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Ubiquitin-Dependent and -Independent Proteasomal Degradation of apoB Associated With Endoplasmic Reticulum and Golgi Apparatus, Respectively, in HepG2 Cells

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Abstract Studies in hepatocyte cultures indicate that apolipoprotein (apo) B-100 production is regulated largely by intracellular degradation and the proteasome pathway is a major mechanism for the degradation. In the present study, we have examined the detailed itinerary of apoB degradation through its secretory pathway in HepG2 cells. We found that ubiquitin-dependent proteasomal degradation of apoB largely occurred on the cytosolic surface of rough and smooth endoplasmic reticulum (ER) and that a small proportion of apoB was dislodged from the secretory organelles into the cytosolic compartment where it underwent ubiquitination for proteasomal degradation. The transmembrane conformation of apoB persisted as the protein was transported through the Golgi apparatus. We further demonstrated that proteasomal degradation of apoB was associated the Golgi apparatus but Golgi-associated apoB was not ubiquitinated, indicating an ubiquitin-independent proteasomal degradation of apoB is associated with this organelle. We conclude that apoB undergoes proteasomal degradation while going through different compartments of the secretory pathway; further, ER-associated proteasomal degradation of apoB in the ER is ubiquitin-dependent whereas that occurring in the Golgi is ubiquitin-independent. J. Cell. Biochem. 89: 1019–1029, 2003.

Key words: ubiquitin; proteasore; apoB; lipoproteins; hepatocytes

As the sole protein component in low density lipoproteins, apolipoprotein (apo)[1] B-100 is an important determinant of atherosclerosis susceptibility [Chan, 1992; Kane and Havel, 1995]. The plasma concentration of apoB-100 is a balance between its production rate in the liver and its removal from the circulation by receptor and non-receptor mediated pathways. ApoB-100 is an obligatory component of the plasma lipoproteins, very low density (VLDL), intermediate density (IDL), low-density lipoproteins (LDL), and lipoprotein (a). Its production by the liver is regulated mainly by intracellular degradation at the translational and posttranslational levels [Borchardt and Davis, 1987; Sato et al., 1990; Sparks and Sparks, 1990; Ginsberg,

Abbreviations used: apo, apolipoprotein; PDI, protein disulfide isomerase; LDL, low density lipoprotein; IDL, intermediate density lipoprotein; VLDL, very low density lipoprotein; ER, endoplasmic reticulum, RER, rough endoplasmic reticulum; SER, smooth endoplasmic reticulum; ALLN, *N*-acetyl-L-leucinyl-L-leucinyl-L-norleucinal; FCS, fetal calf serum; PAGE, polyacrylamide gel electrophoresis; NEM, *N*-ethylmaleimide; CFTR, cystic fibrosis transmembrane conductance regulator.

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1995; Yao et al., 1997; Yeung and Chan, 1998]. We [Yeung et al., 1996; Liao et al., 1998; Liao and Chan, 2000, 2001] and others [Benoist and Grand-Perret, 1997; Fisher et al., 1997; Chen et al., 1998; Mitchell et al., 1998; Zhou et al., 1998; Du et al., 1999] have identified the proteasome pathway as a major mechanism for the degradation of apoB-100, possibly a major means by which the cell removes misfolded apoB. ApoB-100 is one of the largest proteins known and has a complex structure [Chan, 1992; Segrest et al., 1994]. It has been postulated that apoB-100 is prone to misfolding and the misfolded molecule is diverted to the 26S proteasome for degradation. In HepG2 cells in which most of the apoB-100 degradation experiments have been conducted, the addition of the exogenous lipid substrate oleate downregulates the intracellular degradation of apoB-100 and allows more of the newly synthesized protein to be secreted from the cell, presumably by providing additional lipids to facilitate its correct folding and translocation into the lumen of the endoplasmic reticulum (ER) [Dixon et al., 1991]. Nonetheless, even in the presence of optimal amounts of oleate, a significant proportion (20–35%) of apoB-100 is degraded intracellularly before the rest is secreted into the medium.

Our knowledge of the biogenesis of apoB-100 is constantly being modified as we learn more and more of the various components involved in its production, translocation and degradation [Davis, 1999; Chan et al., 2000]. In this study we examined the detailed itinerary of apoB degradation through the secretory pathway in HepG2 cells. We found that ubiquitin-dependent proteasomal degradation of apoB occurs largely on the cytosolic surface of the rough and smooth ER and that a small proportion of apoB is dislodged from the secretory organelles into the cytosol where it undergoes ubiquitination for proteasomal degradation. ApoB-100 is known to exist in a transmembrane conformation and is accessible to digestion by exogenously added proteases in isolated microsomes or ER [Davis et al., 1990; Du et al., 1994; Liao et al., 1998; Davis, 1999]. Here we showed that the transmembrane conformation of apoB persists beyond the ER as the protein is transported through the Golgi apparatus. We further demonstrated that proteasomal degradation of apoB occurs in the Golgi although the Golgi-associated apoB is not ubiquitinated, an observation that suggests an ubiquitin-independent mechanism associated with this organelle. Furthermore, the distribution of accumulated intracellular apoB in HepG2 cells treated with proteasome inhibitors colocalizes with the ER and not the centrosome. Our results suggest that the apoB aggregates resembled Russell bodies [Valetti et al., 1991; Kopito and Sitia, 2000] rather than aggresomes in cells treated with the proteasome inhibitors. Aggresomes are a symptom of "cytoplasmic indigestion" that usually occurs with mutant forms of protein aggregates or with normal proteins whose degradation is blocked by proteasome inhibitors [Johnston et al., 1998; Garcia-Mata et al., 1999; Wigley et al., 1999; Kopito, 2000]. In contrast, Russell bodies are thought to comprise condensed proteins that cannot undergo transport from the ER to the Golgi. They represent a general response of the cell to the accumulation of abundant, nondegradable protein(s) that fail to exit from the ER [Valetti et al., 1991; Kopito and Sitia, 2000].

EXPERIMENTAL PROCEDURE

Materials

Nitrocellulose membrane was from Schleicher & Schuell. *N*-ethylmaleimide (NEM), trypsin, soybean trypsin inhibitor and *N*-acetyl-L-leucinyl-L-leucinyl-L-norleucinal (ALLN) were from Sigma. Gamma-Bind G Sepharose was from Amersham. Lactacystin was obtained from CalBiochem. Mouse monoclonal antibody against ubiquitin, goat polyclonal antibodies against human apoB and apoA-I and rabbit polyclonal antibody against proteasome were from Chemicon. Monoclonal antibody against human apoB (1D1) was kindly provided by Dr. R.W. Milne (Ottawa Heart Institute). Rabbit polyclonal antibody against sec61β was generously provided by Dr. Tom Rapaport at Harvard University. Mouse monoclonal antibodies against protein disulfide isomerase (PDI), BiP and membrin were from Stressgen Biotechnologies Corp. FITC- and Texas Red-conjugated secondary antibodies were from Jackson ImmunoResearch Laboratories, Inc. Texas Red-conjugated concanavalin A was from Molecular Probes. ^{35}S-methionine, methionine, RPMI 1640, and methionine-free RPMI 1640 were from ICN. Tris-glycine gradient gels were from Norvex.

Cell Culture

HepG2 cell lines were from American Type Culture Collection and were maintained at

37°C in an atmosphere with 5% CO_2 and in RPMI 1640 medium (Gibco-BRL, Rockville, MD) containing 10% fetal calf serum (FCS, HyClone, Logan, UT), penicillin (100 U/ml) and streptomycin (100 μg/ml) (Gibco-BRL).

Immunoblot Analysis

Western blot analysis was performed on the cell lysate or immunoprecipitates as described previously [Liao et al., 1998, 1999; Liao and Chan, 2000, 2001]. The cells were washed with cold PBS and lysed in 2% sodium cholate in HEPES-buffered saline (50 mM HEPES, pH 7.4, 200 mM NaCl) containing 1 mM PMSF, 0.1 mM ALLN, 5 mM NEM, and complete protease inhibitors (Boehringer, Mannheim, Germany). Insoluble material in the cell lysate was removed by centrifugation at 10,000*g* for 10 min. ApoB was immunoprecipitated with goat polyclonal anti-apoB antibody. The proteins were separated by SDS–PAGE, transferred overnight onto nitrocellulose membranes, probed with monoclonal anti-ubiquitin or anti-apoB antibodies and detected by enhanced chemiluminescence (ECL kit, Amersham). The relative intensity of the immunoblot bands was quantified by AlphaImagerTM 2000 Documentation & Analysis system (Alpha Innotech Corporation, San Leandro, CA).

Preparation of Cytosol and Microsome

HepG2 cells were cultured in 75 cm^2 flasks in RPMI 1640 medium containing 10% fetal calf serum until about 70%. Cytosol and microsomes were then prepared as described previously [Liao et al., 1998; Liao and Chan, 2000]. In brief, the cells were harvested by scraping the cells into phosphate-buffer saline. The cells were pelleted by low speed centrifugation, and homogenized in 4 ml of 0.25 M sucrose and 10 mM HEPES (pH 7.4) on ice. The homogenate was first centrifuged at 10,000*g* for 15 min at 4°C. The resulting supernatant was centrifuged at 100,000*g* for 60 min to pellet the microsomes. The cytosol (supernatant) was collected. The microsome pellets were suspended in an appropriate volume of 0.25 M sucrose and 10 mM HEPES.

Subcellular Organelle Fraction

Total microsomes were prepared as described above with the exception that 10,000*g* supernatant (3 ml) was layered on 1 ml of 2 M sucrose cushion (2 M sucrose, 10 mM HEPES, pH 7.4) for centrifugation at 100,000*g* for 90 min at 4°C. The resulting microsomes were collected. Subfraction of the total microsomes into rough ER, smooth ER and Golgi complex were prepared according to Banerjee and Redman [1984]. The total microsomes were adjusted to density 1.16 g/ml with an appropriate volume of 2 M sucrose. The total microsome fraction (3 ml) was layered on a 2 M sucrose (3 ml) and layered successively with 1.1 M sucrose (3 ml), 0.6 M sucrose (1.5 ml), and 0.25 M sucrose (1 ml) and centrifuged at 100,000*g* for 18 h at 4°C. Golgi, smooth ER and rough ER banded at 0.6 M/1.1 M, 1.1 M/1.38 M, and 1.38 M/2 M sucrose interfaces, respectively, were collected. The samples were either directly mixed with SDS–PAGE loading buffer and denatured by boiling at 100°C for 5 min in the presence of 5% 2-mercaptoethanol for immunoblot analysis, or digested with trypsin [Liao et al., 1998] and then mixed with equal volume of 4% sodium cholate in HEPES-buffered saline (100 mM HEPES, pH 7.4, 400 mM NaCl) containing 1 mM PMSF, 0.1 mM ALLN, 5 mM NEM, and complete protease inhibitors for immunoprecipitation with the indicated antibodies.

Immunofluorescence Staining

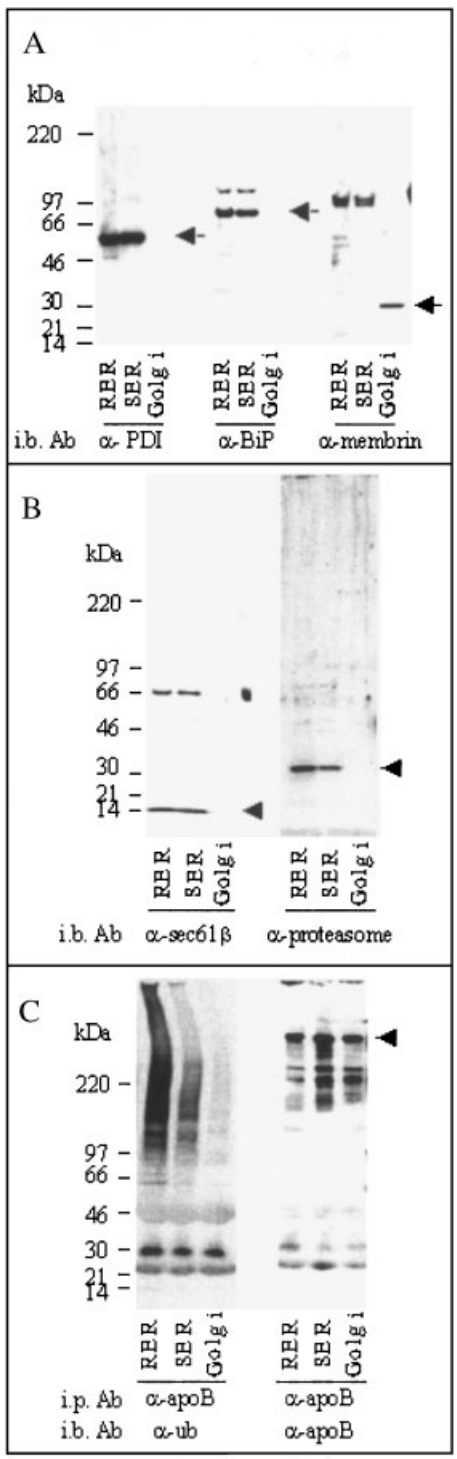
Cells were cultured on 1 cm glass coverslips. The cells were rinsed briefly with ice-cold PBS and then fixed and permeabilized with cold methanol at −20°C for 10 min. Alternatively, cells were fixed at room temperature in 4% paraformaldehyde (30 min) and permeabilized with 0.5% Triton X-100 in PBS (30 min). The cells were then washed, blocked in 10% nonfat milk in PBS for 30 min, incubated with individual primary antibodies in 10% nonfat milk in PBS for 1 h and then washed five times followed by incubation with the corresponding secondary antibodies that were conjugated with FITC or Texas Red. DNA was counterstained by brief exposure to 1 μg/μl DAPI.

RESULTS

Components Involved in the Proteasomal Degradation Pathway Are Associated With the Rough ER and Smooth ER

We first isolated total microsomes from HepG2 cells and fractionated them into rough ER, smooth ER and Golgi fractions. The purity of individual fractions was assessed by immunoblot analysis using antibodies against specific

ER and Golgi markers. As shown in Figure 1A, we found that the ER markers, PDI and BIP, were detected exclusively in the rough ER and smooth ER fractions, and a Golgi marker, membrin [Lowe et al., 1997], was detected only in the Golgi fraction, attesting to the relative purity of the fractions. We have previously shown that ubiquitin-proteasome-mediated degradation occurs on the cytosolic surface of microsomes in HepG2 cells [Liao et al., 1998]. In this study we determined if protein components involved in the proteasomal degradation pathway, such as sec61 and proteasome proteins, are associated with the rough ER, smooth ER or Golgi. We found that sec61β and proteasome proteins were present in both the rough ER and smooth ER fractions, but not in the Golgi fraction (Fig. 1B). This finding is consistent with a recent study in yeast [Enenkel et al., 1998].



ApoB Ubiquitination Is Associated With ER But Not Golgi

To further assess the sites in the secretory pathway where apoB ubiquitination takes place, we immunoprecipitated apoB from the various purified organelle fractions using polyclonal anti-apoB antibody [Liao et al., 1998; Liao and Chan, 2000, 2001], separated the immunoprecipitates on SDS–PAGE and immunoblotted the gels with anti-ubiquitin or anti-apoB antibodies. We found that apoB-ubiquitin conjugates were readily detectible in the rough and smooth ER fractions (Fig. 1C, left panel), but little ubiquitinated apoB was detected in the Golgi fraction. As expected, immunoreactive apoB was present in all organelle fractions in the secretory pathway (Fig. 1C, right panel).

While apoB Ubiquitination Occurs on the Cytosolic Surface of the ER, Transmembrane Conformation of apoB Persists Through the Entire Secretory Pathway

We previously showed that the ubiquitin-proteasome-mediated degradation of apoB occurs on the cytosolic surface of the microsomes [Liao et al., 1998]. We also showed by subcellular fractionation that apoB-ubiquitin conjugates

Fig. 1. Components involved in the proteasomal degradation pathway are associated with the rough ER and smooth ER. Rough ER, smooth ER and Golgi apparatus were prepared from HepG2 cells (see ''Materials and Methods''). Equivalent amount of each compartment was fractionated by SDS–PAGE, immunobloted with antibodies against ER markers (PDI and BiP) or Golgi marker (membrin) (**panel A**) or with antibodies against sec61β and proteasome proteins (**panel B**). Rough ER, smooth ER and Golgi complex were prepared from HepG2 cells treated with ALLN (0.1 mM) for 2 h. Equivalent amount of each compartment was immunoprecipitated with goat polyclonal anti-apoB. The immunoprecipitates were fractionated by SDS–PAGE, immunobloted with mouse monoclonal antibodies against apoB (1D1) or ubiquitin (**panel C**).

were associated with the rough and smooth ER but not the Golgi fraction (Fig. 1). To test whether apoB ubiquitination occurs on the cytosolic surface of the ER, we treated each organelle fraction with trypsin, and then immunoprecipitated the treated and untreated fractions with anti-apoB antibodies. Individual immunoprecipitates were fractionated on SDS–PAGE and immunoblotted with anti-ubiquitin or anti-apoB antibodies. Again, in the untreated fraction we detected apoB-ubiquitin conjugates associated the rough and smooth ER fractions (Fig. 2, left panel, lanes 1 and 3) but little, if any, ubiquitinated apoB in the Golgi fraction (Fig. 2, left panel, lane 5). Trypsin treatment almost completely removed the apoB ubiquitin conjugates in the rough ER (Fig. 2, left panel, lane 2 vs. lane 1) and smooth ER fractions (Fig. 2, left panel, lane 4 vs. lane 3), indicating that apoB ubiquitination takes place on the cytosolic surface of these compartments. Trypsin treatment also removed a substantial proportion of apoB-100 in the rough ER (~89%, Fig. 2, middle panel, lane 2 vs. lane 1) and the smooth ER (~93%, Fig. 2, middle panel, lane 4 vs. lane 3). A substantial, though smaller, proportion of apoB-100 in the Golgi fraction was also accessible to exogenously added trypsin (~68%, Fig. 2, middle panel, lane 6 vs. lane 5). In contrast, albumin, another secretory protein, was completely inaccessible to exogenously added trypsin in the rough ER (Fig. 2, right panel, lane 2 vs. lane 1), smooth ER (Fig. 2, right panel, lane 4 vs. lane 3) or Golgi fractions (Fig. 2, right panel, lane 6 vs. lane 5). These data indicate that, unlike the rough and smooth ERs, apoB ubiquitination is not detectable on the cytosolic surface of the Golgi; in contrast, the transmembrane orientation of apoB appears to span the entire secretory pathway, from the rough and smooth ERs, to the Golgi apparatus.

Proteasomal Degradation of apoB Occurs While apoB Is Being Transported Through the Entire Secretory Pathway

Treatment of HepG2 cells with a proteasome inhibitor, ALLN, led to an accumulation of apoB in the rough (Fig. 3, lane 4 vs. lane 1) and smooth ER (Fig. 3, lane 5 vs. lane 2) as well as Golgi fractions (Fig. 3, lane 6 vs. lane 3) (Fig. 3, left

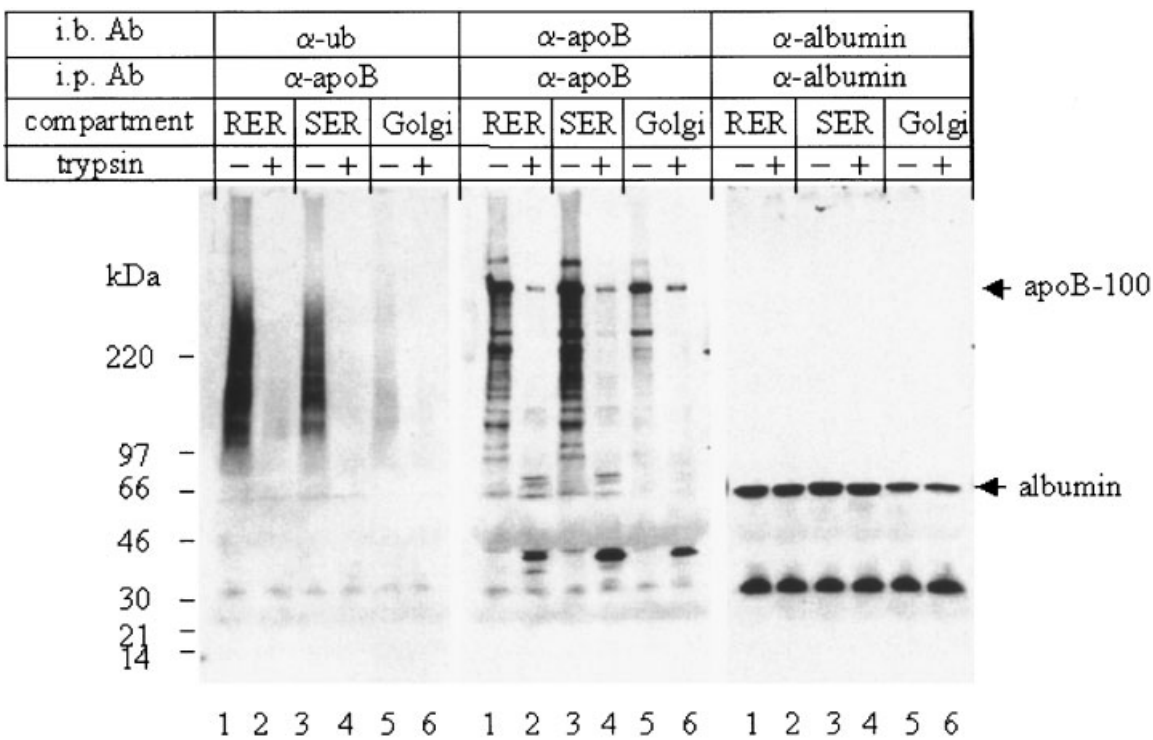


Fig. 2. While ubiquitination of apoB takes places on the cytosolic surface of the rough ER and smooth ER, apoB exists as transmembrane orientation beyond the ER. Rough ER, smooth ER and Golgi complex were prepared from HepG2 cells treated with ALLN (0.1 mM) for 2 h. Equivalent amount of each compartment was trypsinized and immunoprecipitated with goat polyclonal anti-apoB or albumin. The immunoprecipitates were fractionated by SDS–PAGE, immunobloted with mouse monoclonal antibodies against apoB (1D1) or ubiquitin or with polyclonal antibody against albumin as indicated.

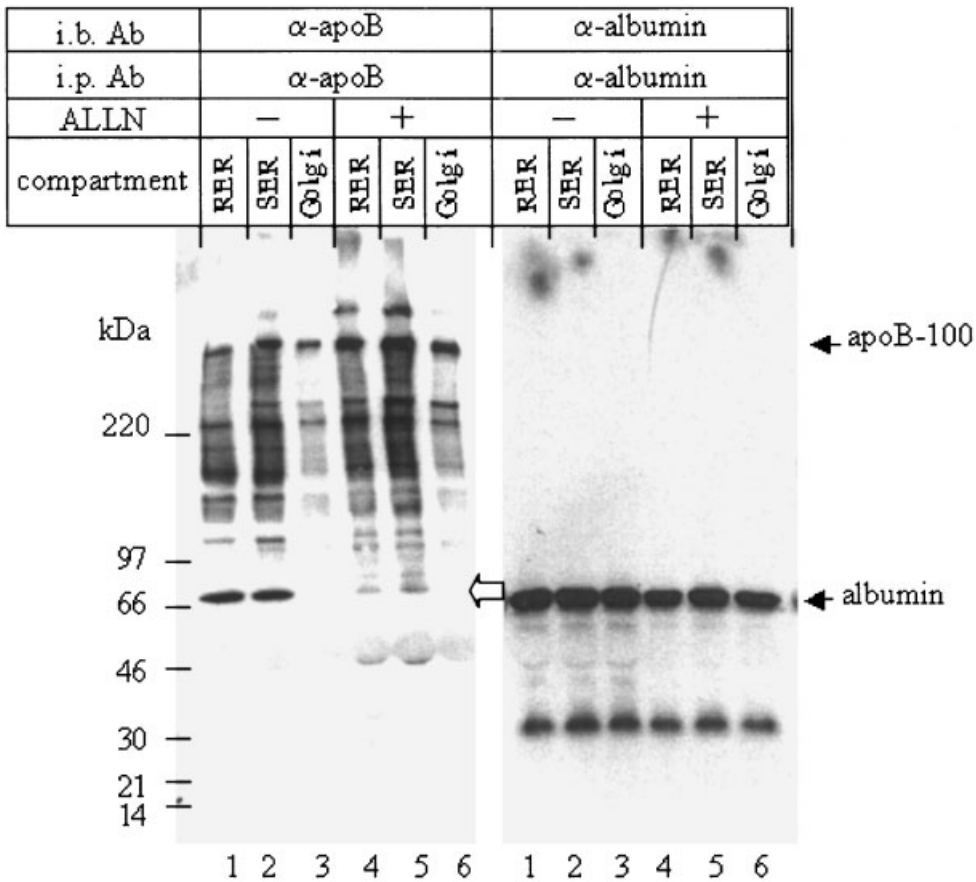


Fig. 3. ApoB accumulation in the ER as well as in the Golgi in response to proteasome inhibitor treatment. Rough ER, smooth ER and Golgi complex were prepared from HepG2 cells treated with ALLN (0.1 mM) or with vehicle (DMSO) for 2 h. Equivalent amount of each compartment was immunoprecipitated with goat polyclonal anti-apoB or albumin. The immunoprecipitates were fractionated by SDS–PAGE, immunobloted with mouse monoclonal antibodies against apoB (1D1) or with polyclonal antibody against albumin as indicated. Open arrow indicates the 69-kDa apoB N-terminal fragment.

panel), but no effect on immunoreactive albumin in any of these compartments (Fig. 3, right panel). These data suggest that apoB undergoes proteasomal degradation throughout the entire synthetic and secretory pathway, in the rough and smooth ERs as well as Golgi apparatus; however, we cannot completely exclude the possibility of the continued trafficking of ubiquinated apoB from the ERs down the secretory pathway to the Golgi apparatus.

It is interesting to note that an ~69 kDa N-terminal apoB fragment identified by a monoclonal antibody 1D1 [as described by Du et al., 1994] was found in the rough and smooth ER fractions (Fig. 3, left panel, lanes 1 and 2, indicated by an open arrow) but not the Golgi fraction (Fig. 3, lane 3). Addition of ALLN increased the intensity of full-length apoB, but markedly reduced that of the 69-kDa (Fig. 3, left panel, lane 4 vs. lane 1 for rough ER; lane 5 vs. lane 2 for smooth ER), indicating that this N-terminal apoB fragment is derived from the proteasomal cleavage of full-length apoB. These data as those of Du et al. [1994] indicate a transmembrane orientation of apoB in the rough and smooth ER with its 69 kDa N-terminus in the luminal side of the ER. Our data further suggest that apoB, while being transported into the Golgi, may be retrogradely translocated into the cytoplasmic compartment (but still associated with the Golgi) for proteasomal degradation or is simply secreted as evidenced by the absence of the 69-kDa N-terminal apoB in the Golgi (Fig. 3, left panel, lanes 3 and 6).

A Small Proportion of apoB Is Dislodged Into the Cytosol Where It Undergoes Ubiquitination for Proteasomal Degradation

We next asked whether apoB could completely dislodge from the secretory pathway

into the cytosol for ubiquitination. To address this question, we separated the cytosol and the microsomes from HepG2 cells. ApoB was immunoprecipitated from both fractions. Immunoprecipitated apoB was separated on SDS–PAGE and analyzed by immunoblotting with anti-ubiquitin and anti-apoB antibodies. The majority of the apoB (full-length as well as smaller fragments) was found to be associated with microsomes but a substantial proportion was detected in the cytosolic compartment (Fig. 4A, lane 3 vs. lane 4). Thus, some apoB was dislodged from the secretory pathway into the cytosol where it underwent ubiquitination, resulting in the presence of apoB-ubiquitin conjugates in this compartment (Fig. 4A, lane 1). It is interesting to note that, consistent with our previous observation [Liao et al., 1998], in the microsomes, the largest apoB-ubiquitin conjugates had apparent molecular masses that were substantially larger than ~550 kDa, size of full-length apoB (Fig. 4A, lane 2). However, essentially all apoB-ubiquitin conjugates isolated from the cytosol had apparent molecular masses smaller than 550 kDa (Fig. 4A, lane 1). The presence of significant amounts of full-length apoB in the cytosol without the simultaneous occurrence of apoB-ubiquitin conjugates with sizes larger than the full-length protein indicates that most of the full-length apoB dislodged in the cytosol was not ubiquitinated yet, or it was rapidly being de-ubiquitinated in this compartment. Comparison between lactacystin-treated cells with vehicle-treated cells revealed that apoB was increased in both the microsomes and in the cytosol fraction (Fig. 4B, lane 3 vs. lane 1 for cytosol; lane 4 vs. lane 2 for microsomes), indicating that, like the ER-associated apoB, the apoB that is dislodged into the cytosol is also degraded by the proteasome pathway.

Intracellular Accumulation of apoB After Proteasome Inhibition

Despite the fact that proteasome inhibitors, such as ALLN and lactacystin, cause the intracellular accumulation of apoB in HepG2 cells, the amount of apoB secreted is generally unchanged [Liao et al., 1999] unless an optimal amount of oleic acid is present in the culture medium [Mitchell et al., 1998]. These observations suggest that the apoB polypeptides destined for proteasome degradation are not competent for secretion even when degradation is blocked by proteasome inhibitors. Thus, the pool of apoB polypeptides that accumulates in HepG2 cells in the presence of proteasome inhibitors may have properties different from those in untreated cells. By immunofluorescence staining, we found that, in parallel analysis,

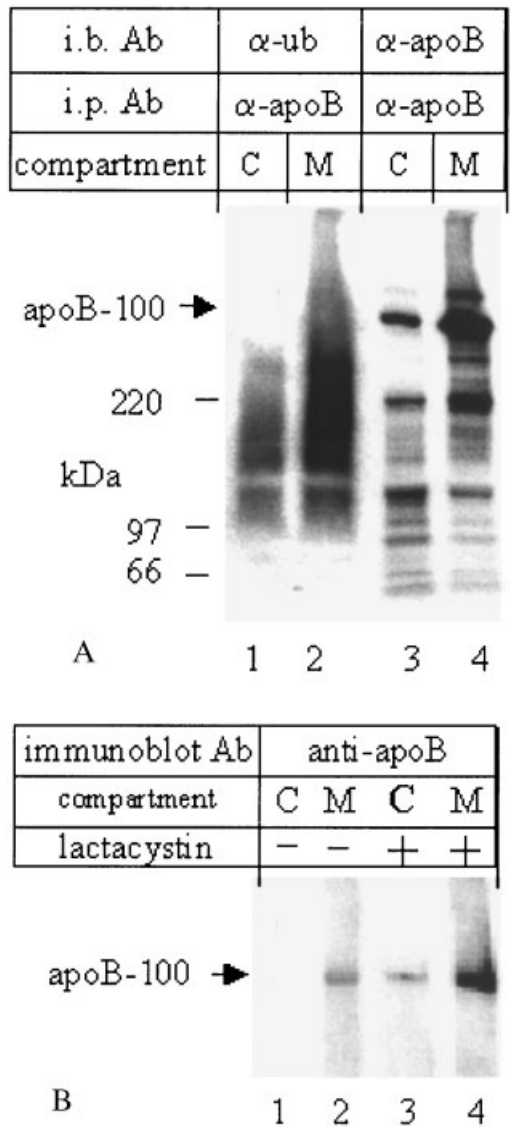


Fig. 4. Ubiquitination and proteasomal degradation of apoB occurs when apoB is associated with the microsomes as well as after the apoB is dislodged into the cytosolic compartment. Total microsomes (M) and cytosol (C) were prepared from HepG2 cells treated with lactacystin (10 μM) for 2 h. Equivalent amount of each compartment was immunoprecipitated with goat polyclonal anti-apoB. The immunoprecipitates were fractionated by SDS–PAGE, immunobloted with mouse monoclonal antibodies against apoB (1D1) or ubiquitin as indicated (**panel A**). In a separate experiment, total microsomes (M) and cytosol (C) were prepared from HepG2 cells treated with lactacystin (10 μM) or DSMO for 2 h. Equivalent amount of each compartment was fractionated by SDS–PAGE and immunobloted with mouse monoclonal antibodies against apoB (1D1) (**panel B**).

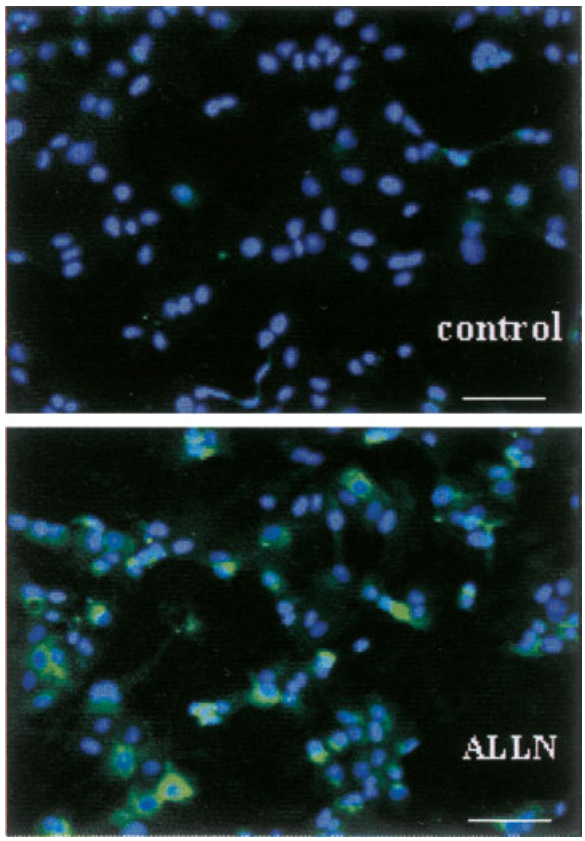


Fig. 5. ApoB accumulation in the cells in response to proteasome inhibitor treatment. HepG2 cells cultured on coverslips were treated with ALLN (0.1 mM) or with vehicle (DMSO) for 16 h. The cells were fixed with paraformadelhyde and permeabilized with Triton X-100 and probed with monoclonal antibody against apoB (1D1) followed with FITC conjugated antibody against mouse IgG (green). Cell nuclei were costained with Dapi (blue). Bar, 100 μM. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com]

apoB accumulated in cells treated with ALLN (Fig. 5) or lactacystin (data not shown) but not in control cells (Fig. 5). The apoB in ALLN-treated cells showed a broad distribution with a punctate appearance. Comparing apoB and concanavalin A (which decorates the ER) distribution in the presence of proteasome inhibition, we found that apoB immunostaining colocalized with concanavalin A (Fig. 6), but not with the centrosome (decorated by a γ-tubulin antibody, data not shown), the usual location of aggresomes, an indication that a large proportion of apoB in the cells treated with the proteasome inhibitor is associated with the ER, in agreement with results of immunoblot analysis of apoB isolated from various subcellular compartments in the secretory pathway (Figs. 3 and 4).

DISCUSSION

The ubiquitin-proteasome pathway has been identified as a major mechanism mediating the intracellular degradation of apoB in HepG2 cells. Degradation by this pathway occurs at both co- and post-translational levels [Liao et al., 1998; Zhou et al., 1998]. We previously reported that ubiquitination and proteasomal degradation of apoB occur on the cytosolic surface of the microsomes [Liao et al., 1998]. In this study, we further defined the ubiquitin-dependent proteasomal degradation of apoB on the cytosolic surface of rough ER and smooth ER. We also found that a proportion of apoB was dislodged into the cytosol where it underwent ubiquitination for proteasomal degradation. The ER is the port of entry and the site where proteins de-

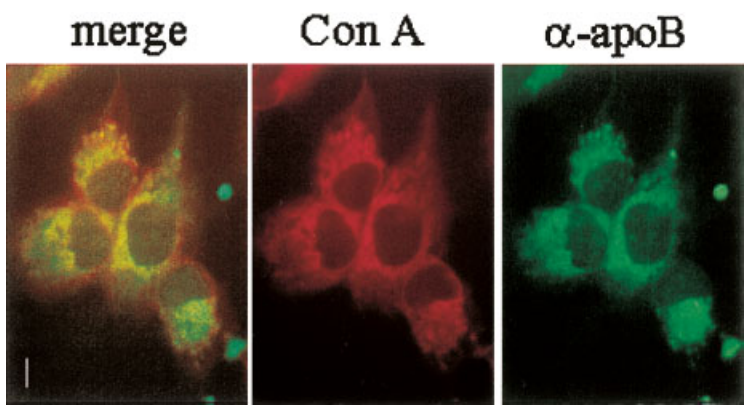


Fig. 6. ApoB is localized in the ER after proteasome inhibitor treatment in HepG2 cells. HepG2 cells cultured on coverslips were treated with ALLN (0.1 mM) for 16 h. The cells were fixed and permeabilized with cold methanol and probed with monoclonal antibody against apoB (1D1) followed with FITC conjugated antibody against mouse IgG (green) and with Texas Red conjugated Con A. Bar, 10 μM. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com]

stined for the secretory pathway are assembled into their properly folded conformation. A stringent quality control is coupled to the folding machinery, ensuring that only properly folded proteins reach the Golgi. It is generally believed that the proteins that fail to attain their correct three-dimensional structure are retained in the ER and are eventually degraded by the cytoplasmic proteasome after retrograde translocation into the cytosol [Kim and Arvan, 1998; Ciechanover et al., 2000a,b]. Our finding that little ubiquitinated apoB went past the ER appears to be consistent with this concept. However, we demonstrated that the transmembrane orientation of apoB persists through the entire secretory pathway including the Golgi where it remained accessible to both exogenously added proteases (Fig. 2) and to the cytosolic proteasomal degradation machinery. While a significant amount of apoB in the Golgi is exposed to the cytosol and degraded by proteasome, we detected little apoB-ubiquitin, indicating that Golgi-associated apoB degradation is largely mediated by an ubiquitin-independent proteasomal degradation pathway. Involvement of a post-ER compartment in the degradation of apoB [Wang et al., 1995] as well as the occurrence of a transmembrane orientation of apoB in the Golgi apparatus [Wilkinson et al., 1993] were previously noted in hepatocyte cultures. Fisher et al. [Fisher et al., 2001] recently reported that omega-3 fatty acids induce apoB degradation involving a post-ER compartment.

When the amount of some misfolded protein exceeds the cellular degradative capacity, the protein can accumulate in a novel, centrosome-associated subcellular structure coined an aggresome [Johnston et al., 1998; Garcia-Mata et al., 1999; Wigley et al., 1999; Kopito, 2000]. Overexpression of cystic fibrosis conductance regulator (CFTR), or inhibition of proteasome activity in cells expressing CFTR, led to the accumulation of stable, ubiquitinated aggregates of CFTR [Johnston et al., 1998; Wigley et al., 1999]. The same phenomenon was observed with other proteins including presenilin-1 [Johnston et al., 1998] and a hybrid protein composed of the green fluorescent protein (GFP) fused to a 250-amino acid fragment of the cytosolic protein, p115 [Garcia-Mata et al., 1999]. These disparate misfolded proteins deposit at and around the centrosome as aggresome and their accumulation is microtubule-based. Aggresome formation was postulated to be a general cellular response to the presence of aggregated, nondegraded proteins [Johnston et al., 1998].

In this study, the apoB that accumulates in proteasome inhibitor-treated HepG2 cells largely colocalized with the ER (by concanavalin A staining) but not with the centrosome (as revealed by γ-tubulin staining), indicating that the apoB aggregate is not associated with aggresomes. The morphological data are consistent with the results of the subcellular fractionation experiments shown in Figs. 3 and 4.

In conclusion, we showed that ubiquitin-dependent proteasomal degradation of apoB occurs on the cytosolic surface of the rough and smooth ER and that a small proportion of apoB is dislodged into the cytosol where it is ubiquitinated. We also found that apoB has a transmembrane orientation through the entire secretory pathway, being partially exposed to the cytosolic degradation machinery both in the ERs and in the Golgi. Though the Golgi-associated apoB appeared not to be ubiquitinated, it underwent degradation that was inhibited by ALLN, presumably via the proteasome-mediated pathway, as it had been shown that the ALLN effect on apoB in HepG2 cells was mediated by inhibition of proteasomal degradation [Yeung et al., 1996; Fisher et al., 1997]. Thus, our data suggest that Golgi-associated proteasomal degradation of apoB is mediated by an ubiquitin-independent pathway. They further indicate that, unlike aggregates of some other proteins such as overexpressed or mutant CFTR, the intracellular aggregates of apoB do not form aggresome structures. These observations on apoB biogenesis underscore the uniqueness of this protein during its production and transit through the secretory pathway in the HepG2 liver cell model.

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