activity of the proteasome, (2) the presence of an apoB100 proteolytic activity of the proteasome resistant to LAC, or (3) a nonproteasomal proteolytic pathway of apoB100 degradation. By studying immunoisolated proteasomes and McA cells treated with LAC and/or MTPI and a variety of protease inhibitors, we determined that the proteasomal component of apoB100 degradation was entirely attributable to the chymotrypsin-like catalytic activity, but only accounted for part of apoB100 degradation induced by MTPI. The nonproteasomal apoB100 degradative pathway was nonlysosomal and resistant to E64d, DTT, and peptide aldehydes such as MG132 or ALLN but was partially sensitive to the serine protease inhibitor APMSF. Furthermore, when the protein trafficking inhibitor, brefeldin A, was used to block endoplasmic reticulum (ER) to Golgi transport in MTPI-treated McA cells, degradative activity resistant to LAC was increased, suggesting that the nonproteasomal pathway is associated with the ER.

Apolipoprotein B100 (apoB100)[1] is an extremely large protein (4536 amino acids) that is a required structural component of liver-derived very low density lipoproteins (VLDL) and low-density lipoproteins (LDL). Elevated plasma levels of these lipoproteins, particularly LDL, are associated with an increased risk of atherosclerosis, focusing attention on the regulation of hepatic apoB100 metabolism. From early studies by a number of laboratories (see refs *1*−*4* for recent reviews), it was concluded that the primary regulation of hepatic apoB100 production was by presecretory degradation. The nature of this degradative process has been studied intensively, and there is now accumulating evidence that there may be multiple and distinct pathways for the degradation of presecretory apoB100 (*1*).

We and others have previously shown in the human hepatocarcinoma cell line, HepG2 (*5*), that when the lipid−ligands that cotranslationally associate with apoB100 are not available in sufficient quantity to form the initial ("primordial") lipoprotein particle, ER-associated apoB100 is directed to proteasomal degradation (*5*−*7*). Although apoB100 ER-associated degradation (ERAD) has been firmly established in the HepG2 system, important remaining questions are whether this is a pathway of general importance in hepatic apoB100 metabolism and which of the multiple proteolytic activities of the proteasome accomplish the degradation.

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[1] Abbreviations: apoB100, apolipoprotein B100; Z, benzyloxycarbonyl; pAB, *p*-aminobenzoate; BFA, brefeldin A; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; LAC, lactacystin; MTP, microsomal triglyceride transfer protein; MTPI, MTP lipid transfer activity inhibitor; ER, endoplasmic reticulum; McA, rat hepatoma McArdle RH-7777 cells; HepG2, human hepatocarcinoma cells. Amino acids in the peptide aldehyde inhibitors and model substrates are indicated using standard single letter designations; CHO indicates the aldehyde derivative of the corresponding amino acid.

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There are two basic ways in HepG2 cells to promote apoB100-ERAD, namely, to limit the availability of the lipid−ligands for apoB100 by either decreasing their synthesis or decreasing their transfer to apoB100 by the microsomal triglyceride transfer protein (MTP). In HepG2 cells, decreased lipid synthesis can be conveniently accomplished by supplying a low concentration (<0.3 mM) of fatty acids in the medium. Notably, a number of other hepatic cell types are less dependent on exogenous fatty acids for lipid synthesis and can maintain an adequate level using other carbon sources (particularly glucose) in the medium. The development of highly specific and potent inhibitors of MTP (*8*), however, has made possible a way to decrease apoB100 production in hepatic cells independent of the type of cell and its lipid synthetic activity.

Because one major objective was to determine whether the proteasomal degradative pathway for apoB100 seen in HepG2 cells also functioned in other cells, we studied rat hepatoma cells (McArdle RH-7777; McA), which phenotypically resemble rat primary hepatocytes more closely than HepG2 cells. For example, they have less dependence on exogenous fatty acids to support lipid synthesis (*9*) and can assemble a higher proportion of apoB100 into lipoproteins of VLDL density (*10*). To reduce the availability of lipid ligands for apoB100 in McA cells and thereby recreate the conditions that induce apoB100 ERAD in HepG2 cells, McA cells were treated with an MTP inhibitor (*8*), and the synthesis and degradation of radiolabeled apoB100 were studied in the absence or presence of a variety of inhibitors of proteolysis.

Here we present evidence that, as in HepG2 cells, there is a proteasome-mediated ERAD pathway for apoB100 in the rat hepatoma cells. Furthermore, of the multiple proteolytic activities of the proteasome, we have established that it is the chymotrypsin-like activity that degrades apoB100. In contrast to the results for HepG2 cells (*5*), however, a substantial (~50%) fraction of the degradation induced by inhibiting MTP cannot be accounted for by proteasomal activity. The nonproteasomal activity was partially sensitive to APMSF and may be associated with the endoplasmic reticulum (ER). This finding shows that, in hepatic cell lines other than HepG2 cells, there are proteasomal and nonproteasomal mechanism(s) for the early and rapid degradation of apoB100 when the assembly of the primordial lipoprotein particles is impeded.

EXPERIMENTAL PROCEDURES

Materials. [^{35}S]Methionine/cysteine (specific activity >1000 Ci/mmol) was purchased from NEN Life Science Products (Boston, MA) as EXPRESS Protein Labeling Mix. Monospecific anti-rat apoB and apoE antisera, raised in rabbits, were provided by Dr. Janet Sparks (University of Rochester). MTP inhibitor (BMS-197636) was kindly provided by Drs. John Wetterau and David Gordon at Bristol-Myers/Squibb. Protein A−Sepharose CL-4B was from Pharmacia LKB Biotechnology Inc. (Uppsala, Sweden). Bovine serum albumin (BSA) was from Sigma (St. Louis, MO). Lactacystin was purchased from the laboratory of Dr. E. J. Corey, Department of Chemistry, Harvard University (Boston, MA). MG132 (Z-LLL-CHO) was purchased from Calbiochem (Cambridge, MA) and ALLN (*N*-acetyl-LLnL-CHO, calpain inhibitor I) from Boehringer Mannheim (Indianapolis, IN). Brefeldin A (BFA) was from Sigma. Z-LLF-CHO, Z-GPFL-CHO, Z-GGF-pAB, and the anti-proteasome antiserum were generous gifts from Dr. Marian Orlowski (Mount Sinai School of Medicine). PSI (Z-IE-(O-*t*Bu)-AL-CHO) was a generous gift of Dr. Sherwin Wilk (Mount Sinai School of Medicine). All other reagents were of the highest purity available and unless otherwise indicated were obtained from Sigma.

Cell Culture. McArdle RH-7777 (McA) cells were obtained from the American Type Culture Collection (Rockville, MD) and expressed predominantly apoB100. Cells were seeded onto 35 or 100 mm dishes coated with 0.01% poly-D-lysine (Sigma) and maintained in Dulbecco's minimum essential medium (DMEM) containing 100 units/mL penicillin, 100 μg/mL streptomycin, 10% fetal bovine serum (FBS), and 10% horse serum (HS). Before each experiment, the cells were washed with phosphate-buffered saline (PBS) and preincubated for 4 h with DMEM containing 0.2% BSA to avoid any possible carryover effects of the high-serum medium on apoB100 metabolism.

Metabolic Labeling. Previous studies with hepatocytes indicate that metabolic labeling for at least 2 h will achieve steady-state isotopic incorporation into apoB100 (*11*). Consequently, for steady-state labeling, the cells were incubated for 3 h with methionine/cysteine-free DMEM containing 100 μCi/mL [^{35}S]methionine/cysteine, 0.5% FBS, 0.5% HS, and various additions as described in the figure legends. For pulse labeling and pulse−chase experiments, the cells were first incubated with treatment medium (methionine- and cysteine-free DMEM containing various additives as indicated in the figure legends) for 1 h, and then 200 μCi/mL [^{35}S]-methionine/cysteine was added to the medium. The incubation was continued for 15 min, followed by incubation for various times in DMEM containing 10 mM unlabeled methionine, 3 mM unlabeled cysteine, and additives described in the figure legends.

Immunoprecipitation (IP) and Quantification of Labeled ApoB100. After metabolic labeling, the media were collected, and the cells were washed with cold PBS. The denaturing IP was performed as previously described (*12*, *13*). Cells were lysed in detergent buffer (0.01 M sodium phosphate, pH 7.4, 0.125 M sodium chloride, 1% Triton X-100, 1% sodium deoxycholate, and 1% lithium dodecyl sulfate) containing protease inhibitors added just before use (2.0 mM benzamidine, 1.0 mM phenylmethanesulfonyl fluoride (PMSF), 20 μg/mL aprotinin, and 20 μg/mL leupeptin). After 30 min incubation at room temperature with shaking, the plates were scraped, and lysates were transferred into tubes and heated at 80 °C for 1 h and then heated at 95 °C for 5 min.

Rabbit anti-rat apoB antiserum was used to immunoprecipitate apoB100 from the conditioned media or from the cell lysates, and protein A−Sepharose CL-4B was used to bind antibody−antigen complexes. After the complexes were precipitated by centrifugation and the supernatants were removed, the pellets were washed (three to five times) with NET buffer (50 mM Tris, pH 7.4, 150 mM sodium chloride, 5 mM EDTA, 0.5% Triton X-100, and 0.05% SDS). The proteins bound to the beads were extracted by heating the samples at 95 °C for 5 min in sample buffer (62.5 mM Tris-HCl, pH 6.8, 8 M urea, 20% glycerol, 5% SDS, and 25 mg/mL DTT) and resolved by 3.5−15% gradient SDS−PAGE.

The ^{35}S-labeled protein bands were quantified by either densitometry or phosphorimager scanning. We have previously shown that quantitative apoB100 recovery is achieved under these conditions (*13*).

Immunoprecipitation of Proteasomes and Measurement of Chymotrypsin-like Activity. Cells were grown to 90% confluence in 100 mm culture dishes, then incubated for 2 h in media prepared as above, but were supplemented with lactacystin (in DMSO) at the indicated concentrations or with DMSO alone. Cell monolayers were washed with PBS and covered with 0.25 mL of 50 mM Tris-HCl, pH 8.0, supplemented with PMSF (1 mM) and E-64 (10 μM). Cells were then detached with a rubber policeman and homogenized by passage through 25 G and 30 G needles, 10 passages for each. Lysates were centrifuged for 5 min at 5000*g* to remove insoluble debris.

To isolate proteasomes from the supernatants, 500 μL of 5% protein A−Sepharose in PBS was added to 10 μL of rabbit anti-proteasome antiserum, and the solution was mixed gently at room temperature for 60 min. The Sepharose beads were washed five times with PBS and resuspended in 500 μL of PBS, and aliquots of supernatant containing equal amounts of protein (as measured by Lowry assay) were added. Typically, approximately 100 μL of supernatant was used. After being mixed gently at room temperature for 60 min, beads were washed with PBS four times and then twice with 50 mM Tris-HCl (pH 8.0) to remove sodium chloride, which is known to depress peptidase activities of the proteasome (*14*). After all liquid was removed, the beads were covered with 96 μL of 50 mM Tris-HCl to which was added 2 μL of DMSO. Tubes were warmed to 37 °C for 5 min, and 2 μL of 50 mM Z-GGF-pAB (final concentration 1 mM), a substrate for the chymotrypsin-like activity of the proteasome (*15*), was added. Reactions were incubated at 37 °C for 60 min and were stopped by the addition of 50% trichloroacetic acid. Activity was determined spectrophotometrically after diazotization.

Statistical Analysis. Equality of means was determined by ANOVA, with multiple comparisons analyzed by the Bonferroni test (GraphPad Prism Software, San Diego).

RESULTS

The Proteasome Participates in the Degradation of ApoB100 in Rat Hepatoma McA Cells. McA cells in serum-free medium were metabolically labeled with [^{35}S]methionine/cysteine for 3 h with or without the proteasome inhibitor lactacystin (LAC, 10 μM). ApoB100 was then immunoprecipitated, resolved by SDS−PAGE, and quantified by a phosphorimager (Figure 1). Note that LAC significantly increased apoB100 recovery from both the media and cells, consistent with the presence of proteasomal degradation under control (C) conditions. Additional wells were treated with the MTP inhibitor (MTPI, 0.1 μM) which, as expected, virtually abolished apoB100 secretion into the media and significantly decreased apoB100 recovery from cells. LAC significantly increased apoB100 recovery in the MTPI-treated cells, though the apoB100 recovery with LAC alone was far higher than with LAC + MTPI. Thus, the degradation induced by MTPI was only partly reversed (by ~50%) in cells treated with LAC.

Several additional experiments were performed to ensure that the results were not attributable to nonspecific effects

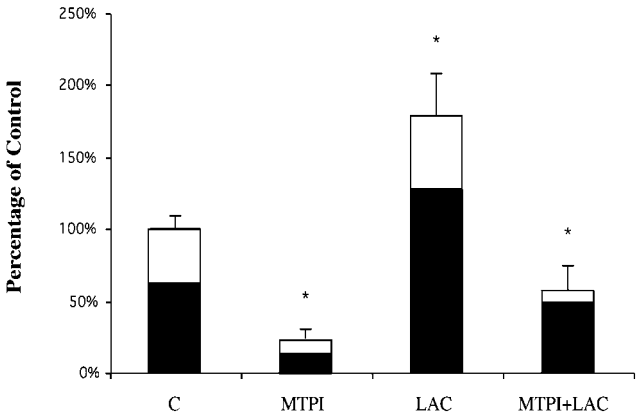

FIGURE 1: Effects of proteasome and MTP inhibition on the recovery of apoB100 in rat hepatoma McA cells. McA cells were labeled with [^{35}S]methionine and cysteine for 3 h with or without LAC (10 μM), MTPI (0.1 μM), or DMSO (control, C). ApoB100 was then immunoprecipitated from cell lysates (open portion of bar) and medium (closed portion), separated by SDS−PAGE, and quantified by a phosphorimager. Data are means ± SEM of eight independent determinations. *, $P \leq 0.01$ compared to control.

of LAC or the MTPI. Neither LAC nor MTPI affected total protein synthesis or the synthesis or secretion of apolipoprotein E (data not shown). Apolipoprotein E has been shown to be secreted independently of intracellular lipid transport and lipoprotein assembly in a variety of hepatic cell types, including McA (*16*, *17*). Thus, these findings indicate that the effects of LAC and MTPI were selective for apoB100.

To confirm that, in the absence of the MTPI, the effect of LAC on apoB100 was due to decreased apoB100 degradation and not to increased synthesis, we performed pulse−chase studies (Figure 2A). Under the control conditions, inhibition of the proteasome indeed delayed the disappearance of apoB100 (e.g., compare the 90 min lysate lanes in Figure 2A). To confirm that the decreased recovery of apoB100 in MTPI-treated cells was also due to increased degradation and that the proteasome was involved, additional pulse−chase experiments were performed. As shown in Figure 2B, addition of the MTPI markedly accelerated the disappearance of apoB100 (compare 0 and 30 min time points in Figure 2, panels A and B). In addition, LAC caused a significant increase in recovery of cellular apoB100 from MTPI-treated cells (Figure 2B). The MTPI almost completely abolished the appearance of apoB100 in the medium, and there was no noticeable effect of LAC on apoB100 accumulation in the media (data not shown).

Because we have previously reported that the inhibition of MTP in HepG2 cells decreased net apoB100 synthesis, either by promoting cotranslational degradation (*12*) or by decreasing apoB100 mRNA translation (*18*), pulse-labeling studies were also performed (Figure 2C). These showed that the MTPI reduced apoB100 net synthesis by ~25%. Recovery of apoB100 from cells was not significantly reduced by LAC, indicating that proteasomal inhibition had no effect on net apoB synthesis. LAC had no effect on apoB100 synthesis in the presence of MTPI, implying that the MTPI effect was probably due to the decrease in apoB mRNA translation we previously reported (*18*). Thus, in contrast to HepG2 cells, in McA cells in the basal or MTP-inhibited states, cotranslational proteasomal degradation of apoB is not a prominent feature of apoB100 metabolism [these results

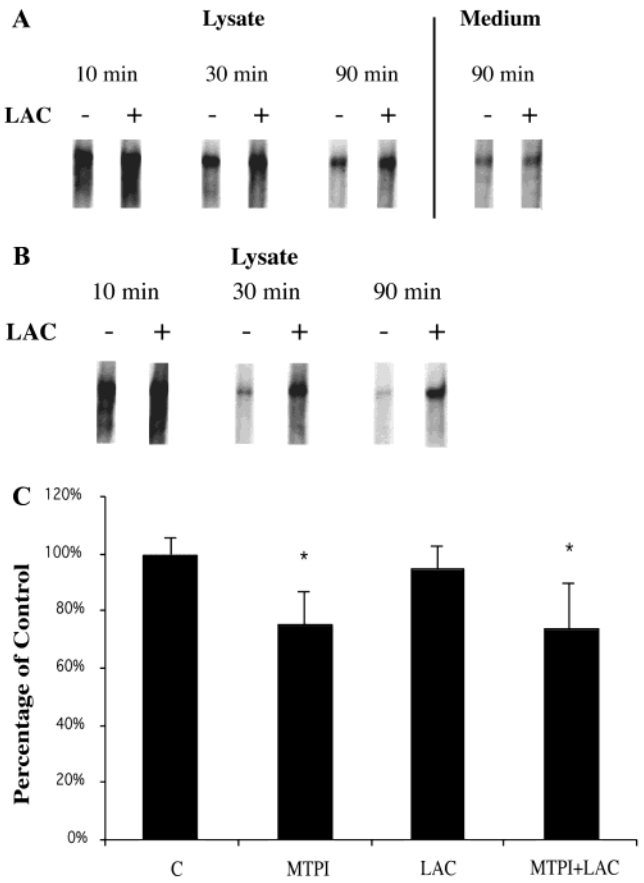

FIGURE 2: Pulse−chase analysis of the effects of lactacystin and MTP inhibition on apoB100 synthesis and degradation. Panel A: Pulse−chase analysis of the effect of LAC on apoB100 degradation. Cells were pretreated for 1 h with LAC (10 μM, "+" lanes) or DMSO (control, "−" lanes), pulsed for 15 min with [^{35}S]methionine and cysteine, and then chased in media containing unlabeled methionine and cysteine for the times indicated. Cellular apoB100 was then isolated and quantifed as above. Panel B: Pulse−chase analysis of the effect of LAC (10 μM) on cellular apoB100 recovery in MTPI (0.1 μM) treated cells. Experiments were performed as above. Essentially no apoB100 was present in media, and consequently only the lysate data are shown. Panel C: Effect of LAC or MTPI on apoB100 synthesis. Cells were preincubated with LAC (10 μM), MTPI (0.1 μM), or DMSO for 1 h, pulsed for 15 min with [^{35}S]methionine and cysteine, and then chased for 10 min with media containing unlabeled methionine and cysteine. Cellular apoB100 was then isolated and quantified as above. Values are means ± SEM for seven determinations. *, $P < 0.01$ versus control.

are also similar to those obtained in rat primary hepatocytes (J. D. Sparks, University of Rochester, personal communication) and in McA studies performed by the laboratory of Z. Yao (*8*)]. Taken together, the findings summarized in Figures 1 and 2 indicate that there is a proteasomal pathway for apoB degradation in McA cells but that the increased degradation of apoB in MTPI-treated cells cannot be completely reversed by a standard proteasomal inhibitor.

Proteasomal Degradation of ApoB100 Is Accomplished by the Chymotrypsin-like Activity. The proteasome has four well-documented catalytic activities (*19*). At the concentrations typically used (10−20 μM), LAC is most active as an inhibitor of the chymotrypsin-like activity (*19, 20*), being up to 50-fold less active toward the other activities. We therefore considered whether its relative ineffectiveness in reversing MTPI-induced degradation was due to the involvement of another catalytic activity of the proteasome, such as the trypsin-like, branched-chain amino acid (BrAAP) or the peptidylglutamyl-peptide hydrolyzing (PGPH) activities, and explored this possibility by using inhibitors selective for specific proteasome activities.

We first tested the effects on MTPI-induced apoB100 degradation of Z-GPFL-CHO, a cell-permeable peptide aldehyde inhibitor selective for the PGPH and BrAAP activities with a K_i value of ∼1 μM (*21*). While this mechanistic class of inhibitors also blocks other types of proteases besides the proteasome, a negative result would still indicate that these activities of the proteasome were noncontributory. As shown in Figure 3A (Z-GPFL-CHO is indicated by "BraapI"), this inhibitor had no effect on apoB100 levels, when added either by itself or in combination with LAC ($P > 0.05$). Thus, the inability of this inhibitor to affect apoB100 recovery argues against involvement of the BrAAP or PGPH activities in MTPI-induced apoB100 degradation. This conclusion was further supported by experiments with the peptide aldehyde inhibitor MG132, which inhibits chymotrypsin-like and BrAAP activities with equal effectiveness (*19*). MG132 was not significantly more effective than LAC in increasing apoB100 recovery from MTPI-treated cells (Figure 3A). This indicated once again that extensive inhibition of both proteasomal activities was no more effective than the inhibition of chymotrypsin-like activity alone.

Direct evaluation of the participation of the trypsin-like activity was difficult because leupeptin, one of the most selective inhibitors of this activity, crosses cell membranes poorly. Nevertheless, we still tried it in an experiment similar to the one described above for the BrAAP inhibitor and did not observe any changes in apoB100 recovery (data not shown). To confirm this result, we exploited the fact that either LAC or MG132 effectively inhibits all proteasome activities at concentrations of 40 μM (*19, 22*; C. Cardozo, unpublished). Thus, incubation of McA cells with increasing concentrations of either proteasome inhibitor would test the contribution to apoB100 degradation of the trypsin-like and other non-chymotrypsin-like activities. As shown in Figure 3B, protection against MTPI-induced apoB100 degradation was achieved at 5−10 μM concentrations of either inhibitor [LAC (lactacystin), MG (MG132)], i.e., concentrations at which LAC inactivates primarily the chymotrypsin-like activity and MG132 inhibits primarily the chymotrypsin-like and BrAAP activities. Notably, no further protection against MTPI-induced apoB100 degradation was observed at higher concentrations of these inhibitors, including the 40 μM concentration known to inhibit all proteasome activities.

A further test of the specific proteasomal activity involved in apoB100 degradation was afforded by the availability of Z-LLF-CHO and of PSI, inhibitors selective for the chymotrypsin-like activity with little activity toward the others (*19, 23*). If apoB100 degradation relied on components in addition to the chymotrypsin-like activity, then such highly selective inhibitors should be less effective when compared to the more broadly acting inhibitors LAC and MG132. However, as shown in Figure 3C, the two selective inhibitors were as effective as LAC in blocking MTPI-induced apoB100 degradation. Taken together, the findings summarized in Figure 3 indicate that the chymotrypsin-like activity is the primary proteasomal activity involved in apoB100 degradation.

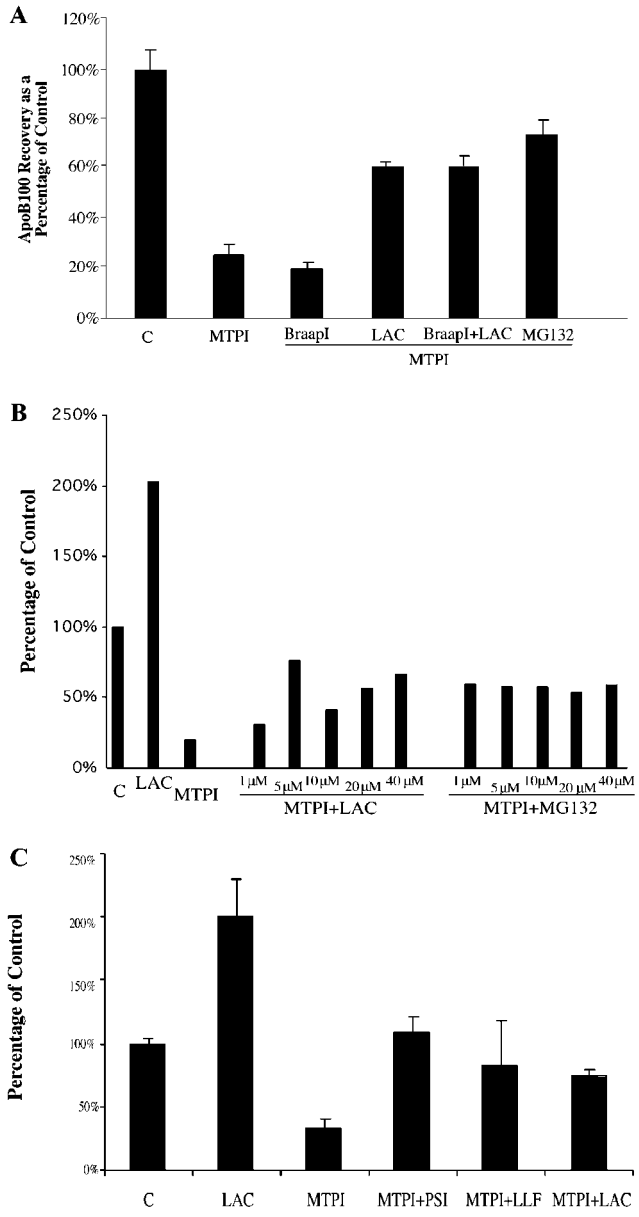

FIGURE 3: Role of different proteasome activities in apoB100 degradation. Experiments were carried out as described in the legend to Figure 1. Panel A: Role of the BrAAP and PGPH components. Cellular apoB100 was isolated and quantitated as described in the legend to Figure 1 after exposure of cells to LAC (10 μM), Z-GPFL-CHO (40 μM), or MTPI (0.1 μM), either alone or in combination as indicated in the figure. Control cells (C) were exposed to an equal amount of the carrier, DMSO. Data are mean values for at least five determinations. Panel B: Effects of various concentrations of LAC or MG132 on recovery of cellular apoB in the presence of MTPI (0.1 μM). Data are the average of two determinations from a representative experiment, which was repeated once again with the same pattern of results. Panel C: Effects on recovery of cellular apoB100 of the chymotrypsin-like activity inhibitors Z-LLF-CHO (10 μM) or PSI (20 μM) as compared to those of LAC (20 μM). Data are means ± SEM for five determinations.

ApoB100 Degradation Involves both Proteasomal and Nonproteasomal Pathways. The involvement of the chymotrypsin-like activity in apoB100 degradation did not explain the inability of LAC or MG132 to completely reverse MTPI-induced degradation, unless neither inhibitor completely blocked this particular activity of the proteasome under the conditions of our experiments. To test this possibility, we immunoisolated proteasomes from cells treated with LAC and measured the residual chymotrypsin-like activity (Figure 4). As shown, treatment of the cells with concentrations of LAC of 5 μM resulted in more than 90% loss of chymotrypsin-like activity. Consistent with Figure 3B, higher concentrations of LAC had no appreciably greater inhibitory effect. Thus, the finding that LAC blocks only about one-half of the MTPI-induced apoB100 degradation cannot be explained by partial inhibition of the chymotrypsin-like activity of proteasomes in McA cells. Therefore, the data in Figures 3 and 4 are best explained by there being a nonproteasomal pathway for apoB100 degradation induced by MTPI in McA cells.

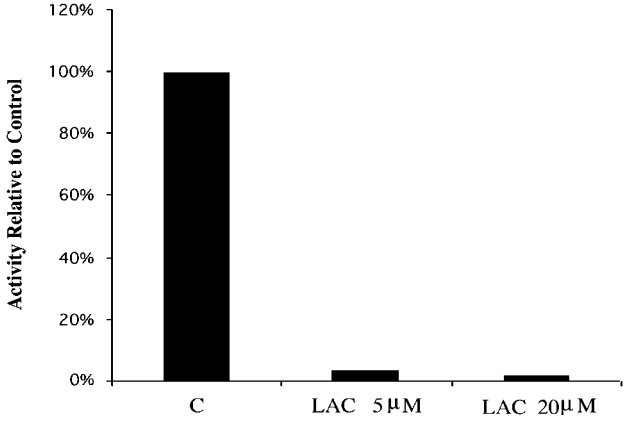

FIGURE 4: Chymotrypsin-like activity of proteasomes isolated from cells exposed to LAC or DMSO. Proteasomes were isolated by immunoprecipitation from cells exposed for 2 h either to LAC (dissolved in DMSO) at the indicated concentrations or to an equal volume of DMSO. Chymotrypsin-like activity was measured using the chromogenic substrate Z-GGF-pAB. Activities are expressed in arbitrary units, where activity of the proteasome from DMSO-treated cells was set to 100. Results from a representative experiment are shown. Data are the averages of two replicate determinations.

The Nonproteasomal Pathway Is ER-Associated. A number of potential candidates for the nonproteasomal component of apoB100 degradation have been suggested and include the lysosome (*24*), the ER p60 and p72 proteins (*25*), and a DTT-inhibited ER proteolytic activity (*26*). Thus, we tested the effects of inhibitors of these proteolytic systems [ammonium chloride and chloroquine, inhibitors of lysosomal function; E64d, a cell-permeable inhibitor of many cysteine proteinases, including ER p60 and p72 (*27*, *28*); and DTT]. None of these agents affects proteasomal activity (*29*, *30*; C. Cardozo, unpublished), and under our experimental conditions, the lysosomal inhibitors (ammonium chloride or chloroquine) and E64d were functionally effective as assessed by their blocking greater than 90% of the degradation of ^{125}I-labeled LDL and Z-LLR-2NA (*31*), respectively. The effectiveness of DTT was indicated by its ability to reduce by more than 50% the cellular recovery of the folded form of albumin (*32*, *33*) under our experimental conditions. On the basis of the results summarized in Figure 5 and results for DTT (M. Pan, H. Wang, and E. Fisher, unpublished), none of these agents significantly ($P > 0.05$) increased apoB100 recovery in MTPI-treated cells, leaving open the relationship among the nonproteasomal pathways described in this report and in the above cited references (see Discussion).

Another approach to determining the nature of the nonproteasomal pathway for the MTPI-induced catabolism is

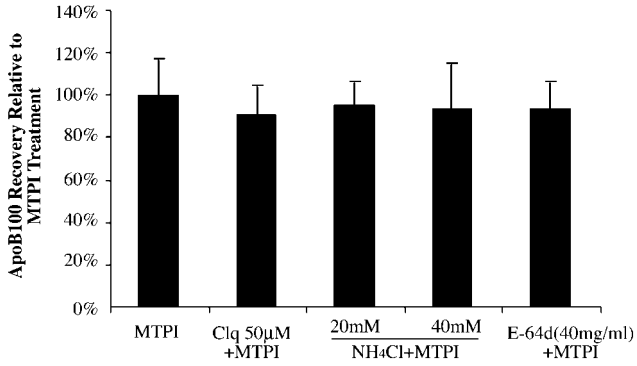

FIGURE 5: Effects of various inhibitors on apoB100 recovery in MTPI-treated cells. Experiments were carried out as described in the legend to Figure 3, using inhibitors of nonproteasomal pathways of protein degradation at the concentrations indicated (Clq, chloroquine). Total apoB100 recovery (media + cellular) was quantified by phosphorimaging or densitometry scanning. Data are means ± SEM for four determinations.

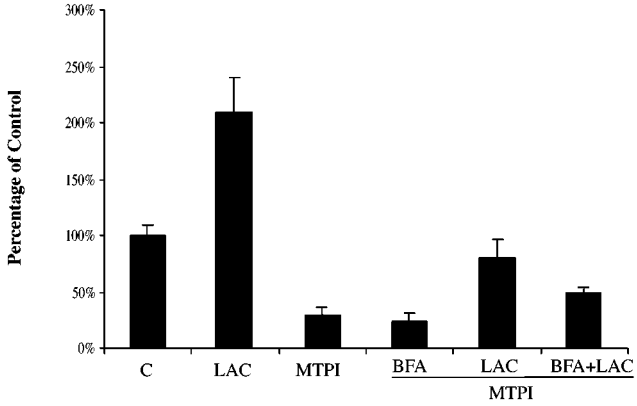

FIGURE 6: Effects of brefeldin A (BFA) on apoB100 recovery in MTPI-treated cells. The basic experimental protocol was as described in the legend to Figure 1. Cellular apoB100 was isolated and quantifed after exposure of cells to BFA (4 μg/mL), LAC (10 μM), or MTPI (0.1 μM). Control cells (C) were exposed to an equal amount of the carrier, DMSO. Data are means ± SEM for three determinations.

to localize the relevant cellular compartment in which apoB100 becomes targeted to it. We hypothesized that, in the presence of MTPI, apoB100 would be incompletely lipidated and have an abnormal conformation, causing it to be recognized by the ER quality control system, which would result in retention (*34*) and targeting to nonproteasomal proteolysis. To test this, McA cells were treated with brefeldin A (BFA), an inhibitor of ER to Golgi traffic. If the pathway were active after apoB100 left the ER, then it should be protected from degradation by BFA. As shown in Figure 6, however, BFA increased apoB100 degradation induced by MTPI. Again, the degradation was only partially reversed by LAC. Thus, the data are most consistent with the pathway for nonproteasomal MTPI-induced degradation beginning in the ER.

The Nonproteasomal Pathway Is Inhibited by APMSF. Our studies thus far have shown that the nonproteasomal pathway is not blocked by cellular poisons such as chloroquine or by an inhibitor of many common cysteine proteases (E64d). In addition, our findings show that none of the peptide aldehyde inhibitors tested is more effective than LAC in blocking MTPI-induced apoB100 degradation, indicating that the proteolytic system is resistant to these agents as well. To

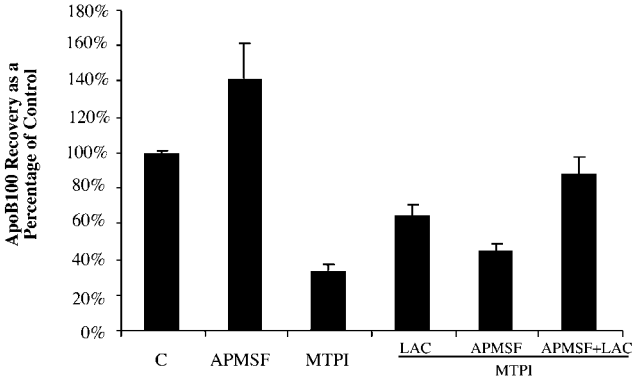

FIGURE 7: Effects of APMSF on apoB100 degradation induced by MTPI. Cellular apoB100 was isolated and quantified after exposure of cells to LAC (10 μM) or MTPI (0.1 μM) as described in the legend to Figure 1. Control cells (C) were exposed to an equal amount of the carrier, DMSO. In addition, some wells contained APMSF. The inhibitor was added to cell culture media three times during the experiments because of the short half-life at neutral pH (less than 10 min). Thus, APMSF (100 μM) was added 15 min before the start of pulse labeling with [^{35}S]methionine and cysteine and again at the start of pulse labeling and 30 min thereafter. Data are means ± SEM for four determinations.

gain further insights into the nature of the nonproteasomal pathway, we tested APMSF, an irreversible inhibitor of serine proteases. As shown in Figure 7, exposure of MTPI-treated cells to APMSF increased recovery of cellular apoB100, and effects of APMSF were additive to those of LAC (multiple comparison statistical analysis showed that the apoB100 recovery in the MTPI-treated groups was in the order of MTPI < MTPI + LAC = MTPI + APMSF < MTPI + APMSF + LAC). APMSF, however, had no appreciable effect on recovery of apoB100 from media (data not shown), consistent with the results of other studies which have shown that, in the absence of sufficient lipidation, apoB100 "rescued" from proteolysis still is not secreted appreciably (e.g., ref *5*).

These findings indicate that the nonproteasomal pathway in McA cells is sensitive to APMSF. Given the brefeldin A (BFA) results above, in which nonproteasomal degradation was increased, the inhibitor would also be expected to protect against apoB100 degradation when ER-Golgi transport is blocked. This was indeed the case; when cells were incubated with both MTPI and BFA, APMSF increased recovery of cellular apoB100 in a manner that was additive to the effect of LAC (recovery values: MTPI/BFA, 20%; MTPI/BFA/APMSF, 32%; MTPI/BFA/LAC, 45%; MTPI/BFA/LAC/APMSF, 58%; mean values based on two separate determinations).

DISCUSSION

An important component of the regulation of apoB100 secretion from hepatic cells is the level of presecretory, intracellular degradation (see refs *2*−*4* for recent reviews). The characteristics of apoB100 degradation have been explored most comprehensively in the human hepatocarcinoma cell line, HepG2. For example, in lipid-deprived or MTP-inhibited HepG2 cells, it has been shown that the proteasome mediates essentially all apoB100 degradation during and after its translation (*5*, *7*, *12*, *35*). Additional studies have suggested that nascent apoB100 could assume a bitopic orientation with respect to the ER membrane, with

sufficient cytosolic exposure of the protein to allow its interaction with cytosolic factors, such as hsp70, ubiquitin conjugating enzymes, and proteasomes (*12*, *36*, *37*). Because HepG2 cells have features distinct from those of mammalian liver, in particular, a severe dependence on exogenous fatty acids to maintain lipid synthesis and a limited ability to fully lipidate apoB100 to form VLDL particles, our initial interest was in determining whether the contribution of the proteasome to hepatic apoB100 degradation was general or was restricted to this particular cell type. The rat hepatoma McArdle RH-7777 is well suited for this purpose. As we have previously shown, relative to HepG2, McA cells are less dependent on exogenous fatty acids to support apoB−lipoprotein assembly and secretion (*9*) and are better able to form VLDL particles (*10*). In addition, by using an inhibitor of MTP, we could create a deficiency of "lipid−ligands" for nascent apoB100, which would be expected to induce proteasomal degradation, based on studies of HepG2 cells (e.g., refs *7* and *38*).

There are three major findings from our studies. The first is that degradation of apoB100 by the proteasome is not confined to HepG2 cells. The second is that, of the multiple catalytic activities of the proteasome, it is only the chymotrypsin-like activity which mediates apoB100 degradation. The third is that the apoB100 degradation induced by inhibiting MTP activity in rat hepatoma cells has a large, nonproteasomal, nonlysosomal component that may be associated with the ER. Each finding will be discussed in turn, below.

ApoB100 in Rat Hepatoma Cells Can Be Degraded by the Proteasome. Recent results in an intestinal cell model (Caco2 cells), in which no evidence for proteasomal degradation of apoB100 was obtained, have raised the issue of the general importance of proteasomal degradation in apoB100 metabolism (*39*). By contrast, in the studies presented above (Figures 1 and 3B), the recovery of apoB100 from McA cells under basal or MTP-inhibited conditions was substantially increased by two inhibitors of proteasomal activity, LAC and MG132. This increased recovery was attributable to decreased degradation, based on pulse−chase studies (Figure 2). While MG132 also inhibits some nonproteasomal serine and cysteine proteinases, LAC is a highly selective, irreversible inhibitor of the proteasome (*20*). The finding that LAC and MG132 had very similar effects on apoB100 recovery (Figure 2B), therefore, suggested that only the proteasome-specific actions of MG132 affected apoB100 degradation.

While our data demonstrate that the role of the proteasome in apoB100 degradation is not restricted to HepG2 cells, our findings also revealed some significant differences between these cells and McA cells. Relative to HepG2 cells (*7*, *12*), there was less evidence of cotranslational proteasomal degradation of apoB100 in McA cells (Figure 2C). Also in contrast to HepG2 cells, in which the inhibition of MTP decreases the apparent synthesis of apoB100 by delaying its translation (*18*) and promoting cotranslational proteasomal degradation (*12*), in McA cells, MTP inhibition was associated with only a mild effect on apoB100 synthesis (Figure 2C). The observation that for McA cells cotranslational proteasomal degradation of apoB100 is not a prominent feature of apoB100 metabolism is similar to those recently obtained in rat primary hepatocytes (J. D. Sparks, personal communication) and in other studies of McA cells (*8*).

The Proteasomal Degradation of ApoB100 Is Accomplished Primarily by Chymotrypsin-like Activity. Proteasomes express at least four peptidase activities. These include a chymotrypsin-like activity cleaving after hydrophobic residues, a trypsin-like activity cleaving after basic residues, a peptidylglutamyl-peptide hydrolyzing (PGPH) activity cleaving after acidic residues, and a branched-chain amino acid preferring (BrAAP) activity cleaving bonds after branched-chain residues such as leucine or isoleucine (*14*, *40*). It is well established that, in both yeast and mammalian systems, the chymotrypsin-like, trypsin-like, and PGPH activities are catalyzed by different active sites localized to different subunits (*41*−*43*). The identity of the subunit(s) catalyzing the BrAAP activity remains uncertain (*19*, *44*, *45*). Studies of yeast mutants with depression or loss of specific peptidase activities implicated the chymotrypsin-like activity as the major factor in the proteasome-dependent degradation of cellular proteins (*42*−*44*).

Our study utilized several peptide aldehyde inhibitors to probe the nature of the activity responsible for apoB100 degradation. While these types of inhibitors are not specific for the proteasome, failure of their effectiveness would still indicate that the targeted catalytic activities of proteasome were not involved in apoB100 degradation. In either control or MTPI-treated cells, however, we failed to find that any of these agents increased recovery beyond that observed with LAC alone.

In contrast, we demonstrate multiple lines of evidence to support a primary role of the chymotrypsin-like activity of the proteasome: (1) highly selective inhibitors of the chymotrypsin-like activity (e.g., PSI) were as effective as less selective agents including LAC and MG132 (Figure 3C); (2) an inhibitor of the BrAAP activity, which has been suggested to be a major proteolytic activity in some settings (*40*), had no effect on apoB100 recovery; and (3) LAC and MG132, less specific at high concentrations, were no more effective than at low concentrations, when particularly LAC primarily inactivates the chymotrypsin-like activity of the proteasome. It should be noted that our experiments tested for the activity involved in initial cleavages of apoB100 and that other activities may well play important roles in subsequent degradation of smaller fragments as has been suggested in other contexts (*40*, *42*, *43*).

It is interesting to compare our results to those recently reported by Skach and colleagues (*46*). Using a cell-free system, they demonstrated that the proteasomal degradation of CFTR utilized multiple catalytic activities (chymotrypsin-like, trypsin-like, and PGPH), and they estimated that only ∼50% of CFTR proteasomal degradation was attributable to the chymotrypsin-like activity. Thus, the catalytic activities mediating proteasomal degradation are likely to depend greatly on properties of the substrate protein.

ApoB100 Degradation Induced by the Inhibition of MTP Activity in McA Cells Involves Nonproteasomal, Nonlysosomal Proteolytic Activities. Our findings indicate the existence of a nonproteasomal pathway for apoB degradation that is induced in MTPI-treated McA cells. The strongest evidence comes from our findings that even high concentrations of LAC or MG132 could block only 50% of apoB100 degradation (Figures 1 and 3B), even though proteasomal activity is essentially abolished under these conditions.

Additional support was provided by the fact that the chymotrypsin-like activity, shown to be responsible for apoB100 cleavage, was almost completely inactivated by LAC (Figure 3B) even at low concentrations, while apoB100 degradation was only partially inhibited under these conditions. The nonproteasomal pathway was not blocked by inhibitors of lysosomal function or by inhibitors of lysosomal proteases such E64d (Figure 5). In addition to inactivating lysosomal cysteine proteases such as cathepsins B and L, E64d also irreversibly inhibits calpains and a number of other cysteine proteases, excluding their involvement in this pathway.

The protease(s) in this pathway was (were) at least partly inhibited by APMSF. It should be noted that APMSF does not inhibit any activity of the proteasome (C. Cardozo, data not shown). This inhibitor is a mechanism-based irreversible inhibitor of serine proteases, suggesting that the protease(s) mediating the nonproteasomal MTPI-induced degradation observed in our study is (are) a member of this protease family. Moreover, none of the peptide aldehydes used were unable to block the pathway, though all are capable of inhibiting selected serine proteases by acting as transition state analogues. One interpretation of these differences in sensitivity to the various inhibitors used is that the specificity of the enzyme(s) involved disfavors substrates with hydrophobic groups in P1 but favors basic amino acid residues in this position. This proposal is supported by the fact that all of the peptide aldehydes in our studies have a bulky hydrophobic group in P1, while APMSF has an amidino moiety that may occupy the S1 subsite of trypsin-like enzymes modified by this inhibitor (*19*, *23*, *47*).

Our finding of a nonproteasomal pathway for apoB100 degradation in MTPI-exposed McA cells is consistent with other studies in the literature using McA cells (e.g., ref *48*) as well as those using isolated rabbit (*49*) and hamster (*50*) hepatocytes. In addition, we recently isolated rat primary hepatocytes (prepared as in ref *48*) and treated them exactly as in Figure 1. Inhibition of MTP reduced apoB100 recovery, and, as with McA cells, lactacystin cotreatment was only partially (~50%) effective in blocking this induced degradation (two separate experiments; $n = 6$ for each treatment). Taken together, the available data all suggest the existence of multiple, nonproteasomal pathways. For example, in the rabbit studies, inhibitors of serine and cysteine proteinases reduced apoB100 degradation in smooth ER and cis-Golgi subcellular fractions, while ALLN (another peptide aldehyde active against the proteasome and many serine and cysteine proteases) caused apoB100 accumulation in the trans-Golgi. Similarly, in studies of apoB100 and apoB48 catabolism in lipid-deprived primary rat hepatocytes, a significant proportion of apoB degradation occurred in an acidified, post-ER compartment and involved an activity or activities inhibited by the cysteine protease inhibitor E64d (*51*).

Results from HepG2 cells have also provided evidence of nonproteasomal pathways of apoB100 degradation. Wang et al. (*52*) found that apoB100 degradation induced by tocotrienol was resistant to inhibition by LAC but was inhibited by ALLN. Studies of permeabilized Hep G2 cells also provided evidence of an ALLN-inhibited pathway localized to the ER which acted primarily on intralumenal apoB100 (*25*, *53*). An ER-associated, ALLN-resistant proteolytic pathway (inhibited by DTT) for apoB100 has also been identified in HepG2 cells (*26*).

A feature of the nonproteasomal apoB100 degradation induced by the inhibition of MTP was that it was further accelerated by BFA, which blocks ER to Golgi transport. This suggests that the responsible protease(s) reside(s) in the ER, consistent with the above cited studies from the laboratories of H. Ginsberg (*26*) and K. Adeli (*25*). Thus, although we and others have evidence consistent with an ER-associated, nonproteasomal degradation of apoB100, the molecular characteristics of the proteolytic pathway(s) involved remain poorly defined. In some important features, they appear to be distinct from each other. For example, agents effective in other contexts, such as DTT (*26*), E64d (*25*), and *o*-phenanthroline (*48*), were all ineffective in blocking the MTPI-induced apoB100 degradation in McA cells (data not shown; the effectiveness of *o*-phenanthroline was indicated by its ability to inhibit ~30% of $n - 3$ fatty acid-induced apoB100 degradation in McArdle cells, consistent with the results in ref *49*). Thus, it is possible that there are several different ER-associated pathways for apoB100 degradation and that there is selective expression of each, reflecting differences in cell types or the specific metabolic conditions.

The results with BFA also imply that a fraction of apoB100 can be translocated into the ER, despite the inhibition of MTPI, consistent with recent results in McA cells from the laboratory of Z. Yao (*8*) and data from apoB translocation studies in a cell-free system employing canine microsomes, which lack MTP (*54*). This would explain why there was only partial protection against degradation by LAC or MG132 in MTPI-treated cells, since the incompletely folded apoB100 molecules would now be shielded from the cytosolic proteasomes.

To explore the translocation status of apoB100 in MTPI-treated McA cells, we have assessed its resistance to the trypsin digestion of isolated microsomes. Consistent with Wang et al. (8), the preliminary experiments (X. Wu, C. Cardozo, and E. Fisher, unpublished) show that, in microsomes isolated from MTPI-treated cells, approximately 80% of the apoB100 is resistant to trypsin degradation. This was comparable to the result from control cells, although, as expected, significantly more apoB100 was recovered from microsomes in the absence of MTPI. Thus, of the apoB100 that escapes the proteasomal pathway in MTPI cells, most appears to be translocated. This pool of apoB100 would be expected to be poorly lipidated and, hence, improperly folded. Its degradation as well as its failure to be secreted (even when degradation was partially blocked) is compatible, then, with the current view that the exit from the ER of proteins with non-native conformations is prevented by multiple quality control mechanisms (*34*).

In summary, when MTP activity in rat hepatoma cells is inhibited, apoB100 is degraded by both proteasomal and nonproteasomal mechanisms. The proteasomal component is accomplished by the chymotrypsin-like activity, and the targeting of apoB100 to the nonproteasomal component, partially sensitive to the serine protease inhibitor APMSF, appears to occur in the ER. Although there were differences in apoB100 metabolism between rat hepatoma and human HepG2 cells, such as reduced cotranslational proteasomal degradation in the former, the results also imply that the role

of the proteasome in the regulated secretion of hepatic apoB lipoproteins is not restricted to a single hepatic cell type. Thus, the relative roles of proteasomal and nonproteasomal mechanisms in cells of hepatic origin most likely depend on the specific cell type and its metabolic state.

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