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Articles

A Microsomal Endopeptidase from Liver That Preferentially Degrades Stearoyl-CoA Desaturase†

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ABSTRACT: A protease was purified some 700-fold from rat liver microsomes by a combination of differential detergent solubilization, hydroxyapatite column chromatography, and gel filtration. The protease exhibits substrate selectivity for stearoyl-CoA desaturase (SCD). The purified protease rapidly degraded SCD while other microsomal proteins including cytochrome b_5 and 11β-hydroxysteroid dehydrogenase were degraded slowly or not at all. The isolated form of the protease has an apparent molecular mass of ~90 kDa. Upon incubation, the 90 kDa form of the protease undergoes rapid conversion to a series of smaller proteins. This conversion is associated with a marked increase in proteolytic activity. Diisopropyl phosphofluoridate (DFP) at high concentration partially inhibited the protease activity. The [^{3}H]DFP-labeled protease was detected as three protein bands of approximately 66 kDa under nonreducing conditions and a single 25 kDa band under reducing conditions. The purified protease was inhibited by dithiothreitol, suggesting the presence of an essential disulfide bond. These results further define the mechanism by which SCD is rapidly and selectively degraded in isolated liver microsomes.

The protein components of the endoplasmic reticulum (ER) are in a constant state of renewal. The mean half-life of an ER protein is approximately 2 days, but the degradation rates of individual protein species of the ER are quite variable (*1*, *2*). The protease(s) involved in the normal turnover of ER proteins and the mechanism by which certain proteins are selected for rapid degradation are unknown. To address these questions, we are investigating the degradation of a short-lived ER protein, stearoyl-CoA desaturase (SCD).

SCD is the terminal component of the microsomal Δ^9 desaturase system and catalyzes the insertion of a double bond into palmityl-CoA and stearoyl-CoA (*3*). The reaction utilizes O_2 and electrons from reduced cytochrome b_5 (cyt b_5) (*3*). The Δ^9 desaturase system is required for the biosynthesis of oleate and palmitoleate, the major monounsaturated fatty acids of membrane phospholipids, triglycerides, and cholesterol esters (*4*). When fatty acids are not available from dietary sources, SCD mRNA, SCD activity, and the concentration of SCD protein in the liver increase up to 50-fold (*5*, *6*). Induction is primarily due to increased transcription of the SCD gene (*6*). Once unsaturated fatty acids are restored in the diet, the SCD protein content of liver microsomes rapidly decreases to preinduction levels (*5*). The half-life of hepatic SCD in vivo and in vitro is approximately 4 h (*5*, *7*). The combination of robust SCD mRNA induction and rapid SCD protein degradation provides elevated but transitory desaturase levels in response to physiological needs.

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SCD consists of a single polypeptide of 358 residues (*8*). The Δ^9 desaturases contain two internal hydrophobic segments separated by a 100-residue middle segment and are predicted to span the ER membrane four times with two short hydrophilic loops in the ER lumen and three cytosolic domains comprising the N-terminus, middle segment, and C-terminus (*9*, *10*). There are eight catalytically essential histidine residues arranged in three clusters in the middle segment and C-terminus (*10*). These histidine residues are conserved in other oxygen-activating non-heme iron enzymes and probably act as ligands for the iron atom(s) contained in these enzymes (*10*). The N-terminus of SCD contains an extended sequence of hydroxylated amino acids and a cluster of lysyl residues (*8*).

In previous studies, we showed that SCD is rapidly and selectively degraded in isolated liver microsomes (*7*, *11*). Microsomal SCD protease activity remains associated with the membrane fraction when cytosolic surface proteins and soluble lumenal proteins are selectively removed (*7*, *11*). More recently, we showed that the N-terminus of SCD contains a sequence determinant for rapid protein degradation in CHO cells (*12*). Three lysyl residues are essential for the function of the N-terminal degradation signal (*12*).

In this report we describe the purification and characterization of a microsomal protease that selectively degrades SCD. The protease was purified 700-fold by a combination of differential detergent solubilization, hydroxyapatite chromatography, and gel filtration chromatography. The protease was isolated as a 90 kDa protein that appears to be a zymogen since incubation leads to increased proteolytic activity and conversion into smaller protein forms.

EXPERIMENTAL PROCEDURES

Materials. Chemicals and reagents were purchased from Sigma unless otherwise noted. CHAPS was from ProChem, Inc. L-[^{35}S]Methionine was from Amersham. [^{3}H]DFP was from New England Nuclear. Hydroxyapatite (Ultragel HA, 60−180 μm bead size) was from LKB. Human apolipoprotein B (apoB) and anti-apoB polyclonal antibody were obtained from Chemicon. The reverse-phase HPLC column (C4, 0.46 × 15 cm) was from Vydac. Centricon-10 and -30 microconcentrators were from Amicon. Restriction and modification enzymes were from Life Technologies. The vector pGEM-11Zf(+) used for the coupled transcription/translation reactions and the coupled transcription/translation system (TNT) was from Promega. The cDNA encoding cyt b_5 inserted in pT7T3D-Pac was from the American Type Culture Collection (ATCC). The cDNA encoding for 11β-hydroxysteroid dehydrogenase (11β-HSD) was a gift from Dr. Anil K. Agarwal, University of Texas Southwestern Medical Center at Dallas.

Construction of Plasmids. The cDNA encoding the rat liver stearoyl-CoA desaturase (SCD) was obtained as described previously (*12*). The cDNAs encoding the full length of rat liver SCD and SCD 45−358 preceded by a Kozak sequence and an *Eco*RI site at the 5′ end and extended at the 3′ end by a *Not*I site were amplified by polymerase chain reaction using the following oligonucleotides: P1 (5′-GAG CGA ATT CCC ACC ATG CCG GCC CAC ATG CTC CAA-3′) and P2 (5′-GAG CGA ATT CCC ACC ATG AGA GAA GAT ATC CAC GAC CCC-3′) for the 5′ end of SCD and SCD 45−358, respectively. The downstream primer was P3 (5′-GTC GCG GCC GCT TTA GCT ACT CTT GTG GCT CCC ATC-3′). The resulting PCR products were digested with *Eco*RI−*Not*I and inserted into *Eco*RI−*Not*I-digested pGEM-11Zf(+).

The cDNA encoding the full length of rat 11β-HSD preceded by a Kozak sequence and an *Eco*RI site at the 5′ end and extended at the 3′ end by a *Not*I site was amplified by polymerase chain reaction using the following oligonucleotides: P4 (5′-GAG CGA ATT CCC ACC ATG AAA AAA TAC CTC CTC CCC GTC CTG G-3′) and P5 (5′-CAG CGG CCG CTA GTT GCT TAC AAA TAG GTC CC-3′). The resulting PCR product was digested with *Eco*RI−*Not*I and inserted into *Eco*RI−*Not*I-digested pGEM-11Zf(+).

The cDNA encoding human cyt b_5 inserted in pT7T3D-Pac was obtained from the ATCC. Contrary to what was reported by the ATCC, this cDNA was incomplete. The sequencing of this insert revealed that the sequence segment Met1−Thr26 was missing. The sequence was then completed by PCR using two long overlapping oligonucleotides, P6 (5′-GCC GTG AAG TAC TAC ACC CTA GAG GAG ATT CAG AAG CAC AAC CAC AGC AAG AGC ACC TGG CTG ATC CTG CAC CAC AAG-3′) and P7 (5′-GAG CGA ATT CCC ACC ATG GCA GAG CAG TCG GAC GAG GCC GTG AAG TAC TAC ACC CTA-3′), adding the sequences AVKYYTLEEIQKHNHSKST and MAEQSDE preceded by a Kozak sequence and an *Eco*RI site at the 5′ end. The cDNA encoding full-length cyt b_5 was then amplified by PCR using oligonucleotides P7 and P8 which contains a *Not*I site (5′-GTC GCG GCC GCT TCA GTC CTC TGC CAT CTA TAG-3′). The PCR product was digested with *Eco*RI−*Not*I and inserted into *Eco*RI−*Not*I-digested pGEM-11Zf(+).

Analysis of Protein Degradation. SCD degradation was assayed by following the disappearance of the [^{35}S]Met-labeled SCD, cyt b_5, and 11β-HSD protein bands by autoradiography after SDS−PAGE. A stock of degradation substrate was prepared by diluting 10 μL of the transcription/translation reaction product containing labeled proteins with 90 μL of 20 mM sodium phosphate, pH 8.0. For each assay, 1−2 μL aliquots of the indicated fractions were added to 10 μL of the diluted TNT reaction product and incubated at 37 °C for the indicated times. For apoB degradation, 2.5 μg of apoB was incubated with 1 μL of the protease fraction at 37 °C for 3 h. Control mixtures were frozen at −20 °C. Degradation was terminated by the addition of SDS−PAGE sample buffer and boiling for 2 min.

Immunoblot Analysis. Preparation of antisera against purified microsomal SCD and a synthetic peptide representing the C-terminus of SCD was described previously (*6*, *7*). Apolipoprotein B (apoB) was detected with commercial polyclonal anti-apoB antibody from Chemicon. Proteins were separated by 10% or 12% SDS−PAGE and transferred onto PVDF membranes. The blots were blocked with 3% albumin. The blots were incubated with antibody overnight at 4 °C. Bound antibody was detected with an anti-IgG−alkaline phosphatase conjugate (Sigma A-3687) and a phosphatase detection kit (Kirkegaard and Perry Laboratory, Gaithersburg, MD).

Preparation of Microsomes and Purification of the SCD Protease. Desaturase-induced rat liver microsomes were

prepared as described (*7*). All subsequent steps were done at 4 °C. Crude microsomes were washed twice with high-salt wash buffer [10 mM Tris−acetate (pH 8.0), 20% glycerol, 500 mM NaCl, 20 mM EDTA, 1 mM DTT] in order to remove trapped cytosolic proteins and peripheral membrane proteins. Microsomes were washed by suspending the pellet in 2.5 mL of high-salt wash buffer/g of liver with a Potter−Elvehjem homogenizer at 4 °C and centrifuged for 90 min at 40000 rpm (125000*g*) at 4 °C in a Beckman Ti45 rotor. After the second high-salt extraction, the microsomes were washed with low-salt buffer [5 mM Tris−acetate (pH 8.0), 10% glycerol, 0.5 mM EDTA, 50 μM DTT] to reduce the salt content of the microsomes. The microsomes were suspended in 2.5 mL of low-salt buffer/g of liver with a Potter−Elvehjem homogenizer at 4 °C and centrifuged for 60 min at 36000 rpm (100000*g*) at 4 °C in a Beckman Ti45 rotor. The washed microsomes were suspended with a Potter−Elvehjem homogenizer in 1 mL of microsomal solubilization buffer [MSB; 10 mM Tris−acetate (pH 8.0), 20% glycerol] per gram of liver. High-salt-washed microsomes (HSWM) refer to microsomes that have been washed twice with high-salt buffer and once with low-salt buffer and suspended in MSB. The HSWM were solubilized by slowly adding Triton X-100 (TX-100) to a final concentration of 2% while stirring at 4 °C. The TX-100 insoluble fraction was collected by centrifugation for 60 min at 38000 rpm (110000*g*) at 4 °C in a Beckman Ti45 rotor. The TX-100 insoluble fraction from 100 g of liver was suspended with a Potter−Elvehjem homogenizer in 15 mL of MSB and solubilized by slowly adding 10% CHAPS and 4 M NaCl sequentially to give final concentrations of 200 mM NaCl and 2% CHAPS while vortexing at 4 °C. The insoluble material was removed by centrifugation for 60 min at 40000 rpm (125000*g*) at 4 °C in a Beckman Ti45 rotor. The solubilized material was applied to an 80 mL column (2.5 × 16 cm) of hydroxyapatite equilibrated in 10 mM NaP buffer [10 mM sodium phosphate (pH 7.5), 1% CHAPS, 5% glycerol]. The column was developed with 200 mL of equilibration buffer, 400 mL of 25 mM NaP buffer, and 200 mL of 50 mM NaP buffer. Individual fractions of the 50 mM NaP eluate were concentrated by ultrafiltration using Centricon-10 or -30 devices. A 0.3 mL aliquot of the concentrated HAP preparation was fractionated by gel filtration FPLC on a Superdex 200 HR 10/30 column (Pharmacia) equilibrated with 10 mM Tris−acetate (pH 8.0), 1% CHAPS, and 200 mM NaCl at a flow rate of 0.3 mL/min. Protein concentrations were determined by the Coomassie colorimetric method (Pierce).

Selective Reduction and Alkylation. The 50 mM NaP eluate from the hydroxyapatite column was concentrated 20-fold by ultrafiltration. The concentrated preparation was alkylated with 10 mM iodoacetamide for 30 min at 37 °C. The alkylated preparation was desalted by ultrafiltration using cold desalting buffer [10 mM Tris-HCl (pH 8.0), 25 mM NaCl, 5% glycerol, 1% CHAPS] as the diluent. Aliquots of the alkylated and desalted preparation were reduced with 0−500 μM DTT for 2 h at 37 °C. The reduced preparations were realkylated with 2.25 mM [^{14}C]iodoacetamide (specific activity 55 mCi/mmol) for 1 h at 25 °C. Alkylation with [^{14}C]iodoacetamide was terminated by adding β-mercaptoethanol. The ^{14}C-labeled preparations were analyzed by SDS−PAGE followed by autoradiography.

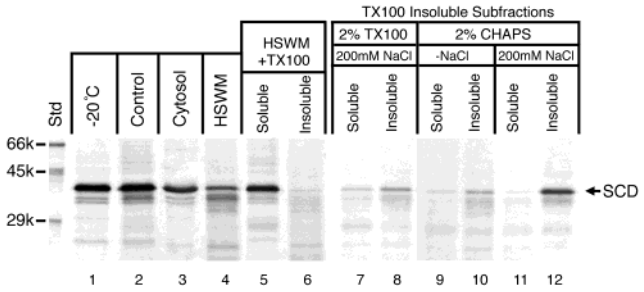

FIGURE 1: Subcellular fractionation and solubilization of the stearoyl-CoA desaturase (SCD) protease. [^{35}S]SCD was synthesized by in vitro transcription and translation. Subcellular fractions were incubated with [^{35}S]SCD for 15 h at 37 °C and analyzed by SDS−PAGE on a gel containing 10% acrylamide. A fluorogram of the gel is shown. Lane 1 shows the [^{35}S]SCD preparation kept at −20 °C. The control in lane 2 is [^{35}S]SCD incubated with buffer. In lanes 7−12, the Triton X-100 (TX-100) insoluble fraction of high-salt-washed microsomes (HSWM) was extracted with 2% TX-100/200 mM NaCl (lanes 7 and 8) or 2% CHAPS ± 200 mM NaCl (lanes 9−12). After salt and detergent were added as indicated, soluble and insoluble subfractions were prepared by ultracentrifugation (125000*g* for 60 min at 4 °C) and incubated with [^{35}S]SCD at 37 °C for 4 h.

Reaction of the Protease with [^{3}H]DFP. Radiolabeling of proteins was done by incubating [^{3}H]DFP (2 μCi, 8.4 Ci/mmol) with 10 μL of the indicated fraction at 4 °C for 16 h. The ^{3}H-labeled preparation was analyzed by SDS−PAGE followed by autoradiography.

RESULTS

Solubilization of a Microsomal Protease That Selectively Degrades SCD. We previously identified a protease activity in isolated liver microsomes with the ability to degrade native SCD (*11*). To further characterize the microsomal protease(s) that degrade(s) SCD, we needed to develop a suitable assay. Purified SCD preparations contain aggregated molecules that are resistant to degradation and lead to a high background signal in assays based on the disappearance of intact SCD. We therefore investigated the degradation of [^{35}S]SCD prepared in vitro by coupled transcription/translation (TNT).

SDS−PAGE and autoradiography of the [^{35}S]SCD in vitro translation product revealed a predominant 37 kDa band consistent with the known molecular mass of native SCD (Figure 1, lane 1). The [^{35}S]SCD was not degraded when the entire TNT reaction mixture was incubated for 15 h at 37 °C in the absence of microsomal protein (Figure 1, lane 2), indicating that the TNT system is free of significant endogenous SCD protease activity.

We next examined selected subcellular fractions for protease activity. [^{35}S]SCD was stable when incubated with cytosol (Figure 1, lane 3). [^{35}S]SCD was degraded when incubated with desaturase-induced rat liver microsomes that had been washed with 500 mM NaCl and 20 mM EDTA, denoted and abbreviated as *h*igh *s*alt *w*ashed *m*icrosomes (HSWM) (Figure 1, lane 4). SCD protease activity was recovered in the insoluble fraction when HSWM were solubilized with 2% Triton X-100 (TX-100) (Figure 1, lane 6). The TX-100 soluble fraction of HSWM did not significantly degrade SCD (Figure 1, lane 5). The TX-100 insoluble fraction degraded SCD much faster than HSWM, indicating that extraction of TX-100 soluble proteins enriched the

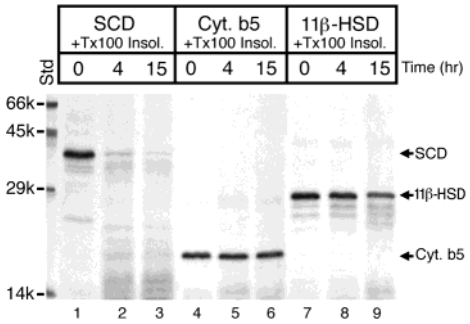

FIGURE 2: SCD is selectively degraded by a protease in the TX-100 insoluble fraction of microsomes. [^{35}S]Methionine-labeled stearoyl-CoA desaturase (SCD), cytochrome b_5 (cyt b_5), and 11β-hydroxysteroid dehydrogenase (11β-HSD) were synthesized by in vitro transcription and translation. Radiolabeled proteins were incubated with the Triton X-100 insoluble fraction of high-salt-washed microsomes at 37 °C for the indicated times. Protein degradation was analyzed by SDS−PAGE and fluorography.

protease activity. The protease that degrades native SCD in isolated liver microsomes also resists solubilization by TX-100 (*7*). Thus, the results in Figure 1 indicate that the subcellular distribution and solubility of the microsomal protease that degrades [^{35}S]SCD are similar to the protease that degrades native SCD. To provide further evidence that [^{35}S]SCD synthesized in vitro is degraded by a mechanism similar to that of native SCD, we compared the rates of degradation of native SCD and [^{35}S]SCD. When [^{35}S]SCD was incubated with desaturase-induced microsomes as the source of both protease and native SCD in the presence of detergent, [^{35}S]SCD was degraded at the same rate as native SCD (data not shown).

To identify conditions that solubilize the SCD protease activity, we extracted the TX-100 insoluble fraction of HSWM, varying the detergent and ionic strength parameters of the solubilization buffer (Figure 1, lanes 7−12). When the TX-100 insoluble fraction was extracted by 2% TX-100 and 200 mM NaCl, both the insoluble and soluble fractions degraded SCD, indicating partial solubilization (Figure 1, lanes 7 and 8). The SCD protease was also partially solubilized by 2% CHAPS at low ionic strength (Figure 1, lanes 9 and 10). The combination of 2% CHAPS and 200 mM NaCl resulted in nearly complete solubilization of the SCD protease (Figure 1, lane 11), as indicated by the absence of SCD protease activity in the insoluble fraction (Figure 1, lane 12).

The specificity of the SCD proteolytic activity in microsomes was characterized by incubating the TX-100 insoluble fraction with other ER proteins synthesized in vitro. Cytochrome b_5 (cyt b_5) and 11β-hydroxysteroid dehydrogenase (11β-HSD) were selected as control proteins of the ER membrane. Cyt b_5, a 20 kDa heme protein, is an essential component of the liver microsomal stearoyl-CoA desaturase system and contains an N-terminal catalytic domain oriented on the cytosolic side of the ER membrane joined to a membrane-binding C-terminal hydrophobic segment by a protease-sensitive hinge region (*3*, *13*). Cyt b_5 is very susceptible to limited proteolysis by a wide spectrum of proteases. Cleavage of cyt b_5 in the hinge region yields a soluble 12 kDa enzymatically active domain (*13*). As shown in Figure 2, [^{35}S]cyt b_5 translated in vitro was resistant to degradation by the TX-100 insoluble fraction. Incubation with sufficient enzyme to completely degrade SCD in the assay in 4 h (Figure 2, lane 2) resulted in no significant degradation of cyt b_5 even when the incubation was extended to 15 h (Figure 2, lanes 5 and 6).

Table 1: Purification of SCD Protease Activity from High-Salt-Washed Liver Microsomes (HSWM)

fraction	volume (mL)	protein[a] (mg)	total activity[b] (units)	specific activity (units/mg)
HSWM	100	1800	12500	7
solubilized TX-100 insoluble HSWM	20	166	9000	54
HAP column eluate (concd 20×)	2.0	10.5	7000	666
Superdex 200 column eluate[c]	0.5	0.1	500	5000

[a] The protein concentration was determined with Pierce Coomassie protein assay reagent. [b] One unit of SCD protease activity is defined as that volume of enzyme solution that degrades 50% of [^{35}S]Met-SCD present in a 10 μL aliquot containing 1 μL of translation reaction mixture and 9 μL of assay buffer (20 mM NaP, pH 8.0) in 4 h. [c] A 0.3 mL concentrated sample was placed on the column.

11β-HSD is bound to the ER membrane by a single transmembrane segment at the N-terminus (*14*). The bulk of 11β-HSD projects into the lumen of the ER (*14*). Unlike cyt b_5, 11β-HSD lacks an extended intradomain region between the membrane-binding segment and the catalytic domain, and the entire molecule is resistant toward proteolysis (*14*). Incubation of [^{35}S]-11β-HSD with the TX-100 insoluble fraction resulted in minimal degradation at 4 h (Figure 2, lane 8) and slight degradation at 15 h (Figure 2, lane 9). Thus, the data in Figure 2 indicate that the TX-100 insoluble fraction contains a protease that selectively degrades SCD.

Purification of a 90 kDa Microsomal Protease from the Triton X-100 Insoluble Fraction of Liver Microsomes. Hydroxyapatite chromatography was investigated as the next step in purification of the SCD protease because this adsorbent is functional in the presence of NaCl and CHAPS. The 2% CHAPS/200 mM NaCl soluble fraction of the TX-100 insoluble material was applied to a hydroxyapatite column equilibrated with 10 mM sodium phosphate (NaP) in the presence of 1% CHAPS. The column was developed with 25 mM NaP and 50 mM NaP buffers. SCD protease activity was recovered in fractions eluted with 50 mM NaP (Figure 3A). SDS−PAGE of active fractions on the ascending side of the 50 mM NaP peak revealed a predominant 90 kDa protein and small amounts of 80, 60, and 45 kDa proteins (Figure 3B). Fractions (112−116) eluting later with 50 mM NaP were less active and contained more of the 60 and 45 kDa proteins.

To confirm the molecular mass of the protease, we characterized the active HAP fractions by gel filtration (Figure 3C−E). Material from the ascending side of the 50 mM NaP eluate was concentrated by ultrafiltration and subjected to gel filtration on a Superdex 200 FPLC column. The activity was recovered in fractions with an approximate molecular mass of 90 kDa (Figure 3C). The SCD protease activity of the active gel filtration fractions correlated with the staining intensity of the 90 kDa protein band (Figure 3D,E). Compared with high-salt-washed microsomes, the most active gel filtration fractions were enriched 700-fold in protease activity (Table 1).

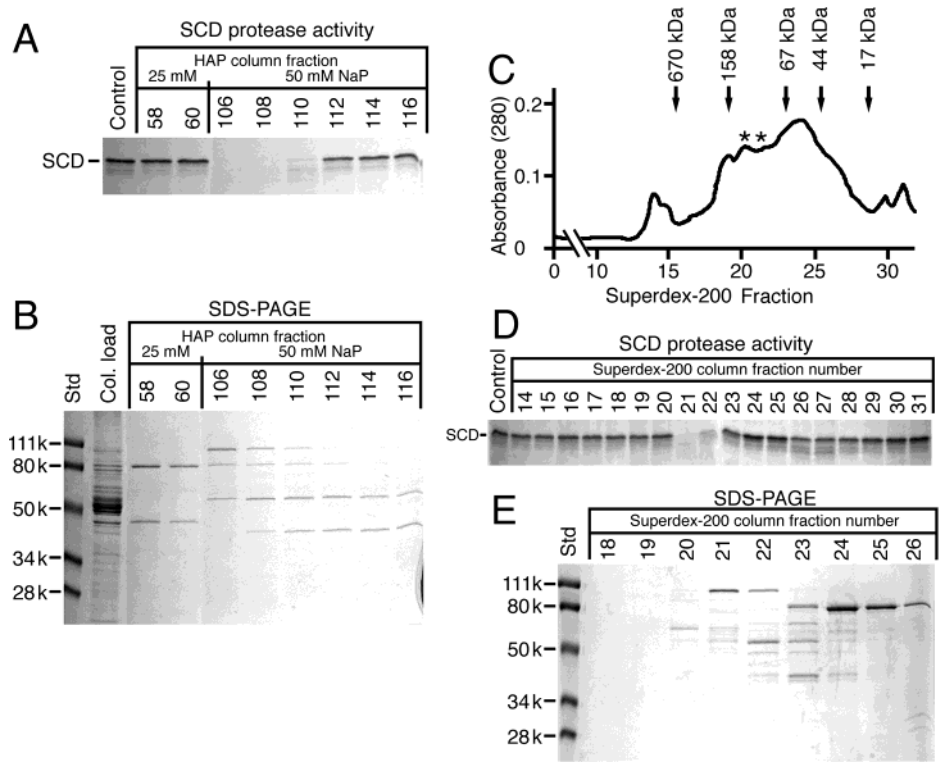

FIGURE 3: Purification of a 90 kDa microsomal protease from the Triton X-100 insoluble fraction of liver microsomes. The Triton X-100 insoluble fraction of high-salt-washed microsomes was extracted with 2% CHAPS/200 mM NaCl, and the soluble fraction was applied to a hydroxyapatite (HAP) column equilibrated with 10 mM sodium phosphate (NaP) buffer containing 1% CHAPS. The column was developed with a stepwise gradient of 10, 25, and 50 mM NaP. The column fractions were concentrated and desalted by ultrafiltration. (A) SCD protease activity of HAP column fractions. (B) Coomassie blue-stained SDS−PAGE gel of the HAP column fractions. (C−E) Gel filtration chromatography. A 300 μL aliquot of the concentrated HAP fraction with the highest specific activity was subjected to gel filtration on a Superdex 200 column. (C) Gel filtration chromatogram with absorbance at 280 nm. Asterisks indicate the positions of fractions with protease activity. Arrows indicate the positions of molecular mass standards. (D) SCD protease activity of the Superdex 200 column fractions. (E) Coomassie blue-stained SDS−PAGE gel of the Superdex 200 column fractions.

The protein compositions of the active fractions at various stages of the purification procedure are shown in Figure 4A. Comparison of the protein profiles of the HAP column fraction and the Superdex column fraction (lanes 5 and 6 in Figure 4A) shows that several new bands appeared in the gel filtration fraction, suggesting that proteolysis occurred after HAP chromatography. This was confirmed when storage of the HAP fraction at 4 °C resulted in the disappearance of the 90 kDa band and the appearance of protein bands around 80 and 66 kDa (Figure 4A, lane 7). To further characterize the stability of SCD Pr, the purified protease was incubated at 37 °C for various lengths of time and then assayed by brief incubation with [^{35}S]SCD. When the protease was incubated at 37 °C, the staining intensity of the 90 kDa protein band rapidly decreased, and the staining intensity of protein bands around 80 and 66 kDa increased slightly (Figure 4B). The proteolytic activity of the preparation increased dramatically when the protease was incubated at 37 °C (Figure 4C).

The 90 kDa Protease Is Inhibited by Diisopropyl Phosphofluoridate, Dithiothreitol, and Leupeptin. To further characterize the SCD protease activity, the effect of various protease inhibitors was investigated (Figure 5A). Degradation of SCD was inhibited 60% by 100 μM leupeptin, suggesting that SCD protease is a serine or thiol protease with Lys/Arg specificity. High concentrations of DFP (5 mM) inhibited activity by approximately 30%. The other protease inhibitors investigated did not show significant inhibition of SCD protease activity.

In the course of developing solubilization and chromatography procedures for the SCD protease, we noted that the degradation of SCD was inhibited by dithiothreitol (DTT). Therefore, DTT was eliminated from the solutions used to characterize the protease activity. High concentrations of NaCl also inhibited the degradation of SCD, and therefore the salt content of column fractions was adjusted to 10 mM Tris-HCl and 25 mM NaCl by ultrafiltration before protease activity was assayed. To characterize the DTT inhibitory effect, SCD was incubated with the active HAP column fractions in the presence of increasing concentrations of DTT (Figure 5B). Fifty micromolar DTT resulted in 50% inhibition of the protease activity, and some 75% inhibition was observed with 1.0 mM DTT.

Specific Radiolabeling of Essential Disulfide Bond(s). A disulfide bond that is essential for protease activity would most readily explain inhibition by DTT. To further evaluate the possible role of a disulfide bond in the SCD protease, we asked whether inhibition by DTT was reversible. When the DTT concentration of the reduced enzyme preparation was decreased to 10 μM by ultrafiltration, most of the protease activity was recovered (Figure 6A, lane 4). This result is consistent with the proposal that the protease contains a disulfide bond that is sensitive to reduction by DTT and able to re-form when the DTT concentration is lowered, but it does not exclude effects unrelated to disulfide bond reduction. When the reduced protease preparation was alkylated with iodoacetamide before ultrafiltration, the protease activity was not recovered (Figure 6A, lane 5),

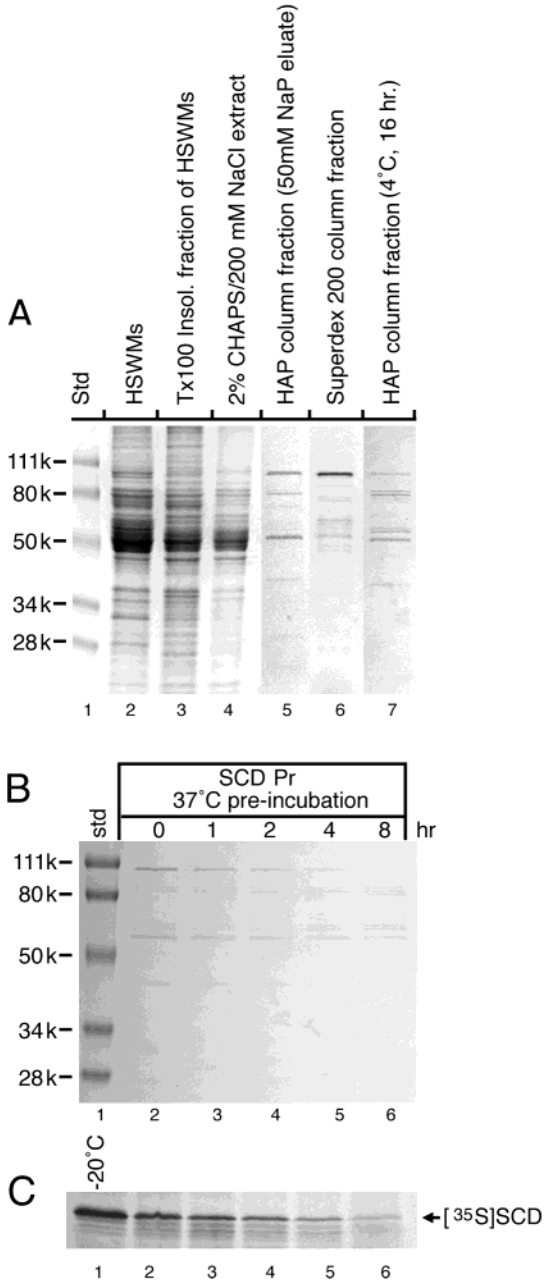

FIGURE 4: Conversion of the 90 kDa protease to lower molecular mass forms and activation of SCD protease activity by incubation at 37 °C. (A) SDS−polyacrylamide gel analysis of the protease purification. The samples are as follows: lane 1, molecular mass markers; lane 2, high-salt-washed microsomes (HSWM) (36 μg); lane 3, TX-100 insoluble fraction of HSWM (TX Insol) (25 μg); lane 4, 2% CHAPS/200 mM NaCl extract of TX Insol (12 μg); lane 5, hydroxyapatite column fraction (50 mM sodium phosphate eluate) (3 μg); lane 6, Superdex 200 column fraction (2 μg); lane 7, hydroxyapatite column fraction after incubation at 4 °C for 16 h. All lanes were run in the presence of β-mercaptoethanol. (B and C) SCD Pr was incubated at 37 °C for the times indicated. (B) Five microliter aliquots of incubated SCD Pr analyzed by SDS−PAGE stained with Coomassie blue. (C) Five microliter aliquots of the samples shown in panel B were incubated with [^{35}S]SCD at 37 °C for 30 min and subjected to SDS−PAGE followed by autoradiography.

suggesting that reoxidation of a labile disulfide bond is essential for recovery of protease activity. Incubation of the protease preparation with iodoacetamide without prior reduction did not inhibit SCD degradation (Figure 6A, lane 3). Taken together, the results depicted in Figure 6A suggest

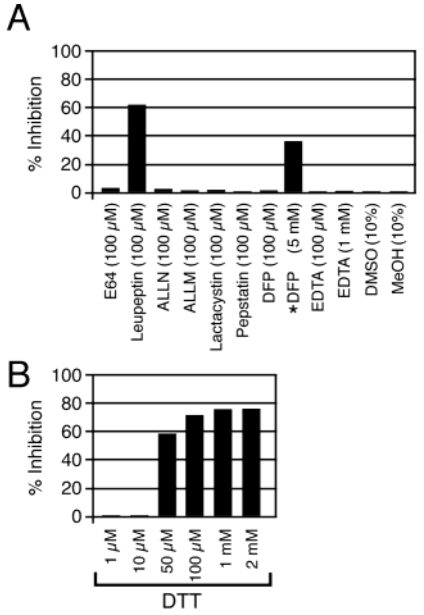

FIGURE 5: Effect of protease inhibitors and dithiothreitol on SCD protease activity. Aliquots of the active hydroxyapatite column fraction were preincubated in the presence of various protease inhibitors on ice for 15 min except for 5 mM DFP (*), which was incubated at 4 °C for 16 h. [^{35}S]SCD prepared by coupled transcription translation was added to the inhibitor/enzyme mixtures and incubated at 37 °C for 4 h. After SDS−PAGE and autoradiography, the data were quantified by scanning densitometry. The inhibitor concentrations indicated are the final concentrations. DMSO and MeOH are inhibitor solvent controls. The data are presented as percentage of inhibition.

that the SCD protease contains one or more disulfide bonds that can be reduced under nondenaturing conditions and are essential for maximum protease activity. To test this hypothesis, a procedure was devised to selectively label the essential disulfide bond(s) in the SCD protease. The protease preparation was alkylated with iodoacetamide. The alkylated preparation was desalted to remove unreacted iodoacetamide and reduced with various concentrations of DTT as indicated in Figure 6B. The alkylated/reduced protease preparations were then incubated with [^{14}C]iodoacetamide to specifically label the newly formed cysteine residues. SDS−PAGE and autoradiography of the [^{14}C]iodoacetamide-labeled protease preparations are shown in Figure 6B. The radioactivity appeared in a single protein band of approximately 90 kDa.

Radiolabeling of the Protease with [^{3}H]DFP. Since the protease activity was partially inhibited by 5 mM DFP (Figure 5A), radiolabeling with [^{3}H]DFP was attempted. Labeling of the protease required prolonged incubation with [^{3}H]DFP at 4 °C. As shown in Figure 4A, lane 7, fragmentation of the protease occurs under these conditions. SDS−PAGE analyses of the [^{3}H]DFP-labeled protease preparation, followed by autoradiography, showed a single radiolabeled band at 25 kDa under reducing conditions and bands at 64 and 66 kDa under nonreducing conditions (Figure 6C). The change in molecular mass caused by reduction indicates that the DFP-labeled form of the protease contains at least two polypeptide chains connected by a disulfide bridge.

Specificity of the 90 kDa Protease. Initial characterization of the substrate specificity of the protease to establish the validity of the [^{35}S]SCD degradation assay was performed using the TX-100 insoluble fraction, which is a relatively crude protease preparation (Figure 4A, lane 3). To confirm

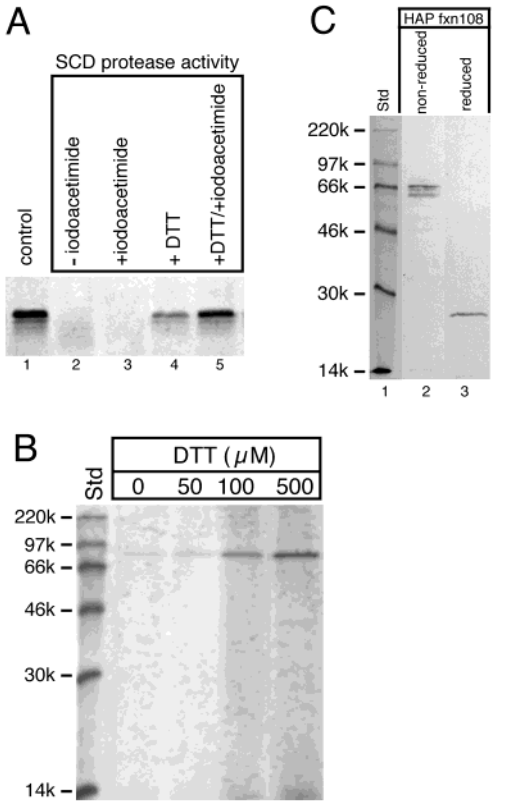

FIGURE 6: Selective reduction and alkylation of SCD protease and specific radiolabeling of the protease with [^{3}H]DFP. (A) Aliquots of the active hydroxyapatite column fraction were reduced (500 μM DTT for 30 min at 37 °C) and/or alkylated (10 mM iodoacetamide for 30 min at room temperature) under nondenaturing conditions. The reduced/alkylated samples were desalted by ultrafiltration, and their SCD protease activity was determined as in Figure 1. (B) Aliquots of the active hydroxyapatite column fraction were alkylated with nonradioactive iodoacetamide, reduced with DTT at the indicated concentrations, and alkylated with [^{14}C]-iodoacetamide as described in Experimental Procedures. The ^{14}C-labeled proteins were separated by SDS−PAGE and visualized by fluorography. (C) Aliquots of the HAP-column active fractions were incubated with [^{3}H]DFP as described in Experimental Procedures. The ^{3}H-labeled proteins were separated by SDS−PAGE under nonreducing and reducing conditions and visualized by fluorography.

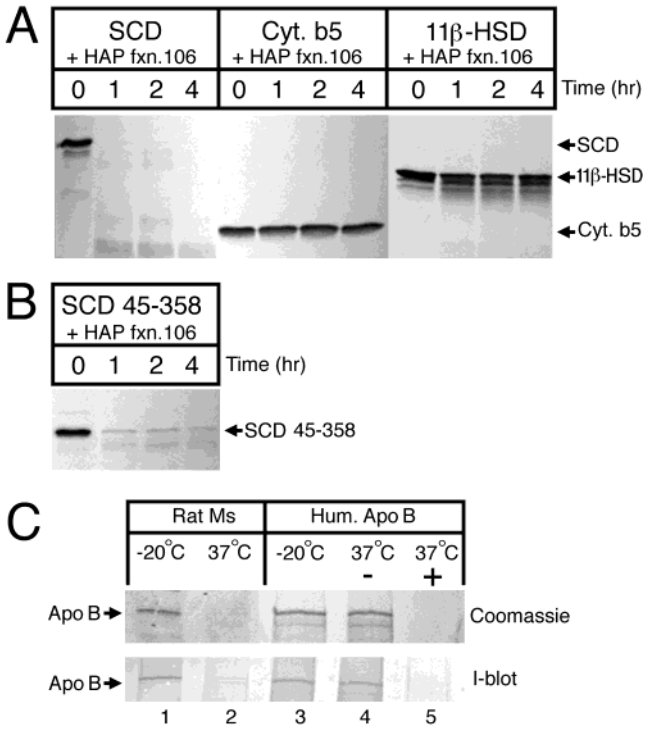

FIGURE 7: Substrate specificity of the purified SCD protease. (A) Stearoyl-CoA desaturase (SCD), cytochrome b_5 (cyt b_5), and 11β-hydroxysteroid dehydrogenase (11β-HSD) and (B) SCD with 44 residues truncated from the N-terminus (SCD 45−358) were synthesized by in vitro transcription and translation in the presence of [^{35}S]methionine. The radiolabeled proteins were incubated with the active hydroxyapatite column fraction at 37 °C for the indicated times. Protein degradation was analyzed by SDS−PAGE and fluorography. (C) Rat liver microsomes in the presence of 1% CHAPS were incubated at 37 °C for 3 h. Human apoB was incubated in the absence (−) or presence (+) of the purified HAP column protease fraction. Initial material is denoted by −20 °C. Protein degradation was analyzed by Coomassie blue staining and immunoblotting with anti-apoB antibody.

the selectivity of the protease after further purification, we carried out degradations using the preparation obtained by hydroxyapatite (HAP) chromatography. The activity of the concentrated HAP preparation is enriched approximately 200-fold relative to the TX-100 insoluble fraction. Under conditions in which SCD was completely degraded in 1 h, cyt b_5 was not degraded, and 11β-HSD was slightly degraded (Figure 7A).

Previously, we reported that N-terminally truncated SCD constructs expressed in CHO cells were degraded more slowly than the full-length protein (*12*). It was therefore of interest whether N-terminally truncated SCD constructs are susceptible to cleavage by the purified SCD protease. As shown in Figure 7B, N-terminally truncated SCD constructs are degraded rapidly by the purified protease.

Ginsberg and co-workers (*16*) reported that after apolipoprotein B (apoB) is translocated into ER lumen, it is degraded by a lumenal DTT-sensitive degradative system. Therefore, it was of interest to determine if the DTT-sensitive SCD protease is capable of cleaving apoB. Incubation of apoB with the SCD protease indeed leads to the degradation of apoB (Figure 7C, lane 5). These results suggest that the protease described in this report may be involved in the degradation of apoB through a DTT-sensitive pathway in HepG2 cells.

DISCUSSION

Our results demonstrate the presence of a 90 kDa protease in liver microsomes that selectively degrades SCD. The SCD protease was isolated from the Triton X-100 insoluble fraction of high-salt-washed liver microsomes. The enzyme was solubilized with 2% CHAPS in the presence of 200 mM NaCl and purified by hydroxyapatite chromatography and gel filtration. The molecular mass estimates provided by gel filtration and SDS−PAGE under reducing conditions are in agreement, indicating that the isolated form of the SCD protease is a single-chain protein of approximately 90 kDa. However, when the purified protease was stored at 4 °C, the SDS−PAGE profile changed dramatically, indicating conversion of the 90 kDa protease into several smaller proteins, by either self-cleavage or cleavage by a contaminating protease. Under reducing conditions, the predominant conversion products of the protease are a pair of doublets around 66 and 80 kDa. Conversion of the 90 kDa protein into these smaller forms is associated with a dramatic

increase in proteolytic activity, suggesting that the 90 kDa form is an inactive proenzyme or zymogen.

The nature of SCD protease conversion was further revealed by labeling with [^{3}H]DFP. Labeling of the protease with [^{3}H]DFP required prolonged incubation at 4 °C, conditions which led to conversion of the 90 kDa protein into smaller forms. As shown in Figure 6C, the form of the protease labeled by DFP is 66 kDa by nonreduced SDS−PAGE. Under reducing conditions, the protease segment labeled by DFP is 25 kDa. This indicates that the 66 kDa (nonreduced) form of the protease is composed of at least two peptides linked by disulfide bond(s). Thus, activation of the 90 kDa protease appears to involve an internal cleavage and removal of protein fragment(s) from one or both ends. The zymogen form of many serine proteases, such as trypsin and chymotrypsin, contains an imperfectly developed active site. As such, DFP labeling of the zymogen form is not detected or is several orders of magnitude lower as compared to the active protease. In the case of trypsinogen, activation of the zymogen involves cleavage of one peptide bond, with consequent loss of a hexapeptide. Activation of chymotrypsinogen involves cleavage of one to four peptide bonds with S−S bonds maintaining the polypeptide chains in a single complex. In contrast, the activation of prothrombin involves the loss of a 30 kDa peptide from the N-terminus. Frequently, however, when zymogen conversion involves internal bond cleavage, the resulting polypetide chains are not released but remain connected by disulfide bridges (*22*).

The proposed structure of the DFP binding form of the protease as a multichain disulfide-linked protein is supported by selective reduction and alkylation experiments. The SCD protease activity in highly purified preparations was partially inhibited by 50 μM DTT. Inhibition by DTT was reversible, but alkylation of the reduced enzyme caused irreversible inhibition. On the basis of these results we propose that the SCD protease contains an intramolecular disulfide bond that is essential for protease activity. The identification of an essential disulfide in the SCD protease suggests that the enzyme is lumenally oriented since disulfide bonds are formed in the ER lumen and are localized predominantly in the extracytosolic domain. This suggests that lumenally oriented segment(s) of SCD may be cleaved by the protease.

Initial attempts to purify the microsomal protease responsible for SCD degradation were hampered by lack of a suitable assay and difficulty in solubilizing the protease. Neither SCD nor the SCD protease was solubilized by extracting crude microsomes with nonionic detergent in low ionic strength buffer, and yet both SCD and the SCD protease were solubilized by the ionic detergent deoxycholate (DOC) or by nonionic detergent in high ionic strength buffer (*7*). These observations suggest that the SCD protease is bound to microsomal membranes in part by ionic interactions. Since DOC interferes with ion-exchange chromatography, we investigated the solubility of the SCD protease in high-salt-washed microsomes. High-salt washing of microsomes increases the solubility in nonionic detergent of many microsomal proteins apparently by disrupting ionic interactions (*15*). We reasoned that salt washing might have the same effect on the SCD protease. In fact, the SCD protease was recovered predominantly in the TX-100 insoluble fraction even after high-salt washing. These observations led to a highly effective step in the purification of the SCD protease. Some 90% of the protein in high-salt-washed microsomes was extracted with 2% TX-100. The SCD protease was recovered with the insoluble membrane remnant and was enriched 5−10-fold in the process.

Insolubility of membrane proteins in nonionic detergents may result from association with large protein complexes or specialized membrane domains and rarely from the formation of large multimeric complexes through self-association. The best example of the latter mechanism is the erythroid/nonerythroid membrane skeletal protein spectrin/fodrin (*18*). A number of microsomal proteins involved in protein translocation across the ER membrane are insoluble in nonionic detergent at low ionic strength due to association with membrane-bound ribosomes (*19*). For example, ribophorins I and II, protein subunits of oligosaccharyltransferase (*17*), were initially identified by their association with ribosomes and isolated in the insoluble membrane remnant of rough microsomes after extraction with nonionic detergent (*20*). Plasma membrane proteins may be insoluble in nonionic detergents because of interaction with the cytoskeleton, membrane skeleton, or detergent-insoluble lipid domains. Membrane proteins with covalent glycosylphosphatidylinositol (GPI) lipid tails are insoluble in TX-100 at 4 °C because they associate with TX-100 insoluble lipid domains (*21*). Of note, GPI-linked membrane proteins are solubilized by TX-100 at 37 °C or octyl β-glucoside (*21*), and these conditions failed to solubilize the SCD protease (data not shown). The solubility of the SCD protease in nonionic detergent was markedly increased by 200 mM NaCl. The most likely explanation for this observation is that the tight association of the SCD protease with microsomal membranes is due in part to ionic interaction(s). The component(s) of HSWM that the SCD protease interacts with has (have) not been identified.

The best characterized pathway for ER protein degradation involves retention in the ER through association with various chaperones, retrograde translocation, and degradation by cytosolic proteasomes (*23*). The proteasome pathway has been implicated in the degradation of a variety of mutated secretory proteins and transmembrane proteins as well as unpaired subunits of multimeric membrane protein complexes (*24*−*26*). While it is now clear that the proteasome pathway is the primary mechanism for the degradation of abnormal ER proteins, there is also evidence for proteasome-independent ER protein degradation in mammalian cells as well as yeast (*27*−*30*). Of particular interest, two laboratories have demonstrated a DTT-sensitive nonproteasomal pathway for the degradation of apolipoprotein B (apoB) in HepG2 cells (*16*, *31*). The DTT-sensitive pathway for apoB degradation occurs in the ER lumen (*16*) and is active in permeabilized HepG2 cells (*31*). Our finding that the purified SCD protease degrades apoB raises the intriguing possibility that two structurally unrelated proteins involved in the assembly of very low density lipoprotein may be regulated by a DTT-sensitive ER protease.

Our results suggest that SCD may be degraded more rapidly than most ER proteins because it is particularly susceptible to cleavage by a 90 kDa serine protease. A recent protease database, MEROPS version 5.7, listed 227 proteases in the rat genome with some 80 serine proteases comprising the largest group (*32*). Although the majority of serine proteases are synthesized with signal peptides that direct their

secretion outside of the cell, some of the serine proteases recently reported may also be associated with cell membranes (*33*, *34*).

Proteolytic activities that are associated with liver ER include the endopeptidase that cleaves signal peptides upon translocation of nascent polypeptide chains, a group of some eight immunologically related proteases of 60−72 kDa that degrade protein disulfide−isomerase and calreticulin (*35*), a 69 kDa endopeptidase with substrate specificity toward paired Arg-Arg residues (*36*), and hepsin (*37*). Hepsin is a 45 kDa serine protease originally cloned from human liver and is implicated in mammalian cell growth and morphology (*38*). All of these peptidases are smaller than SCD protease (M_r 90000) and/or display substrate specificity unrelated to SCD protease. The size of the SCD protease, however, is similar to that of furin, a subtilisin-like membrane-bound serine endopeptidase of M_r 90000. Furin is activated by a multistep process involving excision, internal cleavage, and degradation of the propeptide (*39*). In contrast to SCD protease, however, furin activation requires calcium, and its activity is completely inhibited by DFP (*39*). Furin cleaves substrates at the motif Arg-X-Lys/Arg-Arg↓-. Further enzymatic and molecular characterization of the SCD protease remains to be done in future studies.

REFERENCES

1. Omura, T., Siekevitz, P., and Palade, G. E. (1967) *J. Biol. Chem. 242*, 2389−2396.
2. Klausner, R. D., and Sitia, R. (1990) *Cell 62*, 611−614.
3. Strittmatter, P., Spatz, L., Corcoran, D., Rodgers, M. J., Setlow, B., and Redline, R. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 4565−4569.
4. Ntambi, J. M. (1999) *J. Lipid Res. 40*, 1549−1558.
5. Oshino, N., and Sato, R. (1972) *Arch. Biochem. Biophys. 149*, 369−377.
6. Thiede, M. A., and and Strittmatter, P. (1985) *J. Biol. Chem. 260*, 14459−14463.
7. Heinemann, F. S., and Ozols, J. (1998) *Mol. Biol. Cell 9*, 3445−3453.
8. Thiede, M. A., Ozols, J., and Strittmatter, P. (1986) *J. Biol. Chem. 261*, 13230−13235.
9. Stucky, J. E., McDonough, V. M., and Martin, C. M. (1990) *J. Biol. Chem. 265*, 20144−20149.
10. Shanklin, J., Whittle, E., and Fox, B. G. (1994) *Biochemistry 33*, 12787−12794.
11. Ozols, J. (1997) *Mol. Biol. Cell 8*, 2281−2290.
12. Mziaut, H., Korza, G., and Ozols, J. (2000) *Proc. Natl. Acad. Sci. U.S.A. 97*, 8883−8888.
13. Ozols, J. (1989) *Biochim. Biophys. Acta 997*, 121−130.
14. Ozols, J. (1995) *J. Biol. Chem. 270*, 2305−2312.
15. Heinemann, F. S., and Ozols, J. (1998) *Frontiers Biosci. 3*, 483−493.
16. Wu, X., Sakata, N., Lele, K. M., Zhou, M., Jiang, H., and Ginsberg, H. N. (1997) *J. Biol. Chem. 272*, 11575−11580.
17. Kumar, V., Heinemann, F. S., and Ozols, J. (1994) *J. Biol. Chem. 269*, 13451−13457.
18. Morrow, J. S., and Marchesi, V. (1981) *J. Cell Biol. 88*, 463−368.
19. Gorlich, D., Prehn, S., Hartmann, E., Kalies, K. U., and Rapoport, T. A. (1992) *Cell 71*, 489−503.
20. Kreibich, G., Ulrich, B. L., and Sabatini, D. D. (1978) *J. Cell Biol. 77*, 464−487.
21. Brown, D. A., and Rose, J. K. (1992) *Cell 68*, 533−544.
22. Neurath H. (1975) *Proteases and Biological Control*, pp 51−64, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
23. Ellgard, L., Molinari, and Helenius, A. (1999) *Science 286*, 1882−1888.
24. Kopito, R. R. (1997) *Cell 88*, 427−430.
25. Bonifacino, J. S., and Weissman, A. M. (1998) *Annu. Rev. Cell Dev. Biol. 14*, 19−57.
26. Plemper, R. K., and Wolf, D. H. (1999) *Trends Biochem. Sci. 24*, 266−270.
27. Friedlander, R., Jarosch, E., Urban, J., Volkwein, C., and Sommer, T. (2000) *Nat. Cell Biol. 2*, 379−384.
28. Cabral, C. M., Choudhury, P., Liu, Y., and Sifers, R. N. (2000) *J. Biol. Chem. 275*, 25015−25022.
29. Mziaut, H., Korza, G., Benraiss, A., and Ozols, J. (2002) *Biochim. Biophys. Acta 1583*, 45−52.
30. Kyohei, U., Fukuda, R., Hirata, A., Horiuchi, H., Nakano, A., Ohta, A., and Takagi, M. (2001) *J. Biol. Chem. 276*, 41444−41454.
31. Cavallo, D., Rudy, D., Mohamadi, A., Macri, J., and Adeli, K. (1999) *J. Biol. Chem. 274*, 23135−23143.
32. Rawlings, N. D., O'Brien, E., and Barrett, A. J. (2002) *Nucleic Acids Res. 30*, 343−346.
33. Jeohn, G.-H., Serizawa, S., Iwamatsu, A., and Takahashi, K. (1995) *J. Biol. Chem. 270*, 14748−4755.
34. Hooper, J. D., Clements, J. A., Quigley, J. P., and Antalis, T. M. (2001) *J. Biol. Chem. 276*, 857−860.
35. Urade, R., Nasu, M., Moriyama, T., Wada, K., and Kiko, M. (l992) *J. Biol. Chem. 267*, 15152−15159.
36. Kawabata S., and Davie, E. W. (1992) *J. Biol. Chem. 267*, 10331−10336.
37. Leytus, S. P., Loeb, K. R., Hagen, F. S., Kurachi, K., and Davie, E. (l988) *Biochemistry 27*, 1067−1074.
38. Torres-Rosado, A., O'Shea, K. S., Tsuji, A., Chou, S. H., and Kurach, K. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 7181−7185.
39. Malloy, S. S., Anderson, E. D., Jean, F., and Thomas, G. (1999) *Trends Cell Biol. 9*, 28−35.

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