Protein disulfide isomerases contribute differentially to the endoplasmic reticulum–associated degradation of apolipoprotein B and other substrates

Sarah Grubb\textsuperscript{a}, Liang Guo\textsuperscript{b}, Edward A. Fisher\textsuperscript{b}, and Jeffrey L. Brodsky\textsuperscript{a}

\textsuperscript{a}Department of Biological Sciences, University of Pittsburgh, Pittsburgh, PA 15260; \textsuperscript{b}Departments of Medicine (Cardiology) and Cell Biology and the Marc and Ruti Bell Program in Vascular Biology, New York University School of Medicine, New York, NY 10016

ABSTRACT
ER-associated degradation (ERAD) rids the early secretory pathway of misfolded or misprocessed proteins. Some members of the protein disulfide isomerase (PDI) family appear to facilitate ERAD substrate selection and retrotranslocation, but a thorough characterization of PDIs during the degradation of diverse substrates has not been undertaken, in part because there are 20 PDI family members in mammals. PDIs can also exhibit disulfide redox, isomerization, and/or chaperone activity, but which of these activities is required for the ERAD of different substrate classes is unknown. We therefore examined the fates of unique substrates in yeast, which expresses five PDIs. Through the use of a yeast expression system for apolipoprotein B (ApoB), which is disulfide rich, we discovered that Pdi1 interacts with ApoB and facilitates degradation through its chaperone activity. In contrast, Pdi1’s redox activity was required for the ERAD of CPY\textsuperscript{*} (a misfolded version of carboxypeptidase Y that has five disulfide bonds). The ERAD of another substrate, the alpha subunit of the epithelial sodium channel, was Pdi1 independent. Distinct effects of mammalian PDI homologues on ApoB degradation were then observed in hepatic cells. These data indicate that PDIs contribute to the ERAD of proteins through different mechanisms and that PDI diversity is critical to recognize the spectrum of potential ERAD substrates.

INTRODUCTION
Apolipoprotein B (ApoB) is a large, amphipathic protein that is produced in two isoforms in mammals. ApoB100 (∼540 kDa), synthesized in hepatic cells, is the predominant structural protein in very low density lipoproteins (VLDLs) and low-density lipoproteins (LDLs). A shorter isoform, ApoB48 (containing the amino-terminal 48% of ApoB100) is expressed in enterocytes and assembles into chylomicrons and into very low-density lipoproteins (VLDLs). This article was published online ahead of print in MBoC in Press (http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E11-08-0704) on December 21, 2011. Address correspondence to: Jeffrey L. Brodsky (brodsky@pitt.edu).

Abbreviations used: ApoB, apolipoprotein B; CPY\textsuperscript{*}, carboxypeptidase Y\textsuperscript{*}; ERAD, endoplasmic reticulum–associated degradation; LDL, low-density lipoproteins; PDI, protein disulfide isomerase; VLDL, very low-density lipoproteins.

© 2012 Grubb et al. This article is distributed by The American Society for Cell Biology under license from the author(s). Two months after publication it is available to the public under an Attribution–Noncommercial–Share Alike 3.0 Unported Creative Commons License (http://creativecommons.org/licenses/by-nc-sa/3.0).

“ASCB®,” “The American Society for Cell Biology®,” and “Molecular Biology of the Cell®” are registered trademarks of The American Society of Cell Biology.

crons in all mammalian species and into VLDLs in rodents (Rutledge et al., 2010). Collectively, VLDLs, LDLs, and chylomicrons transport cholesterol and triglycerides through the bloodstream and deliver these essential metabolites to every tissue (Fisher and Ginsberg, 2002; Brodsky and Fisher, 2008; Rutledge et al., 2010). The amount of endogenously produced cholesterol must be tightly regulated since either excess or shortage of this sterol can lead to disease. For example, excess cholesterol can accumulate in coronary arteries and contribute to the development of atherosclerosis (Kannel et al., 1971; Brodsky and Fisher, 2008), the primary cause of coronary artery disease. Conversely, hypobetalipoproteinemia is a disease characterized by decreased plasma concentrations of ApoB and results in nutrient malabsorption, ataxia, and neuromuscular degeneration; the most common form of hypobetalipoproteinemia results from a premature stop codon in ApoB (Linton et al., 1993; Whitfield et al., 2004). Because the amount of secreted ApoB that circulates in the bloodstream correlates directly with serum cholesterol levels (Crooke
et al., 2005; Zimmermann et al., 2006), a better understanding of ApoB secretion is vital for the treatment and prevention of hyperbetalipoproteinemia and coronary artery disease.

ApoB secretion is tightly controlled, in large part through regulated degradation, an important component of which is accomplished via the endoplasmic reticulum-associated degradation (ERAD) pathway (Fisher and Ginsberg, 2002; Brodsky and Fisher, 2008; Rutledge et al., 2010). ERAD involves four major steps: selection of a misfolded protein, retrotranslocation of the protein from the ER to the cytosol, substrate ubiquitination, and proteasome-mediated degradation (Meusser et al., 2005; Vembar and Brodsky, 2008; Xie and Ng, 2010). The ERAD of ApoB is unique in that it is metabolically controlled by lipid availability and occurs cotranslationally (Dixon et al., 1991; Benoist and Grand-Perret, 1997; Zhou et al., 1998). When lipids are abundant, ApoB is cotranslationally translocated into the ER, where it becomes N-glycosylated (Harazono et al., 2005), acquires eight disulfide bonds (Yang et al., 1990; Burch and Herscovitz, 2000), and is lipid loaded via the action of a microsomal triglyceride transfer protein (MTP) complex. The complex contains an M subunit and an ER chaperone/enzyme known as protein disulfide isomerase (PDI; Hussain et al., 2003), which is the founding member of a group of 20 PDI family members in mammals. Upon sufficient lipid loading, ApoB assembles into a pre-VLDL and traffics to the Golgi, where it undergoes further maturation and is secreted (Tran et al., 2002; Olofsson and Boren, 2005; Gusarova et al., 2007; Siddiqi et al., 2010). However, when the intracellular concentrations of phospholipids and triacylglycerols are depleted, ApoB is selected for ERAD, ubiquitinated by the E3 ligase gp78, retrotranslocated, and degraded by the proteasome (Dixon et al., 1991; Yeung et al., 1996; Fisher et al., 1997; Liao et al., 1998; Paryarath et al., 2001; Liang et al., 2003). Because ERAD results in lower intracellular and secreted levels of ApoB, it follows that a decrease in ApoB production will decrease serum lipid and cholesterol levels. Thus we have sought to better define the ERAD pathway for ApoB, an effort that may provide novel therapeutic targets to treat cardiovascular and other, related diseases.

Disulfide bonds, such as those found in ApoB, are formed within the oxidizing environment of the ER by PDI family members (Kleizen and Braakman, 2004; Anelli and Sitia, 2008; Hatahet and Ruddock, 2009; Brodsky and Skach, 2011; Bulleid and Ellgaard, 2011). PDIs are identified by the presence of one or more thioredoxin-like motifs (Cys-X-X-Cys) and can catalyze the oxidation, reduction, and/or isomerization of disulfide bonds. Along with catalytic redox or isomerase activity, some PDI family members also possess chaperone activity due to the presence of a hydrophobic substrate-binding domain (Cai et al., 1994; Song and Wang, 1995; Klappa et al., 1998; Gillece et al., 1999; Ellgaard and Ruddock, 2005; Tian et al., 2006, 2008; Hatahet and Ruddock, 2009). As noted earlier, in mammals there are 20 PDI family members, 1 of which, known as PDI, associates with the M subunit to form the MTP complex. However, with few exceptions (Hatahet and Ruddock, 2009; Jessop et al., 2009; Park et al., 2009; Rutkevich et al., 2010; Brodsky and Skach, 2011), specific functions for each of these proteins have not been assigned. In contrast, in the yeast Saccharomyces cerevisiae there are five PDI family members, which are encoded by the PDI1, MPD1, MPD2, EUG1, and EPS1 genes. PDI1 is the only essential gene among this group (Farquhar et al., 1991). It contains two active-site, thioredoxin-like domains (denoted the a and a’ active sites), each with a CXXC motif and a substrate-binding domain in the b’ domain (Holst et al., 1997; Gillece et al., 1999; Tian et al., 2008). Although PDI1 is essential, the expression of Mpd1 from the PDI1 promoter supports cell viability in pdi1Δ yeast (Norgaard et al., 2001).

Select examples exist in which a clear link between PDI family members and ERAD or an ERAD-like phenomenon has been established. For example, in yeast the chaperone activity of Pdi1p is required for the recognition and degradation of an ERAD substrate, pro-α-factor (pof), which lacks disulfide bonds (Gillece et al., 1999), and Eps1 appears to help target a misfolded membrane protein, Pma1-D378N, for ERAD (Wang and Chang, 2003). In mammalian cells, ERD5, a protein that contains both a J domain that interacts with BiP and six thioredoxin repeat motifs, may reduce disulfide bonds to facilitate ERAD substrate retrotranslocation (Ushioda et al., 2008; Hagiwara et al., 2011). Furthermore, some PDIs bind to select bacterial toxins and viruses prior to their retrotranslocation and delivery to the cytoplasm in infected cells (Magnuson et al., 2005; Forster et al., 2006; Schelhaas et al., 2007; Rainey-Barger et al., 2009; Moore et al., 2010; Taylor et al., 2011).

We previously reported on an in vitro assay that monitors the proteasome-dependent degradation of ER membrane-embedded ApoB48 in the presence of yeast cytosol (Gusarova et al., 2001). Although cytosol prepared from wild-type yeast supported ApoB degradation, lysates from Hsp70 or Hsp90 mutant strains were degradation-deficient. The Hsp70 and Hsp90-dependent degradation of ApoB was also observed in hepatic cells (Fisher et al., 1997; Gusarova et al., 2001). We then developed a yeast ApoB expression system that used a truncated form of the protein, ApoB29, which still associates with lipids in hepatic cells, and we identified the Hsp110 chaperone as an ApoB-stabilizing factor. Here, too, the effect of the human Hsp110 homologue on ApoB was recapitulated in hepatic cells (Hrizo et al., 2007). These data indicate that the yeast system provides a powerful model to isolate and characterize components that play important roles in ApoB biogenesis.

As mentioned, mammalian PDI is one component of the MTP complex that helps load lipids onto ApoB (Hussain et al., 2003), but it is unknown whether PDI targets the protein for ERAD under lipid-poor conditions. Two other mammalian PDI family members, Erp57 and Erp72, can be coprecipitated with ApoB (Adeli et al., 1997; Linnik and Herscovitz, 1998; Hussain et al., 2003; Zhang and Herscovitz, 2003), but their roles during ApoB biogenesis are also unknown. Using both yeast and mammalian cell systems, we report on the specific action of PDI family members during the ERAD of ApoB. We also show that the degradation of ApoB and CPY* (a misfolded version of carboxypeptidase Y that has five disulfide bonds), which is a soluble ERAD substrate, require different Pdi1-encoded functions in yeast: ApoB uses Pdi1’s chaperone activity, whereas CPY* uses the thioredoxin-like motifs. We then discovered that Pdi1 is dispensable for the turnover of another ERAD substrate, the alpha subunit of the epithelial sodium channel (α-ENaC), and with the exception of Mpd1, the genes encoding the remaining three PDI relatives can be ablated without an effect on the ERAD of any of the substrates examined in this study. Finally, we found that two human PDI family members also facilitate the ERAD of ApoB in hepatic cells, although PDI—which is a component of the MTP complex—enhanced ApoB secretion. These data demonstrate the diverse PDI activities that are required during ERAD and that different PDI family members can act either to fold or degrade ApoB.

**RESULTS**

Pdi1 is the major contributor to ERAD in yeast

Disulfide bond formation is critical for ApoB maturation and secretion (Yang et al., 1989, 1990; Burch and Herscovitz, 2000). In addition, as described earlier, PDI is a component of the MTP complex, and two PDI family members, Erp57 and Erp72, have been found associated with ApoB (Adeli et al., 1997; Linnik and Herscovitz, 1998). Although PDI1 is essential, the expression of Mpd1 from the PDI1 promoter supports cell viability in pdi1Δ yeast (Norgaard et al., 2001).
contains seven of the eight disulfide bonds found in full-length ApoB (Yang et al., 1990; Harazono et al., 2005). In each mutant, we found that ApoB was degraded at wild-type levels (Figure 1A). We also measured the ERAD of a well-characterized substrate, CPY* (Wolf and Fink, 1975; Finger et al., 1993). In contrast to ApoB, CPY* was stabilized when MPD1 was deleted, although no stabilization was evident when the other nonessential PDIs were deleted (Figure 1B). In addition, we examined the degradation of pex1, a yeast ERAD substrate that lacks cysteines and that was previously shown to be selected by Pdi1 for degradation (Gillece et al., 1999), as well as α-ENAα, a mammalian protein with seven disulfide bonds whose proteasome- and chaperone-dependent degradation were characterized in yeast (Kashlan et al., 2007; Buck et al., 2010). We found that deletion of the nonessential PDIs also had no effect on the ERAD of these proteins (Supplemental Figure S1). Combined with previous data demonstrating that Eps1 is required for the ERAD of a mutant form of Pma1 (Wang and Chang, 2003), these results indicate substrate specificity among the nonessential PDI family members.

We next asked whether yeast Pdi1 facilitates the ERAD of ApoB. Because previous work indicated that Mpd1 overexpression from the PDI1 promoter supported the growth of a pdi1Δ mutant (Norgaard et al., 2001), we examined ApoB degradation in pdi1Δmpd2Δeps1ΔΔ cells in which Mpd1 is the only PDI family member expressed (strain M4492). In this strain, ApoB was completely stabilized (Figure 2A, open circles). We also examined the degradation of ApoB in a strain in which Pdi1 is the only yeast PDI family member expressed (strain SRH01). In this yeast, ApoB degradation was mostly restored (Figure 2A, open squares). We then measured the degradation of CPY* in these strains, since Pdi1 facilitates the ERAD of CPY* (Gillece et al., 1999; Sakoh-Nakatogawa et al., 2009). As was evident for ApoB, CPY* was also significantly stabilized when Mpd1 is the only PDI family member expressed, and protein degradation was again mostly restored when only Pdi1 was expressed (Figure 2B). Because there was a residual degradation defect when ApoB and CPY* turnover was assessed in SRH01, we suggest that at least one of the other PDI family members contributes during the process of substrate selection and/or retrotranslocation.

The chaperone activity of Pdi1 was previously found to be important for pex1 ERAD (Gillece et al., 1999), but the ability of Mpd1 to support the degradation of this substrate as the only expressed PDI family member has not been investigated. We observed measurable stabilization of pex1 when only Mpd1 was expressed, but degradation was robust when only Pdi1 was expressed (Supplemental Figure S2A). Of interest, although α-ENAα has seven disulfide bonds that are important for protein folding and function ( Firsov et al., 1999; Kashlan et al., 2011), an ERAD defect was

1998; Hussain et al., 2003; Zhang and Herscovitz, 2003). To perform a systematic analysis of the roles of PDIs in the ERAD of ApoB, we first examined ApoB stability in the yeast S. cerevisiae in which the genes encoding the nonessential PDIs were deleted. Our yeast expression system for ApoB was previously used to define several of the requirements for the ERAD of ApoB in hepatic cells (Hrizo et al., 2007). Because the wild-type yeast strain W303 provided a better readout for the extent of ApoB degradation than the BY4742 strain (our unpublished data), we constructed all of our mutants and performed each of the following experiments in this background (Supplemental Table S1).

The gene encoding Pdi1 is essential (Farquhar et al., 1991), so to assess whether any of the other PDI family members were important for the degradation of ApoB, mpd1Δ, mpd2Δ, eug1Δ, or eps1Δ cells were transformed with the ApoB expression vector, and the degradation rate was measured using a cycloheximide chase assay, as described in Materials and Methods. For these studies, we again chose to express ApoB29 from an inducible reporter; ApoB29 is the shortest ApoB isoform whose degradation is metabolically controlled (Wang et al., 1994; Segrest et al., 2001). ApoB29 also

**FIGURE 1:** Mpd1 contributes to the ERAD of CPY* but not ApoB29. (A) Cycloheximide chase reactions were performed as described in Materials and Methods in wild-type (○), mpd1Δ (●), mpd2Δ (□), eug1Δ (▲), or eps1Δ (□) yeast strains expressing ApoB29 (A) or CPY* (B). Chase reactions were performed at 30°C, and lysates were immunoblotted with anti-HA antibody. Anti-Sec61 antiserum was used as a loading control for chase reactions monitoring CPY* turnover, and anti-G6PD antiserum was used as a loading control for chase reactions measuring ApoB29 degradation. Top, quantitative data. Bottom, representative images. Data represent the means of four to six experiments, ± SEM. The lack of visible error bars indicates that the SEM is less than the size of the symbol. **p < 0.01.
absent when Mpd1 was expressed (Supplemental Figure S2B). These data suggest that Pdi1 is not required for the ERAD of all substrates. Normally, ENaC functions as a heterotrimer composed of three subunits—α, β, and γ (Snyder, 2002; Jasti et al., 2007)—but in this expression system α-ENA C is an orphan subunit. Therefore the “decision” to select α-ENA C for degradation may be distinct from other substrates in which intermolecular folding events or protein assembly are monitored. Together these results suggest that Pdi1 function is not imperative for the ERAD of all substrates, and, consistent with previous data (Gillece et al., 1999; Wang and Chang, 2003), our data indicate that some substrates rely in part on the function of other PDI family members during ER quality control.

ApoB and CPY* associate with Pdi1, and their degradation requires either Pdi1’s chaperone activity or the thioredoxin-like motifs

We next determined whether Pdi1 directly associates with ApoB and CPY*. To this end, we used a method in which the ability of Pdi1 to form a mixed disulfide with the ER luminal protein Htm1/Mnl1 was assessed (Sakoh-Nakatogawa et al., 2009). In this protocol, yeast cells expressing ApoB are spheroplasted and lysed, and then proteins are precipitated under conditions in which preformed disulfide bonds are trapped (see Materials and Methods). We therefore precipitated ApoB under these conditions and asked whether Pdi1 bound to this substrate after we added reductant and performed SDS–PAGE. When ApoB was immunoprecipitated, Pdi1 was also present, but an abundant cytosolic protein (glucose-6-phosphate dehydrogenase [G6PD]) that contains a single cysteine was absent (Figure 3A, lane 5). We note that a minor amount of Pdi1 associated with Pdi1-associated with ApoB (lane 6), but neither ApoB nor Pdi1 was precipitated using unconjugated resin, indicating a specific association between Pdi1 and ApoB. Consistent with Pdi1 having a direct effect on the degradation of CPY*, we also observed a Pdi1–CPY* interaction (Figure 3B, lane 5). A prominent association between these proteins was absent in precipitations from lysates that lacked the substrate (lane 6) or in mock precipitations (lanes 3 and 4), although here too a small amount of Pdi1 appears to nonspecifically associate with the resin. Nevertheless, these results strongly suggest that ApoB and CPY* form mixed disulfides with Pdi1.

Because Pdi1 interacts with ApoB and facilitates its degradation, we next investigated which Pdi1-embedded function was important for ApoB ERAD. We first asked whether the α or α' thioredoxin-like active site was necessary. To this end, we used strains in which PDI1 had been deleted but the cells expressed wild-type PDI1 on a plasmid (PDI1CGHC-CGHC), PDI1 on a plasmid with both cysteines in the active site mutated to serines (PDI1SGHS-CGHC), or PDI1 on a plasmid with both cysteines in the α' active site mutated to serines (PDI1CGHC-SGHS). ApoB degradation was then measured by cycloheximide chase analysis. When either active site’s cysteines were mutated, ApoB was degraded at wild-type levels (Figure 4A). In contrast, when either active site was mutated, CPY* was completely stable (Figure 4B), indicating that both thioredoxin-like motifs—and their redox activities—are necessary for CPY* but not ApoB degradation.

Another interpretation of the data presented in Figure 4A is that the α and α' sites
function redundantly to support ApoB degradation; however, it is believed that the two sites are not equivalent (Holst et al., 1997; Hatahet and Ruddock, 2009; Wang et al., 2009; Vitu et al., 2010), and at least one of the thioredoxin motifs must be capable of forming a thiolate to support cell viability (Laboissiere et al., 1995; Chivers et al., 1996). A more likely possibility is that Pdi1’s chaperone activity, which mediates Pdi1’s ability to bind some peptides in the ER (Klappa et al., 1998; Gillece et al., 1999), is necessary for ApoB degradation. To test this hypothesis, we used strains in which PDI1 was deleted but the cells expressed either wild-type PDI1 or with its b’ chaperone domain deleted (PDI1Δ222-302Δ). As shown in Figure 4C, ApoB was completely stable when Pdi1’s chaperone domain was absent. To determine whether the chaperone mutant strain supported the ERAD of other substrates, CPY* degradation was measured. Consistent with previous results (Gillece et al., 1999), no CPY* degradation defect was observed when the Pdi1 chaperone domain was mutated (Supplemental Figure S3A). We also confirmed that the mutant Pdi1 protein was expressed to the same level as wild-type Pdi1 and remained stable over the 90-min chase period (Supplemental Figure S3B). Together these results indicate distinct requirements for Pdi1-embedded activities during ERAD: some substrates (α-ENaC) are Pdi1 independent, some (ApoB and pαF) require the protein’s chaperone activity, and others (CPY*) require both of the enzyme’s thioredoxin-like motifs.

**Yeast expressing only Mpd1 or specific mutant forms of Pdi1 are dithiothreitol sensitive**

We hypothesized that if the chaperone mutant strain (pdi1Δ [PDI1Δ222-302Δ]) was more sensitive to ER stress and less viable, then the stabilization of ApoB observed in these Pdi1Δ222-302Δ-expressing yeast could have resulted from a nonspecific effect on induction of ER stress and compromised cell growth. Therefore the sensitivities of all of the examined mutants to an ER stress-inducing agent, dithiothreitol (DTT), were measured. We first found that the strains with a single deletion of any of the individual nonessential PDIs were DTT insensitive. In contrast, and as a control, ire1Δ yeast, which are unable to induce the unfolded protein response (Cox and Walter, 1996), were inviable when plated on DTT (Figure 5). Strains M4492 (pdi1Δmpd1Δmpd2Δeug1Δeps1Δ [MPDI1]) was significantly sicker than wild-type cells on rich media and was extremely DTT sensitive; however, when Pdi1 was the only PDI family member expressed (strain SRH01, pdi1Δmpd1Δmpd2Δeug1Δeps1Δ [PDI1]), DTT sensitivity was absent (Figure 5). In accordance with previous data (Holst et al., 1997), we also found that mutation of the a active site in Pdi1 (pdi1Δ [PDI1ΔSGHS-CGHC]) led to modest DTT sensitivity. Notably, the b’ chaperone mutant strain (pdi1Δ222-302Δ) was DTT insensitive. These results suggest that ApoB stabilization in the PDI1Δ222-302Δ chaperone-defective strain does not result from unmitigated stress and poor cell growth.

**ApoB degradation is α-mannosidase–like lectin independent**

Pdi1 interacts with and is required for the oxidation of an intramolecular disulfide bond in Htm1/Mnl1 (Clerc et al., 2009; Sakoh-Nakatogawa et al., 2009), which is the ER degradation-enhancing α-mannosidase–like lectin (EDEM) homologue in yeast. As in mammals, Htm1 recognizes misfolded glycoproteins and targets them for degradation (Jakob et al., 2001; Nakatsu- kasa et al., 2001). Recently a mutation in PDI1, pdi1-1, was identified in a screen for yeast that require a functioning unfolded protein response for viability (Gauss et al., 2011). The pdi1-1 mutation harnesses a leucine in place of a proline in the center of...
ApoB’s conformation in the yeast ER, we chose to measure the reactivity of any free cysteines in ApoB to the thiol-modifying reagent maleimide-PEG5000 after ApoB isolation and reduction. Interestingly, the amount of ApoB that could be recovered after this analysis was performed in the pdi1Δ[pdi1222-302Δ] strain—but not in the wild-type strain—was significantly reduced (data not shown). These results suggest that when the chaperone domain of Pdi1 is absent, the conformation of ApoB may be altered.

**Distinct PDI family members contribute differently to ApoB biogenesis in hepatic cells**

To assess whether the knowledge acquired from the use of the yeast system could inform us as to which mammalian chaperones might similarly mediate ApoB quality control, we examined the contributions of select mammalian PDI family members on ApoB biogenesis in hepatic cells. Although there are only five yeast PDI family members, mammals express 20 PDI-like proteins (Hatahet and Ruddock, 2009). To identify which of these PDIs to examine, we first performed a BLAST search with yeast Pdi1 against the human protein database. The three best hits were ERp72, PDI, and ERp57 (our unpublished data). Another criterion for this study was to ascertain which PDIs exhibited overlapping expression patterns with ApoB. PDI, ERp57, and ERp72 are all expressed in the liver (Marcus et al., 1996; also see the Human Protein Reference Database, www.hprd.org/), but the fourth- and fifth-best hits from the BLAST search, PDlp and PDILT, respectively, are not expressed in this tissue. Furthermore, PDI, ERp57, and ERp72 have a similar domain organization to Pdi1 (a-b-b'-a), although ERp72 has an additional thio-reodoxin-like active site (a-a-b-b'-a'; Hatahet and Ruddock, 2009). The crystal structures of yeast Pdi1 and ERp57 have been solved (Tian et al., 2006, 2008; Dong et al., 2009) and share broad overall similarity, having domains organized in a U-shaped conformation, although ERp57 has a more twisted conformation than Pdi1 (Dong et al., 2009). Of interest, PDI and ERp72 are also able to support the growth of pdi1Δ yeast strains (Gunther et al., 1993).

Because Pdi1’s b’ domain was required to facilitate ApoB ERAD in yeast (Figure 4C), we also wanted to examine mammalian PDI family members that harbor substrate-binding domains. PDI, ERp57, and ERp72 all have substrate-binding domains, and specific substrates have been identified for each of these proteins. The chaperone activity of PDI is necessary to refold proinsulin (Winter et al., 2002) and lysozyme (Puig and Gilbert, 1994). ERp57 specifically associates with lectins through a binding site in its b’ domain (Russell et al., 2004), and a range of substrates have been identified for ERp57 in vivo, including, laminin, collagen, and clus-terin (Jessop et al., 2009). Similarly, ERp72 is found within chaperone complexes and interacts with thrombospondin (Kuznetsov et al., 1997) and thyroglobulin (Menon et al., 2007), but, it is unknown whether these interactions occur through the b’ substrate-binding domain. Nevertheless, PDI, ERp57, and ERp72 have previously been shown to precipitate with ApoB, and through its integration into the MTP complex, PDI interacts with ApoB during lipid loading (Adeli et al., 1997; Linnik and Herscovitz, 1998; Zhang and Herscovitz, 2003). However, the roles of PDI, ERp57, or ERp72 as regulators of ApoB degradation in the mammalian ER have not been examined.

To determine whether PDI, ERp57, or ERp72 contributes to ApoB ERAD, we overexpressed each protein in rat hepatoma McA-RH7777 cells, an established cell line for studying ApoB biogenesis (Tanabe et al., 1989). First, to monitor whether each protein could be overexpressed, cells were assayed after they were transfected with a vector control or vectors engineered for
the transient expression of human PDI, ERp57, or ERp72. After 48 h the cells were broken and the lysates were subjected to SDS-PAGE and probed with anti-PDI, anti-ERp57, or anti-ERp72 antibodies. When duplicate samples were analyzed and the signals were quantified relative to a loading control, PDI and ERp72 were overexpressed by approximately sevenfold and sixfold, respectively (Figure 7A), and—assuming similar antibody avidity—the quantity of overexpressed ERp57 appears comparable to that of PDI and ERp72 when overexpressed (Figure 7A).

We hypothesized that if PDI played a strong MTP complex-independent role in mammalian cells during ApoB degradation, then its overexpression would result in decreased recovery of ApoB (Wang et al., 1997). However, if PDI’s only role in ApoB biogenesis was through the MTP complex, we predicted that increased recovery of ApoB would be evident because the MTP complex promotes ApoB secretion (Gordon et al., 1994; Leiper et al., 1994; Haghpassand et al., 1996) and escape from ERAD. No change in ApoB recovery could indicate that a prodegradative role of PDI was balanced by the stabilizing role of the MTP complex. When the amount of radio-labeled ApoB recovered after a 60-min chase in PDI-overexpressing cells was compared with the control, a 37% overall increase in ApoB recovery was evident (Figure 7B, left). These results suggest that the expression of greater amounts of PDI improve MTP complex function and augment ApoB transport and escape from ERAD. As controls for this experiment, we also assessed the secretion of albumin, as described (Gusarov et al., 2007; Hrizo et al., 2007), but found that its biogenesis was unaffected by PDI overexpression (data not shown). In addition, we asked whether PDI overexpression enhanced ApoB secretion because it inhibited ERAD. However, we found that PDI overexpression, regardless of whether the proteasome was active or not (i.e., in the presence of MG-132), led to an increase in the amount of secreted ApoB (Supplemental Figure 4).

If yeast Pdi1 is required for ApoB degradation (Figures 2 and 4), why is the closest homologue in human cells, PDI, not required for ERAD? Instead, human PDI overexpression stabilized the protein and increased its recovery in this experiment. The simplest explanation for this result is that yeast lack the MTP complex, which is required to load lipids onto maturing, ApoB-containing chylomicrons and VLDLs in the ER. In mammalian cells, the loss of MTP function—brought about either by genetic means or through the use of small-molecule inhibitors—is accompanied by increased ApoB degradation (Jamil et al., 1996; Benoist and Grand-Perret, 1997; Zimmermann et al., 2006). Thus our yeast ApoB model most closely resembles the lipid- or MTP complex–deficient state. We conclude that Pdi1 is a prodegradative factor for ApoB in yeast, and yet, when complexed with the M subunit in mammals, the closest Pdi1 homologue, PDI, promotes ApoB folding and secretion.

On the basis of our yeast data, we also hypothesized that overexpression of ERp57 and ERp72 would lead to increased ApoB degradation. As predicted, when either ERp57 or ERp72 was overexpressed, ~33% less ApoB was recovered compared with the vector control (Figure 7B, middle and right). To confirm that ApoB degradation was proteasome mediated and that the effect of ERp57 and ERp72 was via the ERAD pathway and not an alternate degradative system (Pan et al., 2008), we assessed the impact of a proteasome inhibitor, MG132, on ApoB recovery when ERp72 was overexpressed. In this experiment, in the presence of DMSO, less ApoB was again recovered when ERp72 was overexpressed compared with the vector control, but when cells were treated with MG132, ApoB recovery in both the mock and ERp72-overexpressing cells rose (Figure 7C). Overall, these data indicate that PDI is primarily an ApoB-stabilizing chaperone in mammalian cells, most likely through its function as a component of the MTP complex, but that ERp57 and ERp72 facilitate ApoB ERAD. More generally, these
results support the use of the yeast ApoB expression system as a means to identify components that play diverse roles during the ERAD of this protein in mammals.

**DISCUSSION**

ApoB is targeted for degradation unlike any other known ERAD substrate. Under lipid-poor conditions, ApoB is cotranslationally selected for degradation by the Hsp70 and Hsp90 molecular chaperones, as well as a J domain–containing cochaperone, PS8PPK. The apolipoprotein is then ubiquitylated by gp78 while translocon embedded and is retrotranslocated into the cytosol through the Sec61 translocon by the action of the AAA-ATPase p97. Ultimately, this immature form of ApoB is captured and degraded by the proteasome (Dixon et al., 1991; Yeung et al., 1996; Benoist and Grand-Perret, 1997; Fisher et al., 1997, 2008; Yao et al., 1997; Liao et al., 1998; Mitchell et al., 1998; Guszarova et al., 2001; Pariyarath et al., 2001; Liang et al., 2003; Oyadomari et al., 2006). Given these unique attributes, and because of the profound link between ApoB secretion and human disease, we sought to identify and characterize other factors that affect the decision between stabilizing and degrading ApoB.

In this study, we show for the first time that PDI family members play opposing roles during the degradation of an ERAD substrate in mammalian cells. Specifically, we find that ERp57 and ERp72 facilitate the proteasome-mediated degradation of ApoB, but PDI helps stabilize ApoB in hepatic cells, an effect most likely brought about by its membership in the MTP complex. In yeast, which lack the MTP complex, we discovered that Pdi1 facilitates the ERAD of ApoB. We also discovered that the chaperone activity of ApoB is required for degradation. In contrast, the degradation of another substrate (i.e., CPY*) depends on Pdi1’s thio-redox motifs, and destruction of yet another ERAD substrate (i.e., α-ENaC) is PDI independent. These data highlight the complex and unique actions of PDI family members during ERAD.

One surprising aspect of this study is that ApoB forms a disulfide cross-linked species with Pdi1 in the yeast ER (Figure 3). Thus one might predict that the redox or isomerization activity—but not the chaperone activity—of Pdi1 might have been most important for ERAD. It was unexpected that mutating the cysteine residues in the a or a’ site had no effect on the rate of ApoB degradation, although CPY* degradation was blocked. There are two explanations for these data. First, there may be two pools of ApoB. One that has formed cross-links and one that is en route for degradation, an event that may require a noncovalent, chaperone-like association between ApoB and Pdi1. In fact, we note that the ERAD of ApoB is incomplete when chase reactions are conducted in yeast (see, e.g., Figure 1), and so perhaps the remaining, stable material represents the cross-linked pool. Second, Pdi1’s two activities, as a redox enzyme and as a chaperone (LaMantia and Lennarz, 1993; Gilleece et al., 1999), might provide unique functions during ApoB biogenesis. For example, Pdi1 might initially form disulfide bonds with ApoB, which could represent an attempt by Pdi1 and other chaperones to assist protein folding. However, because yeast lack the MTP complex, the chaperone activity of Pdi1 then becomes essential to maintain ApoB’s retrotranslocation competence prior to degradation. Consistent with this view, we find that ApoB can cross-link to Pdi1 even if the chaperone domain or the a or a’ active sites have been mutated; CPY* also cross-links to each Pdi1 species (Supplemental Figure 5). In this model, however, it remains mysterious how the generated disulfide bonds in ApoB might be broken prior to retrotranslocation. Yeast lack ERdj5, which has been proposed to perform this function (Ushioda et al., 2008). So, one is left with the scenario that a yet-to-be-identified enzyme is capable of breaking disulfides in the yeast ER. Alternatively, the reduction of disulfide bonds might be unnecessary for Sec61-dependent retrotranslocation and proteasome degradation. It is worth noting that polypeptide "loops" can be inserted into the medium at the completion of the chase divided by the amount of ApoB-precipitable material recovered from cell lysates and secreted into the medium at the completion of the chase divided by the amount of ApoB-precipitable material recovered from cell lysates and secreted into the medium.
ApoB maturation requires the formation of specific disulfide bonds, and mutations in cysteines that form disulfide bonds in ApoB diminish VLDL assembly and secretion (Huang and Shelnear, 1997; Tran et al., 1998; DeLozier et al., 2001). Mutations in the M subunit of the MTP complex, which contains PDI, can also prevent ApoB maturation, a phenomenon that results in abetalipoproteinemia (Narcisi et al., 1995; Ohashi et al., 2000). Because we observed measurable effects of PDI, ERp57, and ERp72 overexpression on the amount of secreted ApoB, our results may be relevant to explain the relative severity of human diseases such as hypobetalipoproteinemia and atherosclerosis. In most cases, the factor(s) that lead to differences in the circulating lipoprotein levels in the population are mysterious, but it is plausible that variations in the amount or activities of components that control ApoB maturation may be at play. More generally, polymorphisms in ApoB itself are known to contribute to altered levels of secreted VLDLs, as specific nonsense codons in APOB cause hypobetalipoproteinemia (Linton et al., 1993; Whitfield et al., 2004). Overall, we hope that these ongoing studies will provide therapeutic candidates that may be targeted to prevent the catastrophic effects of diseases related to ApoB-containing lipoproteins, in particular atherosclerosis, which is the leading cause of death in Western societies.

MATERIALS AND METHODS
Yeast strains, strain construction, and growth assays
Yeast strains were grown at 26°C using standard conditions for growth, media preparation, and transformation unless otherwise noted (Adams et al., 1997). A complete list of yeast strains used in this study is given in Supplemental Table S1.

To create the PDI single-deletion strains (mpd1Δ, mpd2Δ, eug1Δ, and epl1Δ), PCR-mediated gene disruption was used, using the W303 background (Brachmann et al., 1998). In each case, the KanMX cassette was amplified from the pFA6a-KanMX6 plasmid using primers containing 20 nucleotides of homology to the KanMX cassette (underlined) and 40 nucleotides of homology flanking the gene to be disrupted. To amplify KanMX for the disruption of MPD1 the following primers were used: (forward) 5′-TCC ACT TAA CAC AAT TAG GAG AGA CAA AAT TTG ACA TAT AAG ATT GTA CGT AGA GTG CAC-3′ and (reverse) 5′-TGG GTT TAA TTA TTA GAT AAT CAT TGA ATG AGG AAA CGT ACC ACT GTG CGG TAT TTC ACA CCG-3′. To amplify KanMX for the disruption of MPD2 the following primers were used: (forward) 5′-GTG TAC TGC AAG TAC GTG GCC AAA GTA AAA CAC AAA GGA GAG ATT GTA CGT AGA GTG CAC-3′ and (reverse) 5′-TCG GTA TTC GTA AAG TAA AAG ACA GAG CGA AGC TTA TGT TGT CGG TAT TTC ACA CCA CCG-3′. To amplify KanMX for the disruption of EUG1 the following primers were used: (forward) 5′-ATA TGG CAA TCT CCC AAC AAC CAC CGG CTC ATA TAC CAG ATT GTA CGT AGA GTG CAC-3′ and (reverse) 5′-AGA TGT TAA AAA TAA GTA GCA ATG CAT ATG GTT TAT TTA TTA TGCT GTG CGG TAGT TAT ACA CCG-3′. To amplify KanMX for the disruption of EPS1 the following primers were used: (forward) 5′-AAA AAT ACT ATC TAT AAA AAC AAG TAG CTG TAA GGC AGC AGC CAG ATT GTA CGT AGA GTG CAC-3′ and (reverse) 5′-AGA TAT CAG CAT TCT TTT ATT TTT ATA ACT TAA GCC GTG TCT GTG CGG TAT TTC ACA CCA CCG-3′. The deletions of MPD1, MPD2, EUG1, and EPS1 were confirmed by PCR.

Strain SRH01 was created using a plasmid shuffle. Briefly, plasmid pSG01 (see later discussion) was transformed into strain M4492, kindly provided by the Schmitt lab (Saarland University, Saarbrücken, Germany). Transformants were plated on medium containing 5-fluoroanthranilic acid, which selects for cells that have lost the TRP1 gene. Therefore the MPD1-TRP plasmid from strain M4492 was lost and replaced with a plasmid in which the expression of Pdi1 was driven from its own promoter.

DTT sensitivity was determined by growing cells overnight at 26°C to logarithmic phase in the appropriate selective medium. The number of cells in each culture was normalized. and a dilution series from each culture (1:10, 1:100, 1:1000) was plated on yeast extract/peptone/dextrose solid media (pH 5.5) either containing or lacking a final concentration of 5 mM DTT. The plates were incubated at 30°C for 2 d.

Plasmids
The plasmids used in this study are shown in Supplemental Table S2. To assess the degradation of ApoB29 in yeast, pSLW1-B29 was used (Hrizo et al., 2007). To monitor the degradation of CPY*, we used pRS316CPY*-3HA, which was kindly provided by the Weissman lab (University of California, San Francisco, San Francisco, CA; Bhamidipati et al., 2005).

Plasmids pBH1464, pBH1852, pBH1630, and pCT37 (Holst et al., 1997) were generous gifts from the Schmitt lab (Saarland University). Plasmid pSG01 was constructed by subcloning a PstI–BamHI fragment from plasmid pBH1464 into the same sites in pRS314.

To study the degradation of poxF, we used pSM36 (Kim et al., 2005). To monitor the degradation of α-ENaC in the nonessential PDI deletion strains, we used plasmid pRS426GPD ENaC-3HA (Buck et al., 2010). To measure the degradation of α-ENaC in strains M4492 and SRH01 (Supplemental Table S1) a methionine-repressible α-ENaC plasmid was constructed. To this end, an EcoRI–XhoI fragment corresponding to the coding sequence of α-ENaC and the hemagglutinin (HA) epitope tag from plasmid pRS426GPD α-ENaC-HA was subcloned into the same sites in pRS426MET25 (Mumberg et al., 1994) and was named pRS426MET25 α-ENaC-HA.

To assess the degradation of PrA*-Ab–HA, plasmid pKK223 (Kanehara et al., 2010) was kindly provided by the Ng lab (National University of Singapore, Singapore).

Assays to measure the degradation of ERAD substrates in yeast
To assess the degradation of ApoB29 in yeast, cells transformed with pSLW1-B29 were grown to logarithmic phase (OD600 = 0.4–1.0) overnight at 26°C in synthetic complete medium lacking uracil but supplemented with glucose to a 2% final concentration. The cells were harvested and resuspended in complete medium supplemented with galactose to a final concentration of 2% and were grown for 5 h at 26°C to obtain maximal expression of ApoB29. The cycloheximide chase analysis was performed at 30°C as previously described (Hrizo et al., 2007). Total protein was precipitated as described (Zhang et al., 2001) and immediately resolved by SDS–PAGE before immunoblot analysis. ApoB29 was detected by using an anti-HA-horseradish peroxidase (HRP)–conjugated antibody (clone 3F10; Roche, Indianapolis, IN) antibody. Immunorecords were also probed with anti-G6PD (Sigma-Aldrich, St. Louis, MO) antiserum as a loading control. The G6PD primary antibody was decorated with donkey HRP-conjugated anti–rabbit immunoglobulin G (IgG) secondary antibody (GE Healthcare, Waukesha, WI). The Supersignal West Femto Chemiluminescent Substrate (Pierce, Rockford, IL) was used for the detection of anti-HA ApoB29 in immunoblots, and the Supersignal West Pico Chemiluminescent Substrate (Pierce) was used for the detection of anti-G6PD in immunoblots. The signals were quantified using a Kodak 440CF Image Station and the associated Kodak 1D software (Eastman Kodak, Rochester, NY).

To monitor the degradation of CPY*, cells expressing pRS316CPY*-3HA were grown in the appropriate selective media
overnight at 26°C to logarithmic phase (OD$_{600} = 0.4–1.0$). Cycloheximide chase at 30°C, protein precipitation, and SDS–PAGE were performed as previously described (Tran et al., 2011). Immunooblots were probed with an anti-HA-HRP-conjugated (clone 3F10; Roche) antibody to detect CPY* and with anti-6C61 antisemur (Stirling et al., 1992), which served as a loading control. The anti-6C61 primary antibody was probed with donkey HRP-conjugated anti–rabbit IgG secondary antibody. The Supersignal West Pico Chemiluminescent Substrate was used to detect anti-HA CPY* and immunooblots were also probed with anti-6C61. The resulting signals were quantified as described.

The degradation of the α subunit of ENaC (α-ENaC) was assessed by introducing plasmid pRS426GPD α-ENaC-HA (see earlier discussion and Supplemental Table S2) into the recipient strains that lacked single, nonessential PDI family members. The cells were grown in selective medium overnight at 26°C to logarithmic phase, and cycloheximide chases, protein precipitation, and SDS–PAGE were performed as described (Buck et al., 2010). α-ENaC degradation in strains M4492 and SRH01 was determined by introducing plasmid pRS426MET25 α-ENaC-HA (see earlier discussion), which is engineered for the methionine-repressible expression of α-ENaC. The cells were grown at 26°C in selective medium with 2 mM methionine overnight to logarithmic phase. To obtain maximal expression of α-ENaC, cells were harvested and resuspended to an initial concentration of 0.5 A$_{600}$/ml in selective medium lacking methionine and were grown for another 1.5 h. Cycloheximide chases, protein precipitation, and SDS–PAGE were performed as published (Buck et al., 2010). Immunooblots were probed with an anti-HA-HRP-conjugated antibody (clone 3F10; Roche) to detect α-ENaC, and with anti-G6PD antisemur, which served as a loading control. The anti-G6PD primary antibody was probed with donkey HRP-conjugated anti–rabbit IgG secondary antibody. The Supersignal West Pico Chemiluminescent Substrate was used to detect the anti-HA α-ENaC and anti-G6PD antibodies on the immunooblots, and the signals were quantified as described.

The ERAD of pxF was measured using plasmid pSM36 (see Supplemental Table S2 and earlier discussion). In brief, cells were grown in selective medium at 26°C overnight to logarithmic phase and were harvested and resuspended to 0.5 A$_{600}$/ml. Next the cells were preincubated at 30°C with vigorous shaking for 10 min, and then protein synthesis was stopped by the addition of cycloheximide to a final concentration of 50 μg/ml. At the indicated time points, 1 ml of cells was harvested and frozen in liquid nitrogen. Total protein was precipitated as described (Zhang et al., 2001) and was resolved by SDS–PAGE, followed by Western blot analysis. Immunoblots were probed with an anti-HA-HRP-conjugated antibody (clone 3F10; Roche) antibody to detect pxf and with anti-G6PD antisemur, which served as a loading control. The anti-G6PD primary antibody was probed with donkey HRP-conjugated anti–rabbit IgG secondary antibody. The Supersignal West Pico Chemiluminescent Substrate was used to detect the bound antibodies, and the signals were quantified as described.

Cysteine modification assays
Cysteine were modified by the addition of maleimide-PEG5000 using a previously published protocol (Tetsch et al., 2011) that we adapted for yeast. Briefly, spheroplasts were prepared from cells expressing ApoB29 and were resuspended in 20 mM HEPES, pH 6.8, 150 mM KOAc, 5 mM MgOAc, and 1.2 M sorbitol. Iodoacetamide was added to a final concentration of 10 mM, and cells were incubated at 30°C for 15 min. Following incubation, proteins were precipitated by the addition of TCA to a final concentration of 10% and were incubated on ice for 30 min. The cells were centrifuged (16,000 × g, 15 min) and the pellets were resuspended in denaturing buffer (6 M urea, 200 mM Tris-HCl, pH 8.5, 10 mM EDTA, 0.5% SDS) containing 1 mM DTT and were incubated at 37°C for 1 h. The samples were then divided into two tubes, and again proteins were precipitated with 10% TCA on ice for 30 min. Following centrifugation (15 min, 4°C, 16,000 × g) and removal of residual TCA, the pellets were washed three times with 20 mM HEPES-KOH, pH 7.4, and 50 mM NaCl with protease inhibitors and incubated with anti-HA-conjugated resin (Roche) or Sepharose 6B resin (Sigma-Aldrich) as a negative control for 3 h at room temperature. The immunoprecipitates were washed three times with 20 mM HEPES-KOH, pH 7.4, and 50 mM NaCl and eluted in sample buffer prepared with 120 mM of freshly added DTT for 3 min at 75°C for SDS–PAGE, as described. Prior to the immunoprecipitation, 1% of the lysate was retained and loaded as a control. The samples were immunooblotted with anti-Pdi1 antibody (a kind gift from V. Denic, Harvard University, Cambridge, MA), and the primary antibody was probed with donkey HRP-conjugated anti–rabbit IgG secondary. The Supersignal West Pico Chemiluminescent Substrate was used for detection. ApoB was detected as described.
was amplified using standard PCR conditions and the following primers, which contained a Kozak consensus sequence (in bold) and KpnI or XbaI restriction sites (underlined). PDI was amplified using the primers (forward) 5′-G TAC CCT ACC GCC ACC ATG CTG CGC GCC GCT CTG CTG-3′ and (reverse) 5′-G TAC TCT AQA TTA CAG TTC ATC TTT CAC AGC-3′. Erp57 was amplified using the primers (forward) 5′-G TAC CCT ACC GCC ACC ATG CTG CGC GCC GCT CTG CTG-3′ and (reverse) 5′-G TAC TCT AQA TTA CAG TTC ATC TTT CAC AGC-3′. Erp72 was amplified using the primers (forward) 5′-G TAC CCT ACC GCC ACC ATG CTG CCC GCG GTA TCA AAG CTC TTC CTT GGT CCT G-3′. The engineered restriction sites at the 5′ and 3′ ends allowed for subcloning into the pcDNA3 vector. High-fidelity amplification and cloning was confirmed by sequence analysis.

Rat hepatoma McA-RH7777 cells (American Type Culture Collection, Manassas, VA; CRL-1601) were cultured at 37°C in DMEM supplemented with 10% fetal bovine serum, 10% horse serum, l-glutamine, and penicillin/streptomycin. Cells were transfected with 2 μg of pcDNA3.1 vector (control), pcDNA3.1-hPDI expression vector, or pcDNA3.1-1-Herp57 expression vector, or pcDNA3.1-1-Herp72 expression vector, prepared as described, using FuGENE HD transfection reagent (Roche) according to the manufacturer's specifications. A total of 24 h after the initial transfection a second transfection with 2 μg of DNA was performed. A pulse-chase analysis was performed 48 h after the second transfection, as previously described (Gusarova et al., 2001). The proteasome dependence of ApoB100 degradation and secretion was performed as described (Meex et al., 2011) with either DMSO or the proteasomal inhibitor MG132 (Z-Leu-Leu-Leu-al; Calbiochem) according to the manufacturer's specifications. A total of 24 h after the initial transfection a second transfection with 2 μg of DNA was performed. A pulse-chase analysis was performed 48 h after the second transfection, as previously described (Gusarova et al., 2001). The proteasome dependence of ApoB100 degradation and secretion was performed as described (Meex et al., 2011) with either DMSO or the proteasomal inhibitor MG132 (Z-Leu-Leu-Leu-al; Sigma, St. Louis, MO) added to a final concentration of 25 μM.

ACKNOWLEDGMENTS

We thank Chris Bahur, Jessica Coble, Vlad Denic, Rebecca Hughey, Davis Ng, Karin Römisch, Manfred Schmitt, and Jonathan Weissman for technical assistance, advice, and/or reagents. This work was supported by National Institutes of Health Grants HL58541 to E.A.F. and J.L.B. and GM75061 and DK65161 to J.L.B. We also acknowledge the Core Facilities of the Pittsburgh Center for Kidney Research, Pittsburgh, PA (DK79307).

REFERENCES


