Recent studies have proposed that post-translational degradation of apolipoprotein B100 (apoB) involves the cytosolic ubiquitin-proteasome pathway. In this study, immunocytochemistry indicated that endoplasmic reticulum (ER)-associated proteasome molecules were concentrated in perinuclear regions of digitonin-permeabilized HepG2 cells. Signals produced by antibodies that recognize both α- and β-subunits of the proteasome co-localized in the ER with specific domains of apoB. The mechanism of apoB degradation in the ER by the ubiquitin-proteasome pathway was studied using pulse-chase labeling and digitonin-permeabilized cells. ApoB in permeabilized cells incubated at 37 °C in buffer alone was relatively stable. When permeabilized cells were incubated with both exogenous ATP and rabbit reticulocyte lysate (RRL) as source of ubiquitin-proteasome factors, >50% of apoB was degraded in 30 min. The degradation of apoB in the intact ER of permeabilized cells was much more rapid than that of extracted apoB incubated with RRL and ATP in vitro. The degradation of apoB was reduced by clasto-lactacystin β-lactone, a potent proteasome inhibitor, and by ubiquitin K48R mutant protein, an inhibitor of polyubiquitination. ApoB in HepG2 cells was ubiquitinated, and polyubiquitination of apoB was stimulated by incubation of permeabilized cells with RRL. These results suggest that newly synthesized apoB in the ER is accessible to the cytoplasmic ubiquitin-proteasome pathway and that factors in RRL stimulate polyubiquitination of apoB, leading to rapid degradation of apoB in permeabilized cells.

Apolipoprotein B100 (apoB) is a unique secretory protein because of its large size (540 kDa), requirement for lipids for secretion, and inefficient secretion resulting from rapid post-translational degradation (1, 2). Previous studies showed that >50% of newly synthesized apoB in HepG2, a hepatoma cell line, was degraded within 20 min when cells were cultured under lipid-poor conditions (3). Recent studies have indicated that newly synthesized apoB is degraded post-translationally by a cytosolic proteolytic system, the ubiquitin-proteasome pathway (4–8). This pathway has recently been identified to be involved in the degradation of many different proteins, including proteins within the endoplasmic reticulum (ER) compartment (9–15).

Because apoB is largely located in the ER lumen, the precise mechanism of apoB degradation by the ubiquitin-proteasome pathway has not been worked out. Recently, ER proteins have been shown to undergo retrograde transport from the ER to the cytosol for proteasomal degradation (9, 13, 15–17). Because apoB is located primarily in the ER lumen, it also would require retrograde transport before proteasomal degradation. Recent reports have shown that apoB associates with an ER membrane translocon component, Sec61 (7, 18). ApoB has also been shown to associate with the cytosolic chaperone HSP70 and the ER chaperones calnexin, ERp72, GPR94, calreticulin, and BiP (5, 6, 19, 20). These chaperones may be involved in both normal and retrograde transport of apoB in the ER.

Studies on the ubiquitination of target proteins and their subsequent recognition and degradation by the proteasome are difficult in intact cells and would be even more pronounced with the large apoB molecule (540 kDa). Therefore, to investigate the degradation of apoB in the ER by the ubiquitin-proteasome pathway, we have studied apoB degradation in digitonin-permeabilized pulse-labeled HepG2 cells utilizing rabbit reticulocyte lysate (RRL) as source of ubiquitin-conjugating enzymes and other factors involved in proteasomal degradation (21, 22).

The questions that we sought to answer are as follows. 1) How can newly synthesized apoB that is located largely in the ER lumen be degraded by the ubiquitin-proteasome pathway in the cytosol? 2) Does newly synthesized apoB need to be transported to another location in the cell or are apoB and the proteasome in close proximity in the liver cell?

This study demonstrates that cytosolic components in RRL are required for proteasomal apoB degradation in permeabilized cells with an intact ER membrane. Newly synthesized apoB in the ER is “primed” for rapid degradation by the proteasome, suggesting that efficient retrograde transport of apoB occurs in permeabilized cells.

**Materials—** RRL was obtained from Promega (Madison, WI). ATP, creatine kinase, creatine phosphate, and bovine serum albumin were purchased from Sigma. L-[4,5-3H]Leucine and luminol reagent (ECL kit) were purchased from Nycomed Amersham (Princeton, NJ). Ubiquitin aldehyde, clasto-lactacystin β-lactone, and anti-proteasome α-subunit antibody were obtained from Calbiochem-Novabiochem. Ubiquitin K48R mutant protein was obtained from Boston Biochem (Cambridge, MA). Anti-proteasome α-type subunit (zeta) and β-type subunit (HC10) antibodies were purchased from Affiniti (Mamhead, United Kingdom). Anti-ubiquitin antibody was purchased from Roche Molecular Biochemicals. Monospecific anti-human apoB B4 region antibody was purchased from Serotec Ltd. (Raleigh, NC). The CC3.4 monoclonal antibody to apoB was a gift of Dr. Gustav Schonfeld (Washington University, St. Louis, MO). Monoclonal antibody to p63 (G1/296) was a gift of Dr. Hans-Peter Hauri (Department of Pharmacology, Biocenter of the University of Basel, Basel, Switzerland).

**EXPERIMENTAL PROCEDURES**

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obtained from Amersham Pharmacia Biotech. Minimal essential medium and leucine-free medium (minimal essential medium Selectamine kit) were purchased from Life Technologies, Inc. Anti-mouse and anti-rabbit secondary antibodies conjugated with the fluorochrome Cy3 (indocarbocyanine) or Cy5 (indodicarbocyanine) were obtained from Jackson Laboratory (West Grove, PA). All other chemicals were of the highest purity available.

**Cell Culture**—HepG2 cells were grown on collagen-coated tissue culture dishes (for labeling experiments) or coverslips (for immunocytochemistry) as described previously (23). Briefly, HepG2 cells were cultured in 24- or 6-well tissue culture plates with complete medium containing minimal essential medium with 0.1 mM nonessential amino acids, 100 mM pyruvate, 100 μg/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum. After 4 days of culture in a CO₂ incubator, cells were used for pulse-chase experiments. For immunocytochemistry, HepG2 cells were used at lower confluency (20–30%).

**Permeabilization of HepG2 Cells**—The cell permeabilization procedures were described previously (23). In this study, we used digitonin to perforate the plasma membrane and saponin to perforate both plasma and subcellular membranes. For permeabilization of cells with digitonin for immunocytochemistry, subconfluent HepG2 cells were washed twice with phosphate-buffered saline and treated for 5 min at 4°C with digitonin (60 μg/ml) in intracellular buffer (75 mM potassium acetate, 2.5 mM magnesium acetate, 1.8 mM calcium chloride, and 25 mM HEPES, pH 7.2). After removing digitonin, the cells were washed three times with intracellular buffer and then fixed with 2% paraformaldehyde in room temperature and processed for immunocytochemistry. For permeabilization of cells with saponin for immunocytochemistry, subconfluent cells were washed twice with phosphate-buffered saline, fixed in 2% paraformaldehyde, washed three times with intracellular buffer, and processed for immunocytochemistry. All subsequent steps for saponin-permeabilized cells were performed in intracellular buffer containing 0.1% saponin and 0.1% bovine serum albumin. For permeabilization of cells with digitonin for apoB degradation studies, subconfluent HepG2 cells were washed twice with phosphate-buffered saline, treated for 5 min at 4°C with digitonin (60 μg/ml) in intracellular buffer, washed three times with intracellular buffer, and used immediately.

**Immunocytochemistry**—Digitonin- or saponin-permeabilized cells were incubated for 4 h or overnight with primary antibodies and then incubated for 4 h with the appropriate anti-mouse or anti-rabbit IgG secondary antibodies conjugated with fluorochromes (Cy3 or Cy5) and examined with an MRC-600 confocal microscope (Bio-Rad) as described in detail by Du et al. (23).

**Degradation of ApoB in Permeabilized Cells**—HepG2 cells were preincubated with serum-free medium for 1 h and then pulsed with [3H]leucine (100 μCi/ml) in leucine-free medium for 10 min and chased for 10 min with serum-free medium at 37°C. After labeling, cells were preincubated for 5 min at 4°C and described above and treated with digitonin at 37°C with intracellular buffer (total of 250 μl/well) containing RRL (10–25 μl/well), 2 mM ATP, 100 μg/ml creatine kinase, and 10 mM creatine phosphate. When effects of inhibitors were studied, cells were preincubated with intracellular buffer (250 μl/well) in the absence or presence of inhibitors for 20 min before incubation with RRL and ATP. The incubation buffer (intracellular buffer containing RRL) had also been treated with inhibitors for 20 min and then was added to inhibitor-treated permeabilized cells. After incubation, [3H]ApoB in the cells was extracted with lysis buffer, immunoprecipitated, separated by SDS-PAGE, and detected by fluorography. Film images were scanned by an image scanner (Epson Expression 636) and analyzed by the NIH Image program (Version 1.61) on a Power Macintosh 9600 system (Apple Computer).

**Degradation of ApoB in Vitro**—The degradation of extracted [3H]ApoB was monitored in vitro as described previously (24). Briefly, HepG2 cells were pulsed with 75 μCi/ml [3H]leucine for 10 min and chased for 10 min. Newly synthesized [3H]ApoB was isolated by immunoprecipitation using anti-human apoB antisera and protein A-Sepharose CL-4B (3, 24, 25). Extracted [3H]ApoB (~60,000 dpm/40 μl) in 0.1 M glycine, 0.02 M Tris, and 1% Triton X-100, pH 7.4, was applied to RRL, 2 mg/ml calmodulin, 10 mM creatine kinase, and 10 mM creatine phosphate in a final volume of 90 μl. After incubation at 37°C, 200 μl of electrophoresis sample buffer was added, and it was boiled for 4 min. Samples were separated by SDS-PAGE and visualized by fluorography.

**Immunoblotting**—Unlabeled HepG2 cells were permeabilized with digitonin and incubated for 0 or 30 min at 37°C in intracellular buffer plus rabbit reticulocyte lysate and ATP as described for the radiolabel studies. ApoB in cells was extracted with lysis buffer, immunoprecipitated with anti-human apoB antibody, and separated by SDS-PAGE. Proteins in the gel were transferred to polyvinylidene fluoride Immobilon-P membranes as described previously (23). ApoB and apoB-bound ubiquitin in the membrane were detected by anti-apoB and anti-ubiquitin antibodies and visualized with horseradish peroxidase-conjugated second antibodies and ECL luminol reagent.

**RESULTS**

Recent studies have indicated that the proteasome is largely located in the cytosol and that a small percentage of proteasome molecules are associated with the ER membrane (26, 27). It is not clear whether ER membrane-associated proteasomes are involved in the degradation of ER proteins. To investigate this question, we first studied the locations of apoB and the proteasome in digitonin-permeabilized HepG2 cells using immunocytochemistry.

In a previous study, two domains in apoB were shown to be exposed on the cytosolic side of the ER membrane in subconfluent streptolysin O-permeabilized HepG2 cells (23). In the current experiments, digitonin was used to permeabilize cells because only a maximum of ~50% of the cells in confluent monolayers of HepG2 cells were permeabilized using streptolysin O, whereas almost 100% of the cells can be permeabilized with digitonin (data not shown).

Two anti-apoB antibodies were used for immunocytochemistry. The CC3.4 antibody to apoB recognizes amino acids 690–797 in the N-terminal region of the apoB molecule (28), whereas the B4 anti-apeptide antibody recognizes a more limited region of apoB (amino acids 3221–3240) (23). Although these regions of apoB were shown to be cytosol-facing, a majority of each apoB molecule is located in the lumen of the ER (23). Both apoB antibodies (CC3.4 (Fig. 1A) and B4 (Fig. 1, D and G)) produced bright concentrated staining in the perinuclear regions of cells, with less intense reticular staining in peripheral regions, as previously observed in streptolysin O-permeabilized cells (23).

The 26 S proteasome is primarily a cytosolic protease (11, 12) that consists of a large complex of proteins that compose a central 20 S core and two terminal PA700 complexes (29, 30). We asked the question whether the proteasome would still be present in digitonin-permeabilized cells. For some experiments, cells were permeabilized first and then fixed for immunocytochemistry to allow removal of soluble cytosolic constituents (Fig. 1, A–C). When digitonin-permeabilized cells were probed with a polyclonal antibody to the 26 S proteasome α-subunit, bright signals were observed in the perinuclear regions of cells (Fig. 1B). Monoclonal antibodies to the zeta (α-type) and HC10 (β-type) subunits of the proteasome (Fig. 1, E and H) gave similar signals in the perinuclear regions and more diffuse staining in the peripheral secretory membranes. These results indicate that a significant population of proteasome molecules remains associated with the ER membranes in the perinuclear regions of digitonin-permeabilized cells.

When the signals due to the α-subunit of the proteasome were superimposed with the signals produced by the CC3.4 antibody to apoB (Fig. 1C), there was significant overlap in the areas closest to the nucleus of the cell. There was also substantial overlap of signals from the zeta and HC10 subunits of the proteasome with the signals produced by the B4 region of apoB (Fig. 1, F and D). These immunocytochemical observations suggest that apoB could be a protein that is associated with the ER may be in close proximity to cytosolic domains of apoB in the perinuclear regions of HepG2 cells.

A series of biochemical experiments were performed to investigate the degradation of apoB in digitonin-permeabilized cells. Cells were cultured under standard media conditions without added fatty acid to mimic conditions that allow maxi-
Mal early apoB degradation (3). First, immunocytochemistry was used to determine whether the ER membrane was intact in digitonin-permeabilized cells (23). Whereas a bright signal for the luminal portion of the ER membrane protein p63 was observed when cells were permeabilized with saponin (Fig. 2, right), only a background signal was observed in digitonin-treated cells (Fig. 2, left). These data indicated that ER membranes were not permeabilized by digitonin treatment.

The degradation of apoB in permeabilized cells was measured as described under “Experimental Procedures” and shown in Fig. 3. Digitonin treatment itself did not decrease the content of [3H]apoB in HepG2 cells (Fig. 4A, lanes 1 and 2). When digitonin-permeabilized cells were incubated in buffer at 37 °C with no additions, [3H]apoB was observed to be relatively stable (Fig. 4A, lane 3). This observation is similar to our early observation that apoB was stable in preparations of intact microsomes (25). This result suggests that there is a requirement for cytosolic components for the degradation of apoB in permeabilized cells.

Previously, we utilized RRL to study apoB degradation in vitro (24) and observed that the degradation of isolated apoB was primarily dependent upon the ubiquitin-proteasome system present in RRL. Therefore, we added RRL to permeabilized cells to provide a source of the ubiquitin-proteasome system and other factors. Other additions (such as ATP) were made as noted in the figure legends. In contrast to permeabilized cells incubated in buffer alone, newly synthesized apoB was rapidly degraded in digitonin-permeabilized cells incubated at 37 °C in buffer containing RRL and the ATP-generating system (ATP) (Fig. 4A, lane 4). ApoB degradation was not observed when permeabilized cells were treated with a preparation of boiled RRL, suggesting that the required factors were heat-sensitive (data not shown). As previously observed (24) when apoB was extracted from HepG2 cells and incubated with RRL and ATP, apoB degradation was dependent on RRL, but occurred at a much slower rate (Fig. 4B, lane 4). Approximately 75% of apoB is degraded in digitonin-permeabilized cells incubated with RRL and ATP.

FIG. 1. Proteasome and cytosol-facilitating domains of apoB co-localize in the ER. HepG2 cells were either permeabilized with digitonin (60 μg/ml) for 5 min, washed, and fixed with 2% paraformaldehyde (A–C) or fixed first, permeabilized, and washed (D–I) before processing for immunocytochemistry. Permeabilized cells were incubated with anti-apoB antibodies (A, CC3.4 (1:200 final dilution); D and G, B4 (1:50 dilution) and anti-proteasome antibodies (B, α-subunit; E, zeta (α-type subunit); H, HC10 (β-type subunit); all at 1:200) as described under “Experimental Procedures.” Cells were then incubated with the appropriate anti-mouse or anti-rabbit IgG secondary antibodies (1:100) conjugated with fluorochromes (Cy3 or Cy5) and examined with an MRC-600 confocal microscope. C, F, and I are superimposed pictures of A and B, D and E, and G and H, respectively. The areas of co-localization of apoB and proteasome are yellow.

FIG. 2. Digitonin permeabilization does not perforate the ER membrane. HepG2 cells were permeabilized with digitonin (60 μg/ml) for 5 min (left) or with saponin (0.1%) treatment (right). Cells were washed with intracellular buffer three times and fixed with 2% paraformaldehyde. Fixed cells were incubated with anti-p63 antibody (1:1000), followed by incubation with anti-mouse IgG (1:100) conjugated with Cy3. Cells were examined with an MRC-600 confocal microscope.
Proteasomal Degradation of ApoB in Permeabilized Cells

Fig. 3. ApoB degradation in permeabilized HepG2 cells. HepG2 cells were pulse-labeled with [3H]leucine, chased, permeabilized with digitonin (60 μg/ml), washed, and incubated with RRL at 37 °C for 0 or 3 h. [3H]ApoB was isolated from HepG2 cells that had been pulse-labeled for 10 min with [3H]leucine for 10 min, chased for 10 min, permeabilized with 60 μg/ml digitonin for 5 min, washed with intracellular buffer, and incubated for 0 or 1 h with RRL and ATP at 37 °C. [3H]ApoB in the cells was extracted, immunoprecipitated, and analyzed by SDS-PAGE (3–15%). Lane 1, no digitonin treatment (control); lane 2, digitonin treatment only; lane 3, digitonin treatment plus incubation at 37 °C with buffer alone; lane 4, digitonin treatment plus incubation at 37 °C with RRL and ATP. The asterisk denotes a 150-kDa apoB degradation product in lane 4, shown is the in vitro degradation of extracted apoB. ApoB was isolated from HepG2 cells that had been pulse-labeled for 10 min with [3H]leucine and chased for 10 min with serum-free medium. [3H]ApoB was incubated with RRL and ATP at 37 °C for 0 or 3 h. [3H]ApoB was analyzed as described under “Experimental Procedures.” Lane 1, no incubation (control); lane 2, incubation at 37 °C with buffer alone; lane 3, RRL added, but no incubation; lane 4, incubation at 37 °C with RRL and ATP. The mobilities of molecular mass markers and plasma low density lipoprotein are indicated to the right of each gel.

Permeabilized Cell

Fig. 4. [3H]ApoB is degraded in permeabilized cells in the presence of RRL and ATP. A, HepG2 cells were pulse-labeled with [3H]leucine for 10 min, chased for 10 min, permeabilized with 60 μg/ml digitonin for 5 min, washed with intracellular buffer, and incubated for 0 or 1 h with RRL and ATP at 37 °C. [3H]ApoB in the cell was extracted, immunoprecipitated, and analyzed by SDS-PAGE (3–15%). Lane 1, no digitonin treatment (control); lane 2, digitonin treatment only; lane 3, digitonin treatment plus incubation at 37 °C with buffer alone; lane 4, digitonin treatment plus incubation at 37 °C with RRL and ATP. The asterisk denotes a 150-kDa apoB degradation product in lane 4, shown is the in vitro degradation of extracted apoB. ApoB was isolated from HepG2 cells that had been pulse-labeled for 10 min with [3H]leucine and chased for 10 min with serum-free medium. [3H]ApoB was incubated with RRL and ATP at 37 °C for 0 or 3 h. [3H]ApoB was analyzed as described under “Experimental Procedures.” Lane 1, no incubation (control); lane 2, incubation at 37 °C with buffer alone; lane 3, RRL added, but no incubation; lane 4, incubation at 37 °C with RRL and ATP. The mobilities of molecular mass markers and plasma low density lipoprotein are indicated to the right of each gel.

A time course study indicated that newly synthesized apoB in permeabilized cells was degraded more rapidly than isolated apoB in the in vitro assay (Fig. 5). After 30 min of incubation, ~70% of apoB was degraded in permeabilized cells versus only 25% in the in vitro assay. This difference in rate occurred despite the fact that less RRL was added to permeabilized cells compared with the amount of RRL added to the in vitro assay. To determine whether the loss of apoB signal was due to degradation of apoB or to release of apoB after ER membrane rupture, the effects of incubation with RRL on newly synthesized albumin were monitored in permeabilized cells. We previously showed that if secretory membranes are ruptured, albumin will be lost from permeabilized cells (23). Incubation of permeabilized cells for 30 min with RRL did not lead to loss of albumin signal (Fig. 6). We also measured release of apoB into the incubation buffer. Only 2–3% of the total cellular apoB counts were immunoprecipitated from the incubation buffer regardless of the presence of RRL (data not shown). These results show that ER membranes remained impermeable during and after incubation of cells with RRL.

The next series of experiments utilized inhibitors of the ubiquitin-proteasome pathway to determine whether the degradation of apoB in RRL-treated permeabilized cells was proteasomal in nature. ApoB degradation in permeabilized cells (Fig. 7) was reduced by incubation with 50 μM clasto-lactacystin β-lactone, a potent inhibitor of the proteasome (31–33). ApoB immunoprecipitated from cells treated with clasto-lactacystin β-lactone (Fig. 7A, lane 3) showed increased signals both for the main apoB band and for a higher molecular mass apoB species that may represent polyubiquitinated apoB (see Fig. 8). ApoB degradation was also inhibited by ubiquitin K48R mutant protein, which perturbs polyubiquitination of proteins (34, 35). Incubation with 50 μM ubiquitin K48R mutant protein (Fig. 7B, lane 3) produced an increased apoB signal without increasing the signal of the higher molecular mass apoB species that had been observed in Fig. 7A (lane 3). This observation is consistent with inhibition of polyubiquitination of apoB by ubiquitin K48R mutant protein. In contrast to apoB degradation in the in vitro system (24), degradation of apoB in permeabilized cells was not significantly affected by ubiquitin aldehyde, an inhibitor of ubiquitin hydrolases (data not shown).

Collectively, these results indicate that apoB degradation in RRL-treated permeabilized cells involves polyubiquitination, followed by efficient proteasomal proteolysis. Interestingly, there were only limited effects of the two inhibitors on the formation of the 150-kDa apoB degradation intermediate (Fig. 5).
Incubation at 37 °C with RRL and ATP; the absence or presence of inhibitors of the ubiquitin-proteasome pathway was analyzed. Meabilized cells were incubated for 0 or 30 min with RRL and ATP in the presence of RRL and ATP at 37 °C. [3H]ApoB in the cells was extracted with lysis buffer, immunoprecipitated, and analyzed by SDS-PAGE (3–15%). Lane 1, digitonin treatment only; lane 2, digitonin treatment plus incubation at 37 °C with buffer alone; lane 3, digitonin treatment plus incubation at 37 °C with RRL and ATP.

FIG. 7. Degradation of [3H]ApoB in permeabilized cells is reduced by a proteasome inhibitor, clasto-lactacystin β-lactone, and by ubiquitin K48R mutant protein. After preincubation with/without inhibitors as described under “Experimental Procedures,” permeabilized cells were incubated for 0 or 30 min with RRL and ATP in the absence or presence of inhibitors of the ubiquitin-proteasome pathway. Lane 1, digitonin treatment only; lane 2, digitonin treatment plus incubation at 37 °C with RRL and ATP; lane 3, digitonin treatment plus incubation at 37 °C with RRL and ATP plus inhibitor. A, clasto-lactacystin β-lactone (β-LC; 50 μM); B, ubiquitin (Ub) K48R (50 μM). After incubation, apoB was extracted, immunoprecipitated, and analyzed by SDS-PAGE. The asterisk and double asterisks denote 150- and 380-kDa apoB degradation products, respectively.

In the next experiment, we wished to examine the ubiquitination state and degradation of total cellular apoB in RRL-treated permeabilized cells (Fig. 8, A and B). As observed with [3H]ApoB, apoB degradation could be observed by immunoblotting of digitonin-permeabilized cells after addition of RRL and ATP (Fig. 8A, lane 3), but was not observed during incubation without RRL and ATP (lane 2). Immunoblot analysis of the immunoprecipitated apoB showed that apoB in permeabilized cells was ubiquitinated prior to incubation with RRL (Fig. 8B, lane 1). Despite loss of intact apoB (Fig. 8A, lane 3), ubiquitinated apoB increased during incubation with RRL (Fig. 8B, lane 3). The slower gel mobility of the signal produced by anti-ubiquitin antibody suggested that the ubiquitinated apoB formed during incubation with RRL was primarily polyubiquitinated apoB. When incubated without RRL, apoB appeared to be de-ubiquitinated (lane 2). These results indicate that a certain percentage of apoB is ubiquitinated in intact HepG2 cells and that, during incubation of RRL-treated permeabilized cells, ubiquitination of apoB is maintained or possibly increased, leading to the accumulation of polyubiquitinated apoB.

**DISCUSSION**

Co-localization of Proteasome with ApoB—In mammalian cells, a majority of the cellular proteasome was shown to be present in the cytosol (26, 27, 36). Immunogold electron microscopy studies indicated that 69–83% of proteasomes in rat hepatocytes were located in the cytoplasm, whereas the ER contained ~11–14% of the gold particles (26, 27). A study in yeast showed that proteasomal subunits labeled with green fluorescent protein accumulated mainly in the nuclear envelope-ER network (37).

We did not measure what percent of total cellular proteasomes is represented by the signals produced by anti-proteasome antibodies in our immunocytochemistry photographs. Whether the cells were permeabilized or fixed first, a large amount of soluble proteasome complexes may have been released into the washes during the permeabilization process. The results of this study provide evidence that populations of proteasomes are associated with the ER membrane and that they are located in regions of the cell that are important in the early metabolism of newly synthesized apoB.

Degradation Studies—ApoB was relatively resistant to degradation in permeabilized cells incubated without RRL and ATP. Therefore, although the ER membrane still retained associated proteasomes, some other factor(s) appeared to be required for efficient degradation of apoB in permeabilized cells. We had previously used RRL as a source of ubiquitin-proteasome components in an in vitro apoB degradation assay (24). In the current study, after addition of RRL and ATP, [3H]ApoB in intact ER membranes of permeabilized cells was degraded much more rapidly than extracted apoB that was not associated with secretory membranes (Fig. 5). The decline in apoB in permeabilized cells represents the degradation of a large fraction of newly synthesized apoB. Similar experiments in intact cells under lipid-poor conditions (3, 25) showed remarkably similar early apoB kinetics (0–30 min), but different later decay (> 30 min) due to the presence of a small, more degradation-resistant apoB pool in intact cells. Because only short pulse-chase periods were used in this study, the apoB degradation was primarily newly synthesized apoB. This pool of apoB in the membrane of the ER was primed for rapid degradation, but required cytosolic components.

The degradation of apoB in permeabilized cells incubated with RRL and ATP was primarily by the ubiquitin-proteasome system, as it was reduced by both clasto-lactacystin β-lactone (Fig. 7A) and ubiquitin K48R mutant protein (Fig. 7B). ApoB in permeabilized cells became polyubiquitinated during incubation with RRL (Fig. 8). The results with ubiquitin aldehyde, which had minimal effects on apoB degradation in permeabi-
HepG2 cells. 

radation of apoB in permeabilized cells. Depending upon lipid availability, apoB can either be assembled into a nascent lipoprotein or be degraded in the ER membrane. Ub, HSP, and MTP denote ubiquitin, heat shock protein, and microsomal triglyceride transfer protein, respectively. Ovals without labels are putative ER luminal chaperones.

Apoptosis is required for rapid proteasomal degradation of apoB. ApoB is synthesized in the ER on membrane-bound ribosomes and co-translationally transported into the ER lumen. Depending upon lipid availability, apoB can either be assembled into a nascent lipoprotein or be degraded in the ER membrane. 

Co-immunoprecipitation with Sec61 and treatments that slowed apoB-lipoprotein assembly increased this association. Because apoB is transported cotranslationally through the translocon, it was not determined whether the apoB found associated with Sec61 was being transported inward or outward. But the observation that the ubiquitination state of apoB associated with the Sec61 complex increased as proteasomal degradation of glycosylated apoB increased is evidence that retro-transport may occur through the translocon.

Recently, it was reported that two limited regions in apoB are important for targeting poorly lipidated apoB molecules for proteasomal degradation. These domains may be used as attachment sites for proteins involved in apoB retro-transport.

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