Apoprotein B degradation is promoted by the molecular chaperones hsp90 and hsp70

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Summary

Apoprotein B (apoB) is the major protein of hepatic-derived atherogenic lipoproteins. The net production of apoB can be regulated by pre-secretory degradation mediated by the ubiquitin-proteasome pathway and cytosolic hsp70. To further explore the mechanisms of apoB degradation, we have established a cell-free system in which degradation can be faithfully recapitulated. Human apoB48 synthesized in vitro was translocated into microsomes, glycosylated, and ubiquitinylated. Subsequent incubation with rat hepatic cytosol led to proteasome-mediated degradation. To explore whether hsp90 is required for apoB degradation, geldanamycin (GA) was added during the degradation assay. GA increased the recovery of microsomal apoB48 approximately 3-fold and disrupted the interaction between hsp90 and apoB48. Confirming the hsp90 effect in the cell-free system, we also found that transfection of hsp90 cDNA into rat hepatoma cells enhanced apoB48 degradation. Finally, apoB48 degradation was reconstituted in vitro using cytosol prepared from wild type yeast. Notably, degradation was attenuated when apoB48-containing microsomes were incubated with cytosol supplemented with GA, or with cytosol prepared from yeast strains with mutations in the homologues of mammalian hsp70 and hsp90. Overall, our data suggest that hsp90 facilitates the interaction between ER-associated apoB and components of the proteasomal pathway, perhaps in cooperation with hsp70.
Introduction

Apoprotein B (apoB) is a very large (540kDa) protein and is essential for the assembly and secretion of hepatic lipoproteins. ApoB is the major protein component of low density lipoprotein (LDL), the atherosclerosis-causing particle that transports cholesterol in the blood. During the translation of apoB in the liver, lipids are transferred to the nascent protein by the ER-resident microsomal triglyceride transfer protein (MTP) to form a “primordial lipoprotein”. In hepatocytes and hepatocarcinoma-derived cell lines, apoB either undergoes assembly with lipids and secretion or is subjected to ER retention and intracellular degradation (1-3). We have previously shown that this degradation in HepG2 cells is accomplished by the ubiquitin-proteasome pathway and involves cytosolic hsp70 (4-6). The degree of proteasomal degradation appeared to be regulated by the availability of the lipid-ligands for apoB (4).

There are a number of ER lumenal and ER transmembrane proteins (7) that have also been reported to undergo proteasomal degradation. This process has been referred to as ER-associated degradation (ERAD) (8,9) and for a number of substrate proteins, a common scenario appears to be that the nascent protein undergoes translocation, followed by retrotranslocation and release into the cytosol, where it is degraded by the proteasome. Though the route by which apoB is targeted to the proteasome is not completely defined, certain features of its degradation may differ from this model. In particular, apoB can assume a bitopic topology during or shortly after translocation, resulting in domains that can be accessed by cytosolic or ER lumenal factors, with degradation by the proteasome
accomplished without the requirement of apoB to be fully retrotranslocated (6).

As noted above, the molecular chaperone hsp70 facilitates ERAD of apoB in HepG2 cells (4). Molecular chaperones play important roles in assisting the folding and assembly of proteins, facilitating protein translocation across a variety of intracellular membranes, and the targeting of misfolded proteins for degradation, including secretory or transmembrane proteins destined for ERAD (9,10). The most abundant chaperone in the eukaryotic cytosol is hsp90, which has been shown to be involved in the maturation of signal-transduction proteins, such as steroid hormone receptors (11,12). The effect of hsp90 can be specifically inhibited by geldanamycin (GA), an ansamycin antibiotic that competes for the ATP binding site (13). A positive role for hsp90 in the maturation of CFTR has recently been proposed (14) based on data showing that proteasomal degradation was accelerated by the disruption of hsp90 association with CFTR by the addition of GA to the cell culture medium. Alternatively, other recent studies have suggested that hsp90 can destabilize and promote the ERAD of mutant proteins (e.g., CFTR Δ508 (15) and insulin receptor ΔEx13-IR (16)).

Given this recent controversy, we were particularly interested in investigating the role of hsp90 in the ERAD of apoB. In order to define the mechanisms by which apoB enters the ubiquitin-proteasome pathway and the cytosolic factors involved (including hsp90), we sought to develop a cell-free degradation system. One particular advantage of such a system would be the ability to examine the interactions of chaperones with apoB without changing their steady-state levels. In this report, we summarize the characteristics of the system and the evidence obtained from it suggesting that hsp90
promotes apoB degradation. Importantly, the results from the cell-free system were consistent with those from hepatoma cells transfected with hsp90 cDNA.
Experimental Procedures

Materials – The plasmid encoding apoB48 was from V. Lingappa (UCSF); ubiquitin-HA protein and proteasome inhibitor PSI were from Z. Ronai and S. Wilk, respectively, Mount Sinai School of Medicine. Rat hepatic cytosol was purified from Sprague-Dawley rat liver (17). Yeast cytosols (from strains JN516 [SSA1], JB67 (ssa1-45), and G313N (18-20)) were prepared as described earlier (8). The plasmid encoding human hsp90β was provided by W. Sessa (Yale University). Mouse anti-HA monoclonal antibody (HA11) was purchased from BabCo (Berkeley, CA). Anti-hsp90 (SPA-835 and SPA-845) and anti-grp-94 (SPA-850) antibodies were obtained from StressGen (Victoria, Canada). Protein A sepharose and γ-Bind-plus-Sepharose were purchased from Amersham Pharmacia Biotech (Piscataway, NJ). Ubiquitin aldehyde, lactacystin and MG132 were purchased from Calbiochem (San Diego, CA). Peptide N-glycosidase F (PNGF) was obtained from Roche Molecular Biochemicals (Indianapolis, IN). Cell culture media and related supplies were purchased from Gibco BRL (Rockville, MD). Other reagents were purchased from Sigma (St. Louis, MO) unless otherwise stated.

Cell-free transcription/translation and quantification of apoB48 – ApoB48 was synthesized in a coupled transcription-translation reaction (2 h, 30°C) using the SP6-TNT kit (Promega, Madison, WI) and 0.5mCi/ml [35S] Protein Labeling Mix (1000Ci/mmol, New England Nuclear, Boston, MA) in the presence of dog pancreatic microsomes (DPM; Promega). To assess the characteristics and the level of labeled apoB48, samples were mixed with gel loading buffer (contents: 125mM Tris-HCl pH6.8, 20% SDS, 20% glycerol, 5% β-mercaptoethanol, 2.5% bromophenol blue), the proteins
were resolved by SDS-PAGE, and a fluorogram was made from the resulting gel. The intensities of the apoB48 band signals were measured by densitometry of the fluorograms.

Trypsin treatment to assess apoB48 topology – The reaction mixture at the end of the transcription-translation procedure was treated with trypsin for 1h at 0°C at a final concentration of 0.1mg/ml. Trypsin inhibitor at final concentration 0.2mg/ml was added for 10 min to stop the reaction. The concentrations of trypsin and typsin inhibitor were optimized in pilot experiments.

Peptide N-glycosydase F (PNGF) treatment to assess apoB48 glycosylation – At the end of the transcription-translation procedure, the reaction mixture was layered onto SH buffer (0.25 M sucrose, 5mM HEPES, pH 7.4) and centrifuged at 100 000 x g for 30 min at 4°C. The pelleted microsomal membranes were resuspended in PBS. To 10µl of this suspension, the following were added: 1µl 10% 2-mercaptoethanol, 2.5µl of 10% Triton X100, 1µl of 100mM PMSF, 1µl of PNGF (0.1unit/µl), 4.7µl of H2O, 0.8µl of 5% SDS. This mixture was then incubated for 16 h at 4°C.

Flotation analysis of microsome-associated apoB48 – The association of apoB48 with microsomes was confirmed by flotation of the products of an in vitro transcription/translation reaction through a sucrose gradient (21). In brief, an in vitro transcription/translation was performed as described above, after which the reaction was diluted into 100µl of MSB (50mM HEPES pH 7.6, 150mM NaCl, 5mM EDTA, 1mM DTT, and supplemented with PMSF, leupeptin, and pepstatin according to the manufacturers specifications) and then mixed with 300µl of 2.3 M sucrose in MSB. This
mixture was overlaid onto 300µl of 2.3 M sucrose in MSB in a centrifuge tube. Solutions of 1.5 M sucrose (600µl) and 0.25 M sucrose (500µl) in MSB were overlaid and the discontinuous gradient was centrifuged at 100 000 x g for 5 h at 4°C, after which 150µl aliquots were successively removed from the top of the gradient. Proteins in the fractions were resolved by SDS-PAGE and the radiolabeled apoB48 was detected by phosphorimager analysis of a dried gel.

ApoB48 degradation assay – At the end of the transcription-translation procedure, the reaction mixture was layered onto SH buffer (0.25M sucrose, 5mM HEPES, pH 7.4) and centrifuged at 100 000 x g for 30min at 4°C. The pelleted microsomal membranes were resuspended in 50% (vol/vol) of the appropriate lysate and 50% of 2xPh buffer (40mM HEPES pH 7.4, 220mM KCl, 10mM MgCl₂). At the following final concentrations, 2mM ATP, 10mM creatine phosphate, and 100µg/ml of creatine kinase were added to the complete mixture. Aliquots of the mixture were incubated for 2 h either on ice (control) or at 37°C. At the end of the incubation period, gel loading buffer for SDS-PAGE was added directly to the samples. In some experiments, the proteasome inhibitors (dissolved in DMSO) indicated in the Results were added to the degradation mixture just prior to the 2 h incubation. An equivalent volume of DMSO was added to the control sample.

In some experiments, the following variations were made. To determine the effect of depleting hsp90 on the recovery of apoB48 after the 2 h degradation assay, 100µl samples of rat hepatic lysate were pre-treated with 15µg of hsp90 antibody and 500µl of 5% protein A-sepharose (to collect the chaperone-antibody complexes). The control lysates were treated similarly, except that the chaperone antibodies were omitted. The
control and depleted lysates were then used for the degradation assay. To examine the dependence on the ubiquitinylation of apoB48 for degradation in RRL, just prior to its use in the degradation assay, RRL was supplemented with ubiquitin aldehyde (ubal), an inhibitor of deubiquitinylation (22), at a concentration range of 0-20 \(\mu\)M.

**Effect of geldanomycin (GA) on apoB48 degradation** — At the end of the transcription-translation procedure, the reaction mixture was layered onto SH buffer and centrifuged at 100 000 x g for 30 min at 4°C. The pelleted microsomal membranes were resuspended in 50% (vol/vol) of the rat hepatic lysate and 50% of 2xPh buffer. At the following final concentrations, 2mM ATP, 10mM creatine phosphate, and 100\(\mu\)g/ml of creatine kinase were added to the complete mixture. The mixture was halved and GA (National Cancer Institute, Bethesda, MD) dissolved in DMSO was added to one aliquot at a final concentration of 30\(\mu\)M; an equal volume of DMSO was added to the other aliquot. Samples were taken every 30 min during a 2 h incubation (37°C) period. At the end of the incubation, gel loading buffer for SDS-PAGE was added directly to the samples and the apoB48 content analyzed as above.

**ApoB48 ubiquitinylation assay** — At the end of the transcription-translation procedure, the reaction mixture was layered onto SH buffer and centrifuged as above. The pelleted microsomal membranes were resuspended in 50% (vol/vol) of the appropriate lysate and 50% of 2xPh buffer. To this mixture ATP\(\gamma\)S and HA-tagged ubiquitin protein were added to final concentrations of 5mM and 0.1mg/ml, respectively. The mixture was incubated at 37°C for 1 h. Ubiquitinylated apoB48 was immunoprecipitated with anti-HA antibody.
**Cell culture studies**– Rat hepatoma McA-RH7777 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum, 10% horse serum, 2mM L-glutamine, 100 U/ml penicillin and 100µg/ml streptomycin in 5% CO₂ at 37°C. The medium was changed every 3 days. LipofectAMINE PLUS reagent (Gibco BRL) was used to transfect McA-RH7777 cells with either the pcDNA3 vector (control) or pcDNA3-hsp90β plasmid according to the protocol of the manufacturer. After 48 h, cells were pulse-labeled with [³⁵S] Protein Labeling Mix (NEN) (100 µCi/ml) and chased in label-free DMEM medium, containing 0.5% fetal bovine serum, 0.5% horse serum, 2mM L-glutamine, 10mM L-methionine, 3mM L-cysteine. Cell lysates and conditioned media were analyzed by immunoprecipitation with anti-apoB antibody or anti-rat albumin antibody (Bethyl laboratories, Montgomery, TX). The amount of labeled immunoprecipitated proteins was measured by SDS-PAGE/fluorography.

**Immunoprecipitation and Western blotting**– Samples for immunoprecipitation were diluted in buffer, containing 50mM Tris-HCl (pH 7.5), 150mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, and incubated with appropriate antibodies at 4°C for 2 h followed by incubation with Protein A or G sepharose at 4°C for 2 h. The beads were washed 3 times with the same buffer and immunoprecipitated material was released by heating at 100°C in SDS-PAGE sample buffer. For Western blots, proteins were first resolved by SDS-PAGE, then transferred to a PVDF membrane (New England Nuclear, Boston, MA). Typically, the primary and secondary antibodies were used at 1:1000 and 1:10 000 dilution, respectively, with final detection of signal by the Western Blot Chemiluminescence Reagent Plus (New England Nuclear, Boston, MA).
Results

Biogenesis of apoB48 in vitro - For our studies, we have used the apoB48 species which is expressed in the intestine of all mammals and in the rodent liver. Though shorter than apoB100 (expressed only in mammalian liver), apoB48 still displays in cell culture studies many similar properties with respect to translocation, membrane integration and degradation (23). ApoB48 (MW ~240kD) was synthesized from its cDNA by in vitro transcription/translation in rabbit reticulocyte lysate (RRL) (Figure 1). Translation performed in the presence of dog pancreatic microsomes (DPM) resulted in the appearance of two products (Figure 1, lane 2), implying translocation and post-translational processing. Insertion of apoB48 into the microsomal lumen was confirmed by a protease protection assay. At the end of the transcription/translation procedure, the reaction mixture was incubated with trypsin in the presence or absence of Triton X100 (Figure 2A). Note that the upper band was protected from trypsin digestion in the absence of Triton X100 (Figure 2A, lane 2), indicating that this product translocated across the microsomal membrane, with the lower band representing untranslocated apoB48. This interpretation is consistent with the data reported by Rusinol and colleagues (24). In the presence of TritonX100 (used to destroy membrane integrity), the protein represented by the upper band was digested by trypsin (Figure 2A, lane 3). Thus, the protease resistance in the absence of Triton X100 resulted from proper integration of apoB48 and not from protein aggregation or inadequate protease activity.

To investigate whether the upper band in Figure 1 represented apoB48 that was modified by glycosylation, microsomal associated apoB48 was separated from the
reaction mixture by centrifugation and treated with N-glycosidase F (PNGF). The effect
of PNGF treatment is shown in Figure 2B. Note that PNGF reduced the apparent
molecular weight of the upper band to that of the lower band (lane 2 vs. 3). For
comparison, the total reaction (lane 1) and supernatant after pelleting the microsomes
(lane 4) are also displayed. This reduction of apparent molecular weight of the upper
band by PNGF treatment not only demonstrates modification of apoB48 by
glycosylation, an event also known to occur with apoB in intact cells (25), but also
confirms that in the presence of DPM, there was translocation of apoB48. Treatment with
sodium carbonate at pH 11.5 (26), however, released less than 5% of apoB48 from the
microsomal membrane (data not shown).

To confirm that apoB48 was associated with the microsomal membrane, an in
vitro transcription/translation reaction in the presence of DPM was performed, after
which the microsomes were floated through a sucrose gradient as previously described
(21). As shown in Figure 3, we found that the majority of apoB48 had migrated into
fractions of lower density than that at which it was loaded onto the gradient (denoted by
the arrow), indicative of it being membrane associated.

Together, the sodium carbonate and sucrose gradient data imply that translocated
apoB48 strongly interacts with membrane lipid or protein components, consistent with
previous results using isolated microsomes or intact cells (5,6,27).

ApoB48 is ubiquitinylated and degraded in the cell-free system - Recent studies have
shown that degradation of apoB in HepG2 cells is mediated by the ubiquitin-proteasome
pathway (e.g., (4,28,29)). To determine whether nascent apoB48 was ubiquitinylated in
vitro, HA-tagged ubiquitin was added at the end of the transcription/translation procedure and the incubation was continued at 37°C for 1 h in the presence of ATP and an ATP regenerating system (Figure 4). Ubiquitinylated apoB48 was immunoprecipitated with anti-HA-tag antibody (Figure 4, lane 4). That the high molecular weight material represents bona fide modification of apoB by ubiquitinylation was supported by 3 results: 1) if the antibody to HA tag was omitted, no labeled material was detected (Figure 4, lane 5); 2) when the reaction was depleted of ATP by the ATP-hydrolyzing enzyme apyrase, ubiquitinylation was not observed (Figure 4, lane 3); and, 3) in the absence of the apoB48 translation product, no labeled material was recovered by the anti-HA antibody (Figure 4, lane 6). For comparison, the contents of the transcription/translation reactions (with or without apoB48 cDNA) prior to immunoprecipitation are displayed (in lanes 1 and 2, respectively). Thus, apoB48 become ubiquitinylated in the cell-free system and this process, like in intact cells, is ATP-dependent.

Overall, the data in Figures 1-4 demonstrate that the early events during apoB biogenesis are faithfully reproduced in the cell-free system, and suggest that this represents a valid model for the study of the mechanisms leading to apoB degradation. Towards this goal, microsomal-associated apoB48 was studied in the next series of experiments because we have previously shown in HepG2 cells that apoB destined for proteasomal degradation remains stably associated with microsomes (4-6). Thus, at the end of transcription/translation, microsomes were pelleted and resuspended in RRL or rat hepatic cytosol. The latter cytosol was utilized to study apoB48 degradation because it derives from the tissue in which it is normally expressed.
Aliquots of each reaction mixture were incubated on ice (control condition) and at 37°C for 2 h in the presence of ATP and an ATP regeneration system. Independent of the source of the cytosol, after 2 h, the majority (~75%) of apoB48 was degraded compared to the control (Figure 5, panels A and B, lanes 2 vs. 1). To determine whether this degradation was mediated by the proteasome, the experiments were repeated in the presence or absence of the proteasome inhibitors lactacystin, Cbz-leu-leu-leucinal (MG132), or Cbz-ile-glu(O-t-Bu)-ala-leucinal (PSI) (30). Note that proteasome inhibitors did not significantly reduce degradation in RRL (Figure 5, panels A and C), consistent with the data for CFTR reported by Xiong and colleagues (31), who showed that lactacystin is ineffective and a high concentration of MG132 (200 μM) is only partially effective at blocking degradation of ubiquitinated substrates in RRL. Based on their results, they hypothesized that RRL contains a factor or factors that interfere with the actions of some proteasome inhibitors. Because of this limitation, to support the interpretation that in RRL, apoB48 degradation is accomplished by the ubiquitin-proteasomal pathway and not by another protease, we performed degradation assays in which RRL was supplemented with ubiquitin aldehyde (Ubal), an inhibitor of deubiquitinating enzymes. Ubal has previously been reported to decrease apoB degradation in semi-permeabilized HepG2 cells (32) and exerts a negative effect on proteasomal degradation by interfering with the removal of ubiquitin chains from substrates prior to their entry into the proteasome (e.g., see (22)). The addition of Ubal to RRL resulted in a dose-dependent increase in the recovery of apoB48 at the end of the 2 h degradation assay, from 50% to 80% and 93% at 0, 5 and 20 μM of Ubal, respectively.
Overall, the results from these and the prior (31) studies imply that the degradation of apoB48 in RRL is accomplished by the ubiquitin-proteasome pathway, but the response of proteasomes in RRL to some inhibitors may be anomalous.

In contrast, the proteasome inhibitors effectively inhibited degradation in rat hepatic cytosol (Figure 5, panels B and D). In addition to the increased recovery of apoB48 in the presence of these inhibitors, further evidence for a role of the ubiquitin-proteasome pathway was suggested by a reduction in apoB48 degradation when the cytosol was pre-treated with the ATP-depleting agent apyrase (data not shown). Consistent with the rat hepatic lysate studies, similar results were obtained using human hepatic cytosol (data not shown).

Hsp90 is required for apoB degradation – As reviewed in the Introduction, hsp90 has been shown to either promote or inhibit the interaction of substrate proteins with components of the ubiquitin-proteasome pathway. In light of this controversy, and our finding that in the cell-free system apoB48 can be ubiquitinylated and degraded by proteasomes (Figures 4 and 5), we next explored whether hsp90 plays a role in apoB degradation.

Cytosolic hsp90 has been shown to be the specific target for the ansamycin benzoquinone antibiotics (33,34), particularly geldanamycin (GA). Therefore, GA, the most potent drug from this class antibiotics (35), was added to the degradation mixture and apoB recovery was monitored every 30 min during the subsequent 2 h incubation. The results shown in Figure 6 indicate that the recovery of apoB48 was increased over 3-fold in the presence of GA. It has been previously shown with purified proteasomes, GA
did not reduce the degradation of the model substrate SLLVT-AMC (36), suggesting that GA did not function simply as a proteasome inhibitor in the cell-free degradation assay, but directly interfered with a pro-degradative effect of hsp90 on apoB48.

The homologue of hsp90 in the ER, grp94, which also binds the same class of antibiotics (37), has been shown to interact with apoB100 in HepG2 cells (38). In that study, grp94 was found to interact with apoB100 upon a reduction of the ATP level by apyrase treatment. Note that under our conditions, ATP and an ATP regeneration system are added (Experimental Procedures), making it unlikely that GA's effect was mediated by disrupting an apoB48-grp94 interaction. Nonetheless, to show that the GA effect was specific for cytosolic hsp90, we determined whether either chaperone interacts with apoB48. Using antibodies to either hsp90 or grp94, we attempted to co-immunoprecipitate apoB48 and the results are presented in Figure 7. As shown, apoB48 could be co-immunoprecipitated with hsp90, but not with grp94 (Figure 7A, lane 3 vs. 5). Notably, GA added to the incubation mixture disrupted the interaction of apoB48 with hsp90 (lane 4). The interaction between apoB48 and hsp90 and its disruption by GA was independent of the antibody (anti-hsp90 or anti-apoB) used for the immunoprecipitation (compare Figure 7A to 7B). These results could not be explained by a perturbation of the general pool of hsp90, because Western blotting (Figure 7C) indicated comparable signal intensities for hsp90 in samples untreated and treated with GA. Taken together, the results shown in Figure 6 and 7 imply that hsp90 interacts with apoB48 and promotes apoB48 degradation.

Effect of GA on ubiquitinylation of apoB48 – Because ubiquitin marks proteins for
proteasome-mediated degradation, we wished to determine whether GA treatment mediated the increased recovery of apoB48 by a decrease in its ubiquitinylation.

Microsome-associated apoB48 was incubated at 37°C for 1 h in rat hepatic lysate with HA-ubiquitin, in the presence or absence of GA. Because GA inhibits apoB48 degradation, apoB48 recovery increases (Figure 6). Therefore, to control for this in the analysis of the amount of ubiquitinylated apoB48 (ub-apoB48), the content of apoB48 in each sample was first determined by SDS-PAGE/densitometry. Then, aliquots containing equivalent amounts of apoB48 were immunoprecipitated with an antibody against the HA epitope. As shown in Figure 8, ubiquitinylated apo48 (ub-apoB48) appeared as a smear reaching to the top of the gel; as confirmed by densitometry, there is no significant decrease in the ub-apoB48 signal in the sample treated with GA (Figure 8, lane 3 vs. lane 2).

Transfection with hsp90 cDNA increases apoB degradation in rat hepatoma cells – To establish in intact hepatic cells the relevance of our results implicating hsp90 in apoB48 degradation, rat hepatoma McArdle-RH7777 cells were transfected with hsp90 cDNA. These cells express primarily the apoB100 form of native apoB, the recovery of which we have shown to be increased by lactacystin treatment (39). We took this transfection approach rather than treating the cells with GA because treatment of cells with ansamycin antibiotics not only directly affects protein-chaperone interactions, but increases the level of multiple heat shock proteins by promoting the activation of heat shock transcription factor (HSF1) (40,41). For example, treatment of yeast with ansamycin antibiotics inhibits ERAD due to a secondary increase in BiP concentration (J.L. Brodsky,
unpublished data).

After transfection either with pcDNA3 vector (control condition) or pcDNA3-hsp90β plasmid, cells were pulse-labeled with [35S]-methionine/cysteine for 15 min and chased in isotope-free medium for 30 and 90 min. Cell lysates and conditioned media were then analyzed by immunoprecipitation with antibodies to apoB and rat albumin (as a control). Successful transfection of the cells and expression of the plasmids were confirmed by RT-PCR detection of the plasmid region encoding neomycin antibiotic resistance (data not shown).

As shown in Figure 9 (panel B), transfection with the hsp90 cDNA did not affect albumin synthesis or secretion. In contrast, the total recovery of apoB100 from lysate and medium was lower at the 30 min (21% reduction) and 90 min (48% reduction) chase time points in the cells transfected with the hsp90 cDNA (Figure 9, panel A). These results are consistent with the data from the cell-free system, and support a pro-degradative role for hsp90 in apoB degradation in the liver.

In addition to studying the effects of increasing hsp90 on apoB degradation in hepatic cells, it would have been desirable to also determine the consequences of decreasing the level of a chaperone. Perhaps because of the abundance and relatively long half-lives of many chaperones, it has been difficult to achieve major decreases in intact cells. For example, a ribozyme approach undertaken independently by two laboratories resulted in no more than a 25-30% reduction in cellular grp94 levels (42,43). Thus, we tried to immunodeplete hsp90 from rat hepatic lysate (Experimental Procedures) and to use the depleted lysate in the cell-free degradation assay. Compared
to control rat hepatic lysate, there was a 4-fold increase in apoB recovery from the reaction mixtures containing lysate that had been depleted of ~75% of its hsp90. This increase in apoB recovery is again consistent with the transfection results and the other data from the cell-free system that hsp90 is a pro-degradative factor for apoB.

**Degradation of microsomal apoB48 in yeast cytosols** – The availability of specific hsp70 and hsp90 chaperone mutants in yeast (18-20,44) prompted us to explore whether yeast cytosol would support apoB48 degradation. Cytosols from wild type yeast or from isogenic strains with either a temperature-sensitive mutation in hsp82 (the yeast homologue of mammalian hsp90) and deleted for the gene for the constitutive hsc90 homologue, hsc82, or a temperature-sensitive mutation in the hsp70 homologue ssa1 (ssa1-45) were prepared from cells shifted for 1 h to the non-permissive temperature. These cytosols were then used in the degradation assay (Experimental Procedures).

As shown in Figure 10 (panel A, lane 2 vs. lane 1; panel E), after the 2 h incubation, 68% of apoB48 was degraded in the presence of wild type yeast cytosol. Note that GA (lane 3) blocked this degradation. The degradation of apo48 in yeast cytosols was also reversed by proteasome inhibitors (data not shown). Thus, the characteristics of apoB48 degradation in the presence of yeast cytosols were quite similar to those in the presence of rat hepatic cytosol (Figures 5 and 6).

The experiments were then repeated using the cytosols from the mutant strains. The results clearly show that apoB48 is almost completely protected from degradation when the hsp82 or ssa1 mutant cytosols were used (Figure 10, panels B, C, and E), similar to the effect of GA on wild type cytosol.
It is known that hsp90 acts in association with several different co-chaperones, which bind to hsp90 and organize it into discrete subcomplexes (12). In particular, hsp70 has been found in such a complex, associated with hsp90 through the Hop protein (45). We previously found that hsp70 promotes apoB degradation (4). Thus, the protection of apoB48 from degradation when only one of these chaperones is mutated suggested that hsp70 and hsp90 may have cooperative effects on apoB degradation. To test this hypothesis, cytosols from the two mutant yeast strains were mixed at a 1:1 ratio and used in the degradation assay. The degradation of apoB48 was restored to wild type levels, and under these conditions, apoB48 was protected when GA was added to the reaction (Figure 10, panels D and E).
Discussion

The majority of apoB is subjected to proteasome-mediated ERAD in cells of hepatic origin that are either deprived of exogenous fatty acids (which stimulate lipid synthesis) or deficient for microsomal triglyceride transfer (MTP) activity (4,28,29). It is assumed that the association of apoB with lipid-ligands is required for the achievement of its native conformation, and when the concentration or transfer of lipids is reduced, apoB is detected by quality control mechanisms that prevent the exit of malfolded proteins from the ER (46).

That chaperones may have important functions in apoB degradation stems from the growing recognition that these molecules influence the ERAD process of other proteins (for a review, see (9)) and from our demonstration that increasing the expression of hsp70 in fatty acid–deprived human liver-derived HepG2 cells promoted the proteasomal degradation of apoB (4,47). Further hints that hsp70 and other chaperones may be involved in the quality control of apoB come from Linnik and Herscovitz (38), who showed in HepG2 cells that BiP/Grp78, calreticulin, Erp72 and grp94 co-immunoprecipitate with apoB, and from Olofsson and colleagues, who have found recently that BiP, PDI, calreticulin and grp94 are associated with apoB-containing lipoprotein particles in rat hepatoma cells (48).

Because of the limited ability in intact hepatic cells to modulate the interactions of chaperones without affecting their steady state levels, we developed a cell-free degradation assay system in order to best explore the roles of these proteins in apoB ERAD. The previous report that apoB48, synthesized in a coupled transcription-
translation system, was translocated into dog pancreatic microsomes (24) suggested the feasibility of this goal. Under such conditions, there is neither MTP activity nor active lipid synthesis, thereby mimicking the lipid-ligand deficient state in hepatic-derived cells deprived of fatty acids or lipid transfer activity. In the present studies, we have extended the previous results by showing that the nascent protein was modified by glycosylation and ATP-dependent ubiquitinylation, both known to occur under native conditions (4,25,28,49). By isolating the microsome-associated apoB48 and demonstrating its proteasome-mediated degradation after resuspension in hepatic cytosol, we have established a cell-free system in which ERAD of apoB can be reconstituted. As with other mammalian and yeast ERAD substrates studied in similar systems (e.g., (8,50,51)), this now allows us to decipher the molecular requirements for the degradation of apoB by the ubiquitin-proteasome pathway.

As noted in the Introduction, we were interested in studying the potential influence of hsp90 on apoB degradation, given its abundance in mammalian cells and its controversial role as a protective or susceptibility factor in the proteasomal degradation of other proteins (14-16). Multiple lines of evidence implicate hsp90 as a pro-degradative factor for apoB. First, there was increased recovery of apoB in the cell-free degradation assay when GA was added to the rat hepatic cytosol (Figure 6). GA is widely considered to be a specific inhibitor of protein interactions with members of the hsp90 family (e.g., (34,37)) and in fact, was used in two of studies cited above (14,15).

Further support that GA was operating through a specific hsp90-mediated mechanism was our finding that it disrupted the interaction between hsp90 and apoB48
(Figure 7) without perturbing the total pool of hsp90. Importantly, in spite of the interaction of apoB and grp94 in HepG2 cells (38), there was no evidence for this in the cell-free system, consistent with the suggestion that grp94 plays a role in the maturation of lipoproteins containing apoB escaping ERAD (48).

The pro-degradative effect of hsp90 on apoB in the cell-free assay was also found in rat hepatoma cells, McArdle-RH7777, transfected with hsp90 cDNA (Figure 9). This cell line is a standard model of hepatic mammalian lipoprotein metabolism and also exhibits ERAD of apoB (39,52). Although the cells produced predominately apoB100, the small amount of apoB48 in the control cells was also reduced in the hsp90 cDNA-transfected cells (data not shown). These effects on cellular apoB were not attributable to a non-specific effect of hsp90 or the transfection procedure, as albumin recoveries from the cell and medium were not significantly changed and the control cells were subjected to the same transfection protocol. Overall, these results imply that the cell-free system is an accurate reflection of the role of hsp90 in intact cells of hepatic origin.

The availability of yeast strains with mutations in the homologues of mammalian hsp90 and 70, hsp82 and ssa1, respectively, has allowed us to confirm and to examine further the roles of these chaperones in apoB degradation (Figure 10). The suitability of this system was first evidenced by the fact that the degradation of microsome-associated apoB48 in the presence of the wild type yeast cytosol had characteristics resembling those observed with rat hepatic cytosol; i.e., >60% of apoB48 was degraded, which was reversed by the proteasome inhibitor (data not shown) or GA. Second, consistent with the results summarized here and our previous studies implicating a pro-degradative effect
of hsp70 (4,47), cytosols from the strains with either chaperone mutated did not support significant degradation. This implied that both chaperones are required for the proteasomal degradation of apoB. This requirement was specific, as supported by our finding that degradation was reconstituted in the complementation (mixing) experiment. Other studies have shown that hsp90 and hsp70 cooperate. For example, both chaperones participate in the activation of hormone binding by glucocorticoid receptor (53). Multichaperone complexes containing hsp90 and hsp70 have also been shown to mediate refolding of denatured luciferase and β-galactosidase in a cooperative manner (54,55).

How do we envision hsp90 and hsp70 to function in the ERAD pathway for apoB? Based on our recent studies (5,6,47,56), we find no evidence for a complete retrotranslocation into the cytosol of apoB destined for proteasomal degradation, as for MHC Class I molecules in CMV-infected cells (57) or mutant pre-pro alpha factor (8); rather, there appears to be an extraction process in which factors interact with apoB domains as they appear on the cytosolic surface of the ER membrane. It has been proposed that hsp70 (58) can facilitate such an extraction, perhaps by binding to a domain and serving as a molecular motor or a ratchet. This would be consistent with our finding that increased expression of hsp70 decreased the secretion of apoB-containing lipoproteins even when lipid synthesis was stimulated in HepG2 cells (4), suggesting a competition between the cytosolic factors targeting apoB to ERAD and the ER lumenal factors promoting lipoprotein assembly and exit from the ER.

Increased expression of hsp70 enhances apoB ubiquitinylation (4,47). In contrast, GA did not change apoB ubiquitinylation (Figure 8), implying that the hsp90 acted after
ubiquitin tagging of apoB48. Because hsp90 has been shown to be associated with the proteasome, it has been proposed recently that it may facilitate the unfolding of substrates into the relatively narrow mouth of the 19S cap of the proteasome (59,60). In this scenario, we envision hsp70 and hsp90 working sequentially - with hsp70 being an early participant as an "extraction" and, perhaps, an ubiquitinylation factor, and hsp90 as a factor acting later to facilitate the association with and entry into the proteasome of the ubiquitinylated apoB. Thus, for maximal degradation both chaperones should be expected to be required for apoB degradation. Because of the length of apoB (2152 and 4536 amino acids for apoB48 and apoB100, respectively) and the nature of an extraction process, however, it is likely that at steady state in the hepatic cell, both chaperones are bound to apoB targeted for ERAD, as we have recently observed in HepG2 cells ((6) and R. Pariyarath and E. Fisher, unpublished results). The participation of both chaperones in apoB degradation is consistent also with the recent finding that hsp70 and hsp90 have roles in the folding and ubiquitinylation of wild type and ΔF508 CFTR in mammalian cells (15) and that hsp70 facilitates the degradation of CFTR in yeast (21).

In summary, we have established a powerful and convenient cell-free system to study the ERAD pathway for apoB. With this system, and confirmed in transfection studies, we have now obtained clear evidence that both hsp90 and hsp70 promote apoB ERAD. The effects of these two chaperones and other cytosolic factors on this process can now be studied by using purified factors or cytosols from yeast strains with relevant mutations, singly or in combination, to allow the biochemical characterization and the kinetic ordering of events related to the targeting of apoB to the proteasome.
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References

1. Borchardt, R. A., and Davis, R. A. (1987) *J Biol Chem* **262**(34), 16394-402

2. Dixon, J. L., Furukawa, S., and Ginsberg, H. N. (1991) *J Biol Chem* **266**(8), 5080-6

3. White, A. L., Graham, D. L., LeGros, J., Pease, R. J., and Scott, J. (1992) *J Biol Chem* **267**(22), 15657-64

4. Fisher, E. A., Zhou, M., Mitchell, D. M., Wu, X., Omura, S., Wang, H., Goldberg, A. L., and Ginsberg, H. N. (1997) *J Biol Chem* **272**(33), 20427-34

5. Mitchell, D. M., Zhou, M., Pariyarath, R., Wang, H., Aitchison, J. D., Ginsberg, H. N., and Fisher, E. A. (1998) *Proc Natl Acad Sci U S A* **95**(25), 14733-8

6. Pariyarath, R., Wang, H., Aitchison, J. D., Ginsberg, H. N., Welch, W. J., Johnson, A. E., and Fisher, E. A. (2001) *J Biol Chem* **276**, 541-550

7. Bonifacino, J. S., and Weissman, A. M. (1998) *Annu Rev Cell Dev Biol* **14**, 19-57

8. McCracken, A. A., and Brodsky, J. L. (1996) *J Cell Biol* **132**(3), 291-8

9. Brodsky, J. L., and McCracken, A. A. (1999) *Semin Cell Dev Biol* **10**(5), 507-13

10. Hayes, S. A., and Dice, J. F. (1996) *J Cell Biol* **132**(3), 255-8

11. Pratt, W. B., and Toft, D. O. (1997) *Endocr Rev* **18**(3), 306-60

12. Caplan, A. J. (1999) *Trends Cell Biol* **9**(7), 262-8
13. Prodromou, C., Roe, S. M., O’Brien, R., Ladbury, J. E., Piper, P. W., and Pearl, L. H. (1997) *Cell* **90**(1), 65-75
14. Loo, M. A., Jensen, T. J., Cui, L., Hou, Y., Chang, X. B., and Riordan, J. R. (1998) *Embo J* **17**(23), 6879-87
15. Fuller, W., and Cuthbert, A. W. (2000) *J Biol Chem* **275**(48), 37462-8
16. Imamura, T., Haruta, T., Takata, Y., Usui, I., Iwata, M., Ishihara, H., Ishitaki, M., Ishibashi, O., Ueno, E., Sasaoka, T., and Kobayashi, M. (1998) *J Biol Chem* **273**(18), 11183-8
17. Bonifacino, J. S., Dasso, M., Harford, J. B., Lippincott-Schwartz, J., and Yamada, K. M. (1998) *Current Protocols in Cell Biology* (Morgan, K. S., Ed.), John Wiley & Sons, Inc.
18. Becker, J., Walter, W., Yan, W., and Craig, E. A. (1996) *Mol Cell Biol* **16**(8), 4378-86
19. Nathan, D. F., and Lindquist, S. (1995) *Mol Cell Biol* **15**(7), 3917-25
20. Fliss, A. E., Benzeno, S., Rao, J., and Caplan, A. J. (2000) *J Steroid Biochem Mol Biol* **72**(5), 223-30
21. Zhang, Y., Nijbroek, G., Sullivan, M. L., McCracken, A. A., Watkins, S. C., Michaelis, S., and Brodsky, J. L. (2001) *Mol. Biol. Cell* **12**, 1303-14.
22. Shaeffer, J. R., and Cohen, R. E. (1996) *Biochemistry* **35**(33), 10886-93.
23. Davis, R. A., Thrift, R. N., Wu, C. C., and Howell, K. E. (1990) *J Biol Chem* **265**(17), 10005-11
24. Rusinol, A. E., Jamil, H., and Vance, J. E. (1997) *J Biol Chem* **272**(12),
25. Adeli, K. (1994) *J Biol Chem* **269**(12), 9166-75

26. Fujiki, Y., Hubbard, A. L., Fowler, S., and Lazarow, P. B. (1982) *J Cell Biol* **93**(1), 97-102

27. Rustaeus, S., Stillemark, P., Lindberg, K., Gordon, D., and Olofsson, S. O. (1998) *J Biol Chem* **273**(9), 5196-203

28. Yeung, S. J., Chen, S. H., and Chan, L. (1996) *Biochemistry* **35**(43), 13843-8

29. Benoist, F., and Grand-Perret, T. (1997) *J Biol Chem* **272**(33), 20435-42

30. Lee, D. H., and Goldberg, A. L. (1998) *Trends Cell Biol* **8**(10), 397-403

31. Xiong, X., Chong, E., and Skach, W. R. (1999) *J Biol Chem* **274**(5), 2616-24

32. Sakata, N., and Dixon, J. L. (1999) *Biochim Biophys Acta* **1437**(1), 71-9.

33. Stebbins, C. E., Russo, A. A., Schneider, C., Rosen, N., Hartl, F. U., and Pavletich, N. P. (1997) *Cell* **89**(2), 239-50

34. Grenert, J. P., Sullivan, W. P., Fadden, P., Haystead, T. A. J., Clark, J., Mimnaugh, E., Krutzsch, H., Ochel, H. J., Schulte, T. W., Sausville, E., Neckers, L. M., and Toft, D. O. (1997) *J Biol Chem* **272**(38), 23843-50

35. Thulasiraman, V., and Matts, R. L. (1996) *Biochemistry* **35**(41), 13443-50

36. Goasduff, T., and Cederbaum, A. I. (2000) *Arch Biochem Biophys* **379**(2), 321-30.

37. Chavany, C., Mimnaugh, E., Miller, P., Bitton, R., Nguyen, P., Trepel, J.,
Whitesell, L., Schnur, R., Moyer, J., and Neckers, L. (1996) *J Biol Chem* 271(9), 4974-7

38. Linnik, K. M., and Herscovitz, H. (1998) *J Biol Chem* 273(33), 21368-73

39. Wang, H., and Fisher, E. A. (1997) *Circulation* 96, 1543

40. Zou, J., Guo, Y., Guettouche, T., Smith, D. F., and Voellmy, R. (1998) *Cell* 94(4), 471-80

41. Lawson, B., Brewer, J. W., and Hendershot, L. M. (1998) *J Cell Physiol* 174(2), 170-8

42. Little, E., and Lee, A. S. (1995) *J Biol Chem* 270(16), 9526-34.

43. Nganga, A., Bruneau, N., Sbarra, V., Lombardo, D., and Le Petit-Thevenin, J. (2000) *Biochem J* 352 Pt 3, 865-74.

44. Bohen, S. P., and Yamamoto, K. R. (1993) *Proc Natl Acad Sci U S A* 90(23), 11424-8

45. Chen, S., Prapapanich, V., Rimerman, R. A., Honore, B., and Smith, D. F. (1996) *Mol Endocrinol* 10(6), 682-93

46. Ellgaard, L., Molinari, M., and Helenius, A. (1999) *Science* 286(5446), 1882-8

47. Zhou, M., Fisher, E. A., and Ginsberg, H. N. (1998) *J Biol Chem* 273(38), 24649-53

48. Stillemark, P., Boren, J., Andersson, M., Larsson, T., Rustaeus, S., Karlsson, K. A., and Olofsson, S. O. (2000) *J Biol Chem* 275(14), 10506-13

49. Chen, Y., Le Caherec, F., and Chuck, S. L. (1998) *J Biol Chem* 273(19),
50. Shamu, C. E., Story, C. M., Rapoport, T. A., and Ploegh, H. L. (1999) *J Cell Biol* **147**(1), 45-58

51. Wilson, C. M., Farmery, M. R., and Bulleid, N. J. (2000) *J Biol Chem* **275**(28), 21224-32

52. Wu, X., Sakata, N., Lele, K. M., Zhou, M., Jiang, H., and Ginsberg, H. N. (1997) *J Biol Chem* **272**(17), 11575-80

53. Rajapandi, T., Greene, L. E., and Eisenberg, E. (2000) *J Biol Chem* **275**(29), 22597-604

54. Schumacher, R. J., Hurst, R., Sullivan, W. P., McMahon, N. J., Toft, D. O., and Matts, R. L. (1994) *J Biol Chem* **269**(13), 9493-9

55. Freeman, B. C., and Morimoto, R. I. (1996) *Embo J* **15**(12), 2969-79

56. Liang, J., Wu, X., Fisher, E. A., and Ginsberg, H. N. (2000) *J Biol Chem* **275**(41), 32003-10

57. Wiertz, E. J., Tortorella, D., Bogyo, M., Yu, J., Mothes, W., Jones, T. R., Rapoport, T. A., and Ploegh, H. L. (1996) *Nature* **384**(6608), 432-8

58. Glick, B. S. (1995) *Cell* **80**(1), 11-4

59. Wagner, B. J., and Margolis, J. W. (1995) *Arch Biochem Biophys* **323**(2), 455-62

60. Verma, R., Chen, S., Feldman, R., Schieltz, D., Yates, J., Dohmen, J., and Deshaies, R. J. (2000) *Mol Biol Cell* **11**(10), 3425-39
Footnotes

Abbreviations:

apoB  apoprotein B
ER     endoplasmic reticulum
DPM    dog pancreatic microsomes
GA     geldanamycin
Figure legends

Figure 1. In vitro synthesis of apoB48 in the cell-free system. ApoB48 was synthesized in the absence (-) or presence (+) of dog pancreatic microsomes in a coupled transcription-translation reaction in which rabbit reticulocyte lysate (containing [35S]-methionine/cysteine) was programmed with apoB48 mRNA. The reaction mixture was then separated by SDS-PAGE and the resulting fluorogram is displayed. The migration of the 207 kDa marker is indicated.

Figure 2. Topology and glycosylation of in vitro synthesized apoB48. Panel A. Trypsin digestion of apoB48. ApoB48, synthesized in a coupled transcription-translation reaction in which rabbit reticulocyte lysate (containing [35S]-methionine/cysteine) was programmed with apoB48 mRNA in the presence of dog pancreatic microsomes (lane 1), was treated with trypsin (0.1mg/ml, 1 h, on ice) in the absence (lane 2) or presence of Triton X100 (lane 3). Panel B. Glycosylation of apoB48. ApoB48 was synthesized as in panel A in the presence of dog pancreatic microsomes (lane1). Microsome-associated apoB48 was collected by centrifugation and incubated (4ºC, 16 h) with (lane 2) or without (lane 3) the glycosidase PNGF (lane2). Lane 4 contains the untreated supernatant after centrifugation (i.e., non-microsome-associated apoB48). Samples were resolved by SDS-PAGE and representative fluorograms are displayed in both panels. The migration of the 207 kDa marker is indicated.

Figure 3. In vitro synthesized apoB48 is microsome-associated. ApoB48, synthesized in
a coupled transcription-translation reaction in which rabbit reticulocyte lysate
(containing $[^{35}\text{S}]$-methionine/cysteine) was programmed with apoB48 mRNA in the
presence of dog pancreatic microsomes, was analyzed by floatation through a
discontinuous sucrose gradient as described in the Experimental Procedures. “T”
indicates ~1% of the diluted reaction prior to the gradient, and the numbered fractions
represent SDS-PAGE analysis of portions of 150 µl aliquots taken from the top (fraction
1) to the bottom (fraction 13) of the gradient after centrifugation. The arrow denotes the
position at which the sample was loaded.

Figure 4. Ubiquitinylation of apoB48 in the cell-free system. ApoB48 was synthesized
in the presence of dog pancreatic microsomes in a coupled transcription-translation
reaction in which rabbit reticulocyte lysate (containing $[^{35}\text{S}]$-methionine/cysteine)
was programmed with apoB48 mRNA (lane1). In a control reaction, the apoB48 cDNA
was omitted (lane 2). Unlabeled methionine and cysteine were added at the end of the
reaction in 100-fold excess relative to the corresponding labeled amino acids present at
the beginning of reaction. To aliquots of this mixture were added 0.1mg/ml of HA-
tagged ubiquitin and either 10 units/ml of apyrase (lane 3) or 2mM ATP, 10mM creatine
phosphate, and 100µg/ml of creatine kinase (lanes 4, 5 and 6). The mixtures were then
incubated at 37°C for 1 h and immunoprecipitated with (lanes 3, 4, and 6) or without
(lane 5) anti-HA antibody. Samples were resolved by SDS-PAGE and a representative
fluorogram is displayed.
Figure 5. **Degradation of microsome-associated apoB48 in rabbit reticulocyte lysate and rat hepatic cytosol.** ApoB48 was synthesized in the presence of dog pancreatic microsomes in a coupled transcription-translation reaction in which rabbit reticulocyte lysate (containing $^{35}$S-methionine/cysteine) was programmed with apoB48 mRNA. The reaction mixture was layered onto a sucrose cushion and centrifuged at 100 000 x g for 30 min (Experimental Procedures). The pelleted microsomal membranes were resuspended in 50% (vol/vol) of rabbit reticulocyte lysate (panels A and C) or rat hepatic cytosol (panels B and D) containing ATP and an ATP regenerating system. Aliquots of each mixture were then incubated on ice (lane 1) or at 37°C for 2 h (lane 2 - without proteasome inhibitor; lanes 3-5 - with the indicated proteasome inhibitors, each at 50µM). Gel loading buffer was then directly added and apoB48 recovery assessed by SDS-PAGE/fluorography (panels A and B). The apoB48 bands from repeated experiments were scanned with a Molecular Dynamic Densitometer and plotted as the mean signal intensity ± SD (n=3) for each lane (panels C and D).

Figure 6. **Geldanamycin (GA), an inhibitor of hsp90, blocks the degradation of microsome-associated apoB48.** Panels A and B: ApoB48 was synthesized in the presence of dog pancreatic microsomes in a coupled transcription-translation reaction in which rabbit reticulocyte lysate (containing $^{35}$S-methionine/cysteine) was programmed with apoB48 mRNA. Microsomes were isolated and resuspended in rat hepatic cytosol as in Figure 5, except that the mixture was divided, and to each half either GA (30µM, in DMSO) or DMSO alone was added. Samples were taken every 30 min during 2 h
incubation at 37º C. SDS-PAGE and densitometric analyses were performed as in Figure 5. Representative fluorograms are shown in panels A and B. The apoB48 signal intensity at each time point was measured in 3 separate experiments and is plotted as the mean ± SD in panel C.

Figure 7. Co-immunoprecipitation analysis of microsome-associated apoB48 and the chaperones hsp90 and grp94. Panel A: ApoB48 was synthesized and microsomes were isolated and resuspended in rat hepatic cytosol with or without GA, as in Figure 5, except that the final mixtures were incubated at 37ºC for 1 h. Then, equal aliquots were taken for immunoprecipitation with antibodies to apoB (lanes 1 and 2), hsp90 (lanes 3 and 4), or grp94 (lanes 5 and 6). The immunoprecipitates were resolved by SDS-PAGE and the resulting fluorogram is displayed. Panel B: Western blot of the material in lanes 1 and 2 of panel A using the hsp90 antibody. Panel C: Western blot of an aliquot of the sample analyzed in lanes 3 and 4 of panel A using the hsp90 antibody.

Figure 8. Effect of GA on the ubiquitinylation of microsome-associated apoB48 in rat hepatic cytosol. ApoB48 was synthesized in the presence of dog pancreatic microsomes in a coupled transcription-translation reaction in which rabbit reticulocyte lysate (containing [35S]-methionine/cysteine) was programmed with apoB48 mRNA. Microsomes were isolated as in Figure 5 and were resuspended in a 1:1 mixture of rat hepatic cytosol:2xPh buffer (Experimental Procedures). This mixture was supplemented with 0.1mg/ml of HA-tagged ubiquitin and either 10 units/ml of apyrase (lane 1) or 5mM
ATPγS (lanes 2 and 3). Either DMSO (lanes 1 and 2) or GA (lane 3; 30µM in DMSO) was then added and the samples were incubated for 1 h at 37°C. Samples containing equivalent amounts of apoB48 were then analyzed by immunoprecipitation with the anti-HA antibody, followed by SDS-PAGE and fluorography. The migration of the 207 kDa marker is indicated.

Figure 9. Transfection with hsp90 cDNA increases apoB100 degradation in rat hepatoma cells. Panels A and B: Rat hepatoma (McArdle-RH7777) cells were transfected either with pcDNA3 (control; lanes 1 and 3) or pcDNA3-hsp90β plasmid (lanes 2 and 4). Forty-eight hours after transfection cells were pulse-labeled with [35S]-methionine/cysteine for 15 min and chased in isotope-free medium for 30 min (lanes 1 and 2) or 90 min (lanes 3 and 4). Cell lysates and conditioned media samples containing an equal amount of trichloroacetic acid-insoluble radioactivity (cpm) per mg of total protein were immunoprecipitated with antibodies to apoB (panel A) or albumin (panel B) and the immunoprecipitates analyzed by SDS-PAGE and fluorography. The lack of apoB100 in the conditioned medium samples at the 30 min time point was expected based on previous data that it takes approximately 40 min for newly synthesized apoB100 to be secreted by hepatic cells (e.g., (2)). Data were reproducible in three separate experiments and typical fluorograms are shown.

Figure 10. Degradation of microsome-associated apoB48 in cytosols from wild type and mutant yeast strains. Panels A-D: ApoB48 was synthesized and the microsomes were
isolated as in Figure 5. The pelleted microsomal membranes were resuspended in yeast cytosols (wild type, WT; mutant hsp82 [yeast homologue of mammalian hsp90]; mutant ssa1 [homologue of hsp70]; or, an equal mixture of cytosols containing mutant hsp82 and ssa1) and then mixed 1:1 with 2xPh buffer containing ATP and an ATP regenerating system (Experimental Procedures). Aliquots of each mixture were incubated at either 4°C (control condition) or at 37°C for 2 h. As indicated, some samples also contained GA (30µM). At the end of the incubation, gel loading buffer was added and the samples analyzed by SDS-PAGE and fluorography. Representative fluorograms are shown in panels A-D. The densitometric signal intensities from repeated experiments are plotted in panel E and are expressed as means ± SD (n=3).
Fig. 1

microsomes

207

1 2
Fig. 2

A.

|           | 1 | 2 | 3 |
|-----------|---|---|---|
| Trypsin   | - | + | + |
| Triton X-100 | - | - | + |

B.

| PNGF | 1 | 2 | 3 | 4 |
|------|---|---|---|---|
|      | + | - |   |   |
Fig. 3

The image shows a gel with lanes labeled from T to 13. Lane T is labeled apoB48.
ATP
apyrase
anti-HA antibody
apoB48 plasmid

apoB48

ub-apoB48

1 2

3 4 5 6

+ + + + +
+ + + + +
+ + + + +
+ + + + +
Fig. 5

A. 

B. 

C. 

D.
Fig. 7

A. Ab apoB hsp90 grp94
  GA - + - + - +

apoB48
  1 2 3 4 5 6

B. GA - +

Hsp90

C. GA - +

Hsp90
Fig. 8

| GA   | - | - | + |
|------|---|---|---|
| ATPγS| - | + | + |
| apyrase | + | - | - |

Ub-apoB48

207
Fig. 9.

A.  

| Chase, min | Cell |  |  |  | Medium |  |  |  |
|------------|------|---|---|---|--------|---|---|---|
| 30         | 30   | 30 | 30 | 30 | 30     | 30 | 30 | 30 |
| hsp90-tf   | -    | +  | -  | +  | -      | +  | -  | +  |
| apoB100    |      |    |    |    |        |    |    |    |
| 1          | 2    | 3  | 4  | 1  | 2      | 3  | 4  | 4  |

B.  

| Chase, min | hsp90-tf |  |  |  |  |  |  |  |
|------------|----------|---|---|---|---|---|---|---|
| 30         | -  | +    | -  | +    | -  | +   | -  | +    |
| 90         | -  | +    | -  | +    | -  | +   | -  | +    |
| albumin    |      |      |    |      |    |      |    |      |
| 1          | 2    | 3  | 4  | 1  | 2      | 3  | 4  | 4  |
Fig. 10

A.  

| GA | T,°C |
|----|------|
| -  | 4    |
| -  | 37   |
| +  | 37   |

B.  

| GA | T,°C |
|----|------|
| -  | 4    |
| -  | 37   |
|    |      |

C.  

| GA | T,°C |
|----|------|
| -  | 4    |
| -  | 37   |
|    |      |

D.  

| GA | T,°C |
|----|------|
| -  | 4    |
| -  | 37   |
|    |      |

E.  

|        | WT     | hsp82  | ssal   | hsp82 / ssal |
|--------|--------|--------|--------|--------------|
| apoB48 recovery, % |        |        |        |              |
| 0°C   |        |        |        |              |
| 37°C  |        |        |        |              |
| 37°C +GA |        |        |        |              |
Apoprotein B degradation is promoted by the molecular chaperones hsp90 and hsp70
Viktoria Gusarova, Avrom J. Caplan, Jeffrey L. Brodsky and Edward A. Fisher
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