A Plasminogen-like Protein Selectively Degrades Stearoyl-CoA Desaturase in Liver Microsomes

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SUMMARY

Stearoyl-CoA desaturase (SCD) is an integral membrane protein of the endoplasmic reticulum that is rapidly and selectively degraded when isolated liver microsomes are incubated at 37°C. We previously reported the purification of a 90-kDa microsomal protein with SCD protease activity, and characterized the inhibitor sensitivity of the protease. Here we show that the 90-kDa protein is a microsomal form of plasminogen (Pg), and that the purified SCD protease contains a spectrum of plasmin-like derivatives. The 90-kDa protein was identified as Pg by mass spectrometry of its tryptic peptides. The purified SCD protease reacted with Pg antibody, and immunoblotting demonstrated enrichment of Pg by the purification procedure established for the SCD protease. Analysis of microsomes by zymography demonstrated a single band of proteolytic activity at 70-kDa corresponding to the mobility of Pg in nonreduced polyacrylamide gels. When microsomes were incubated at 37°C prior to zymography, an intense band of proteolytic activity developed at 30-kDa. The purified SCD protease displayed a spectrum of proteolytic bands ranging from 70- to 30-kDa. Degradation of SCD by the purified protease and by microsomes was inhibited by bdellin, a plasmin inhibitor from the medicinal leech Hirudo medicinalis. To explore the role of Pg in the degradation of SCD in vivo, we examined SCD expression and degradation in microsomes isolated from Pg deficient (Pg-/-) mice. Compared with microsomes from wild-type littermate control mice, liver microsomes from Pg-/- mice had significantly higher levels of SCD. Degradation of SCD in microsomes from Pg-/- mice was markedly diminished, whereas liver microsomes from control mice showed rapid SCD degradation similar to that observed in rat liver microsomes. These findings indicate that SCD is degraded by a protease related to Pg, and suggest that plasmin moonlights as an intracellular protease.
INTRODUCTION

The biophysical properties of membranes are critically dependent on the degree of desaturation of their hydrocarbon core (1). When environmental (dietary) sources are scarce, the pool of unsaturated fatty acyl-CoA precursors required for the assembly of membranes and other lipid-based structures is maintained by synthesis of oleoyl-CoA (18:1) and palmitoyl-CoA (16:1) in the endoplasmic reticulum (ER) by \( \Delta^9 \) stearoyl-CoA desaturase (SCD). The rate of unsaturated fatty acyl-CoA synthesis is directly related to the concentration of SCD in the ER (2); no allosteric mechanisms of SCD regulation have been identified. The concentration of SCD in the ER membrane is determined by the rates of SCD synthesis and degradation. Transcriptional regulation of SCD synthesis has been studied extensively (3,4). SCD degradation is less well understood, but is critical to the regulation of SCD since the concentration of an enzyme can be reduced rapidly only if it is short-lived. Hepatic SCD is degraded more rapidly than most ER proteins and has a half-life of about 3-4 hours (5). The physiological need to rapidly degrade SCD is not clear, but this property of SCD has been maintained during evolution since yeast SCD is also rapidly degraded (6). Presumably, excessive availability of unsaturated fatty acyl-CoAs would result in the assembly of membranes that are too disordered and be detrimental.

We have studied the degradation of SCD in two systems. Investigation of SCD-green fluorescent protein chimeras expressed in cultured animal cells identified a 33-amino acid segment at the amino terminus of SCD that is essential for optimal degradation of those constructs (7,8). We have also investigated the degradation of SCD in liver using conventional methods of subcellular fractionation, protein isolation, and reconstitution (9,10). When liver microsomes prepared from rats induced to express high levels of hepatic SCD are incubated at 37°C, rapid and selective degradation of SCD occurs (9). The microsomal protease that degrades
SCD (SCD Pr) is tightly membrane bound, as it cannot be extracted by selective release of lumenal ER proteins or by high salt washing procedures that remove proteins from the cytosolic surface of microsomes (10). [35S]SCD synthesized \textit{in vitro} is also rapidly degraded by desaturase induced liver microsomes, whereas, under the same conditions, several other microsomal proteins synthesized \textit{in vitro} are stable (11). Using specific degradation of [35S]SCD as an assay to monitor the purification of SCD Pr, a 90 kDa protease was isolated from the Triton X-100 insoluble fraction of high salt washed microsomes (11). 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. The purified protease is partially inhibited by diisopropylphosphofluoridate, dithiothreitol, and leupeptin suggesting it is a serine protease zymogen with Arg/Lys specificity and an essential disulfide bond (11). Mass spectrometry of its tryptic peptides indicated that the 90-kDa protein is a microsomal form of Pg. This prompted us to compare SCD Pr with Pg isolated from plasma, to study the activation of Pg in microsomes, and to examine SCD expression and degradation in mice with a genetic deficiency in Pg. In light of recent evidence that Pg is expressed in a broad range of tissues (12), the significance of plasmin as an intracellular protease is discussed.
EXPERIMENTAL PROCEDURES

Materials. Chemicals and reagents, including bdellin, were purchased from Sigma unless otherwise noted. CHAPS was from ProChem, Inc. L-[\textsuperscript{35}S] methionine was from Amersham. 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®) were from Promega. Rat Pg was from Innovative Research, Inc. Polyclonal goat antibody against rat Pg was obtained from Accurate Chemical Scientific Corp. N-glycosidase F (Endo F) was from Calbiochem. Pg deficient (Pg-/−) mice and wild-type littermate controls were female and approximately 3 weeks old, and were obtained from Jackson Laboratories.

Construction of plasmids. The construction of the plasmid and the cDNA encoding the rat liver stearoyl-CoA desaturase (SCD) were described previously (7). Briefly, the cDNAs encoding the full length of rat liver SCD preceded by Kozak sequence and EcoRI site at 5’ end and extended at 3’ end by NotI site was amplified by polymerase chain reaction. The resulting PCR product was digested with EcoRI-NotI and inserted into EcoRI-NotI digested pGEM-11Zf(+).

Analysis of protein degradation. SCD degradation was assayed by following the disappearance of native SCD in either mouse microsomes or desaturase induced rat microsomes by SDS-PAGE and immunoblot analysis or the disappearance of [\textsuperscript{35}S]SCD by autoradiography after SDS-PAGE. [\textsuperscript{35}S]SCD was prepared by coupled transcription/translation as previously described (11). A working solution of [\textsuperscript{35}S]SCD degradation substrate was prepared by diluting 10µl of the transcription/translation reaction product containing labeled proteins with 90µl of 20 mM sodium phosphate, pH 8.0. Assays were initiated by adding an aliquot (1 to 5µl) of the indicated fraction to 10 µl of the diluted TNT® reaction product. Assays were incubated at 37°C for the indicated
times. Control mixtures were maintained at –20°C. Degradation was terminated by the addition of SDS-PAGE sample buffer and boiling for two minutes.

**Immunoblot analysis.** Preparation of polyclonal antisera against SCD was described previously (10). Proteins were separated on 6% or 10% SDS-PAGE and transferred onto PVDF membranes. The membranes were incubated with 3% albumin. The transblots were incubated with antibody against SCD or rat Pg overnight at 4°C. Bound antibody was detected with an anti-IgG-alkaline phosphatase conjugate 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 (10). Subsequent purification of the SCD protease was done at 4°C as described previously (11). Protein concentrations were determined by the Coomassie colorimetric method (Pierce).

**MALDI Mass Spectrometry.** For identification of the 90-kDa protein, gels were stained with Coomassie blue and protein bands were excised with a razor blade. Gel pieces were rinsed in water and in-gel tryptic digest was performed. Peptide digests were concentrated using ZipTip C18 micropipette tips (Millipore Corp.). MALDI-TOF and Post-Source-Decay (PSD) data were obtained on an AXIMA-CFR mass spectrometer using software version 2.0.1 (Kratos Analytical, Manchester, UK). Mass spectra were obtained at a laser power near threshold with pulsed extraction optimized for 2,000 Da. PSD data were obtained at a laser power about 15% higher than threshold. Samples were co-crystalilized with alpha-cyano-4-hydroxy-cinnamic acid (10mg/ml in 1% formic acid, 50% acetonitrile). PSD fragment ions were fitted to a generated curve that was calibrated with PSD fragments from the synthetic peptide, P₁₄R. Data base searches were performed using Protein Prospector version 3.4.1. PSD fragments were searched
against the NCBI nonredundant data base using MS-Tag and 0.2 Da as the parent tolerance and 0.5 Da as the fragment ion tolerance (13).

_Inhibition of SCD Pr by bdellin._ Inhibition of SCD proteolysis was determined by the addition of bdellin to aliquots of either the translation mixture containing [35S]-Met labeled SCD and SCD Pr or to desaturase induced rat microsomes. The samples were incubated on ice for 30 min followed by incubation at 37°C for the indicated times.

_Zymography._ Standard zymography was performed using 0.2% casein copolymerized in a 10% SDS-polyacrylamide gel (14). The presence of Pg activators was determined by Pg zymography using casein (0.2%) and Pg (10µg/ml) copolymerized in a 10% SDS-polyacrylamide gel (15, 16). Electrophoresis was done at 4°C under nonreducing conditions. The gels were washed with 50 mM Tris-HCl, pH 8.0 containing 2.5% Triton X-100 at room temperature for 1 hr and then incubated in 50 mM Tris-HCl, pH 8.0 at 37°C either overnight for standard zymography or 1 hr for Pg zymography. The gels were stained with Coomassie blue.

_Stearoyl-CoA desaturase activity_ The microsomal membrane homogenates were prepared as described above. Reactions were performed at 37°C for 1 h with aliquots of microsomal protein suspension at 8µg/µl and 1.5 nmole of [1-14C]-stearoyl-CoA (62 µCi/µmole), 100 µM of NADH, 50 µM of Tris-HCl buffer, pH 7.4, in a volume of 50 µl. After the reaction, 150 µl of water was added and saponification was carried out in vacuum sealed glass vacules containing 200 µl KOH/ethanol (0.1g/1ml) at 100°C for 20 min. The mixture was then acidified with 200 µl 6N HCl, fatty acids were extracted with heptane, evaporated to dryness and then methylated with methanol/HCl at 25°C for 18 hrs. After evaporation and resuspension in 30 µl chloroform saturated fatty acid and monounsaturated fatty acid methyl esters were separated by 5% AgNO₃-
impregnated TLC using benzene as developing solution. The TLC plates were then subjected to autoradiography. The enzyme activity was determined as nmole-min⁻¹·mg⁻¹ protein.

**HPLC analysis of SCD degradation.** To identify SCD cleavage sites, 20 µg purified microsomal SCD was incubated with a 2 µl aliquot of either SCD Pr fraction or plasmin (1 µg) at 37°C for 12 hr. Rat plasmin was generated from rat Pg by urokinase (1:20 ratio) at 37°C for 3 hr. Peptides were extracted with 0.1% trifluoroacetic acid (TFA) in 25% acetonitrile in water and subjected to HPLC using 0.1% TFA in water (A) and 0.1% TFA in 75% acetonitrile (B) as solvents with a linear gradient of 0-70% B in 50 min. Isolated peptides were sequenced on an Applied Biosystems 470A sequencer.

**Two dimensional difference gel electrophoresis (2-D DIGE).** Rat Pg and SCD Pr were labeled with Cy3 and Cy5 dye, respectively, followed by isoelectric focussing (24 cm, pH 3-10 IPG strip) as per manufacturers instructions (Amersham Biosciences). For the second dimensional separation, the IPG strips were then loaded and run on a 12.5% SDS-PAGE gel. Both samples were run on the same gel and fluorescent images were analyzed.

**Deglycosylation.** Protein samples (2 µg) were diluted in 0.1% SDS, 50 mM β-mercaptoethanol, 0.5% Triton-X100, and 50 mM NaHPO₄, pH7.4, in 50 µl and boiled for 5 min at 100°C. Then 2 µl of N-glycosidase F (10 Units) or H₂O were added to each tube and incubated for 3 hr at 37°C. The samples were subjected to SDS-PAGE and immunobloted with antibodies against Pg. Human Pg was used as a positive control.

**Kinetic Assays.** The kinetic parameters of SCD Pr and rat plasmin were measured with the substrate D-Ile-Phe-Lys p-nitroanilide (Sigma I6886) in an assay buffer (0.05 M Tris•HCl/0.1 M NaCl, pH7.4). The reaction was initiated by addition of enzyme (1 µg in 2 µl aliquot) to assay buffer containing substrate (final concentration 10-1,000 µM) in a total volume of 200 µl in
microtiter plates at 37°C. The generation of amidolytic activity (at 405 nm) was monitored at 37°C for 3 min in a microplate reader (Molecular Devices). The Km values were calculated using Prism software (GraphPad Co.).
RESULTS

We previously reported the purification of a 90-kDa protein that selectively degrades microsomal SCD. In order to identify the 90-kDa protein, tryptic peptides obtained by in-gel digestion of SDS-PAGE gel slices were analyzed by MALDI-TOF mass spectrometry (Fig. 1A). Eleven peptides matching the molecular weight predicted for tryptic peptides of Pg were identified (Table 1). The matched peptides represented the most prominent ions present. No other proteins were matched by data base searches using the predominant ions. The identified peptides span the entire Pg sequence except for the amino terminal 68 residues of the intact secreted form. These results demonstrate that the predominant protein in the 90-kDa gel band is closely related and possibly identical to the circulating form of Pg. A similar tryptic peptide profile was obtained when rat Pg was analyzed by the same method although the yield of some peptides differed (Fig. 1B).

The presence of Pg in the SCD Pr preparation was confirmed by SDS-PAGE and immunoblot analysis. The mobility of SCD Pr was identical to Pg isolated from rat plasma (Fig. 2A). A protein band with mobility identical to Pg was detected in microsomal fractions obtained during purification of SCD Pr by immunoblot analysis with Pg antibody, and the amount of the Pg immunoreactive band increased with progressive purification of SCD protease activity (Fig. 2B). The mobility of both Pg and SCD Pr relative to an 111-kDa protein standard was dependant on the acrylamide concentration of the gels. In 6% SDS-PAGE gels, both Pg and SCD Pr migrated slightly slower than the 111-kDa protein standard (Fig. 2) whereas in 10% SDS-PAGE gels they migrated faster than the 111-kDa protein standard (Fig. 3B). The reason for this difference relative to the standard is not clear, but under both sets of SDS-PAGE conditions, the mobility of SCD Pr was identical to the mobility of Pg. The SDS-PAGE protein profiles of SCD Pr under
reducing and nonreducing conditions were compared with Pg (Fig. 3A and B). Pg had a significantly higher mobility in nonreduced SDS-PAGE gels as compared with reduced gels presumably because its numerous internal disulfide bonds maintain it in a compact conformation even after denaturation in SDS. SCD Pr exhibited the same SDS-PAGE mobility shift under nonreducing conditions. The SCD Pr preparation used in the experiments shown in Fig. 3A and B had been stored briefly at 4°C. We previously showed that the 90 kDa form of SCD Pr is converted to a spectrum of smaller peptides when stored at 4°C (11). A number of minor peptide bands were detected by reduced SDS-PAGE of the stored SCD Pr preparation that were not evident in nonreduced gels. This suggests that upon storage at 4°C certain peptide bonds in SCD Pr are cleaved and the resulting peptides are held together by disulfide bonds.

To evaluate their N-linked carbohydrate status, Pg and SCD Pr were digested with endoglycosidase F followed by SDS-PAGE and immunobloting (Fig. 3C and D). Endoglycosidase F digestion of rat Pg and SCD Pr did not alter their mobility upon SDS-PAGE. Human Pg was used as a positive control in these experiments since it exists in two major glycoforms (17). Human Pg I contains an N-linked oligosaccharide at Asn-289 and an O-linked oligosaccharide at Thr-345. Human Pg II contains only an O-linked oligosaccharide at Thr 345. As expected, SDS-PAGE of human Pg revealed a doublet, and treatment of human Pg with Endo F resulted in depletion of the upper band (form I). Thus, it appears that, in contrast of human Pg, rat Pg and SCD Pr lack N-linked carbohydrate structures. The O-linked carbohydrate status of rat serum Pg is not known.

SCD Pr was further compared with Pg by 2-dimensional gel electrophoresis, and no significant difference in mobility was observed (Fig. 3E).
The amidase parameters of SCD Pr and rat plasmin towards substrate D-Ile-Phe-Lys p-nitroanalide are similar, with Km values of 18.2 µM and 18.3 µM, respectively.

Since SCD Pr appeared to represent a microsomal form of Pg, the ability of Pg and plasmin isolated from rat plasma to degrade SCD was investigated (Fig. 4A). There was no detectable degradation of SCD when it was incubated with 0.6 µM Pg for two hours at 37°C. By contrast, coincubation of SCD with 0.6 µM Pg and urokinase (uPA) or tissue Pg activator (tPA) led to extensive degradation of SCD within two hours (Fig. 4A, lanes 4 and 5). These results indicate that plasmin is able to degrade SCD. The results obtained with Pg alone suggest the Pg preparation was not contaminated with plasmin or Pg activators. The peptides produced by plasmic cleavage of SCD were compared with degradation products produced by incubating SCD with SCD Pr (Fig. 4B and C). As shown, the HPLC profiles of the peptides produced by SCD Pr and plasmin are nearly identical. Sequence analysis of the identified peptides showed cleavage at Lys residues and a single peptide resulting from cleavage at His residue (Fig. 4D). No cleavage at Arg residues were identified, despite the presence of 22 Arg residues and 20 Lys residues in the SCD sequence (9).

The proteolytic activity in microsomes and microsomal fractions obtained during the purification of SCD Pr was analyzed by zymography on casein gels (Fig. 5). Zymography is a method for measuring proteolytic activity in SDS gels impregnated with a protein substrate such as casein or gelatin (14-16). Proteases that remain active in SDS or have the ability to refold into an active conformation when the SDS concentration is lowered can be analyzed by this method. Since most of the SCD Pr activity in the hydroxyapatite fractions is latent (11), zymograms of initial microsomal fractions were compared with zymograms of the same fractions after incubation at 37°C. It was essential to run zymogram gels under nonreducing conditions; no
proteolytic activity was detected in microsomal fractions by zymography under reducing conditions. Interestingly, Pg which was free of plasmin by other criteria (see above) yielded a 70-kDa band of proteolytic activity on casein zymography (Fig. 5, lane 2). This observation suggests that Pg undergoes conversion to plasmin during zymography, or refolds into a conformation with protease activity when the SDS is removed. After Pg was incubated with uPA, a spectrum of proteolytic species from 40-kDa to 70-kDa was detected (Fig. 5, lane 3). The primary proteolytic activity detected in microsomes by casein zymography migrated with an apparent molecular weight of 70-kD. The mobility of this protease is identical to the mobility of the protease detected in Pg (compare lanes 2 and 4 in Fig. 5). When microsomes were incubated at 37°C, the casein hydrolysis activity of the 70-kDa protease increased, and a 30-kDa protease was generated (Fig. 5, lane 5). The same pattern was seen when high salt washed microsomes (HSWM) were analyzed, but a smaller quantity of the 30-kDa protease was produced, and it appeared as a doublet (Fig. 5, lane 7). When HSWM were solubilized with 2% Triton X-100 (TX-100) at 4°C, SDC Pr activity was recovered in the insoluble fraction (11). Zymography of the TX-100 insoluble material showed enrichment of the 70-kDa protease. Incubation of the TX-100 insoluble material led to a decrease in the 70-kDa protease band and generation of the 30-kDa protease along with a spectrum of proteases of intermediate mobility (Fig. 5, lanes 8 and 9). Several proteolytic bands between 30- and 70-kDa were also observed after the TX-100 insoluble material was solubilized with 2% CHAPS/200 mM NaCl (Fig. 5, lane 10). Thus, even at 4°C, the detergent CHAPS appeared to activate conversion of the 70-kDa microsomal protease into smaller forms. After the CHAPS solubilized preparation was incubated at 37°C, intense proteolytic bands were seen at 32-kDa and 45-kDa, and most of the 70-kDa protease was no longer detected (Fig. 5, lane 11). After hydroxyapatite chromatography, the proteolytic pattern in
the SCD Pr preparation was even more complex suggesting ongoing autoproteolysis (Fig. 5, lane 12).

Since it appeared that SCD Pr was a product similar to activated Pg, we analyzed microsomal fractions for Pg activators by zymography using SDS-PAGE gels containing copolymerized casein and Pg (16). By this method, two Pg activators were detected in microsomes and in partially purified SCD Pr preparations with apparent molecular weights of 35-kD and 55-kDa. (Fig. 6A). These bands of activity are microsomal Pg activators because they were not detected in casein gels without Pg and did not align with those of uPA or tPA(Fig. 6B). Several proteolytic bands were detected in Pg activator zymograms of SCD Pr (Fig. 6A, lane 7), however, these bands were also present in casein gels without copolymerized Pg (Fig. 6B, lane 7), indicating that they represent caseinases rather than specific Pg activators.

Further proof that SCD is degraded by a microsomal form of Pg was provided by bdellin inhibition studies. Bdellin, also known as bdellastasin, is a 59 residue, cysteine-rich serine protease inhibitor of the antistasin family which occurs naturally in the medical leech (Hirudo medicinalis) (18). Bdellin is a powerful and relatively specific proteinase inhibitor of trypsin and plasmin and displays no inhibition for a variety of other serine proteases (19). As shown in Fig. 7, 100 µM bdellin inhibited the degradation of native SCD in microsomes and inhibited the degradation of [35S]SCD by purified SCD Pr.

To explore the role of Pg in the degradation of SCD in vivo, we isolated microsomes from Pg deficient (Pg-/-) mice, and compared the levels of SCD protein, SCD enzyme activity, and SCD Pr activity with wild-type control animals (Fig. 8). Liver microsomes from Pg-/- mice had significantly higher levels of SCD protein and SCD enzyme activity than microsomes from the wild-type mice. Degradation of SCD in microsomes from Pg-/- mice was slow, whereas liver
microsomes from control mice showed rapid SCD degradation similar to that observed in rat liver microsomes.

**DISCUSSION**

Hepatic microsomal desaturase activity is regulated by the rapid degradation of SCD. SCD is also rapidly and selectively degraded in vitro when liver microsomes are incubated at 37°C (9). We previously reported that the microsomal SCD protease (SCD Pr) is a 90-kDa zymogen with Arg/Lys specificity (11). The data presented here show that the 90-kDa protein is a microsomal form of Pg. Pg is secreted by hepatocytes, so it is not surprising that it can be isolated from hepatic microsomes which are derived predominantly from secretory organelles including the endoplasmic reticulum. Initially, we were concerned that Pg might also adsorb onto the surface of microsomes during tissue homogenizaton. However, several observations indicate that the source of Pg in our preparations is intracellular. SCD Pr was isolated from microsomes that had been extensively washed with high salt 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. This washing procedure removed more than 95% of the peripheral membrane protein but did not deplete microsomal membranes of Pg or SCD Pr activity. Extensive perfusion of livers to remove plasma proteins prior to homogenization had no effect on the Pg content of microsomes. Most importantly, the observation that Pg-/− mice have elevated SCD activity suggests that Pg degrades SCD in vivo.

SCD Pr activity was recovered in the TX100 insoluble fraction when high-salt washed microsomes were solubilized with 2 % TX100. This suggests that SCD Pr is derived from a microsomal Pg fraction that is bound to the microsomal membrane surface. Earlier work showed that 40% of Pg in rough microsomes is tightly associated with microsomal membranes, and that
the secretion of Pg is delayed relative to albumin (20). The mechanism by which Pg binds the microsomal membrane and is retained in the ER is unknown, however several hypotheses are consistent with current knowledge of ER protein traffic. Post-translational lipidation with palmitate or an isoprenoid derivative would explain membrane binding, and would not change the SDS-PAGE mobility significantly. Palmitoylation and isoprenylation both occur in the ER (21). Pg binds C-terminal lysyl residues in fibrin and cell surface proteins via kringle structures in its N-terminal domain. Thus, interaction with lysyl residues in microsomal proteins is a second possible mechanism for the tight binding of SCD Pr to microsomal membranes. A number of proteins, including C-reactive protein (22) and β-glucuronidase (23), which lack ER retention/retrieval signals are retained in the ER by forming a complex with lumenal esterases 1 and 2. Microsomal esterases are retained in the ER by a C-terminal HIEL motif (24). Nacent secretory glycoproteins are maintained in a monoglucosylated state and retained in the ER by lectins until folding is complete (25). Finally, the current paradigms for protein folding and ER quality control suggest that “incorrect” conformations are recognized and held in the ER by chaperones. A fraction of newly synthesized microsomal Pg molecules may have a unique conformation or structural feature that codes for ER retention. Our results address several of the possible mechanisms for retention of Pg in the ER. MALDI-TOF mass spectrometry of peptides obtained by in-gel tryptic digestion and purification by C18 micropipette tips did not identify post-translational modifications that could explain membrane binding. However, only eleven peptides were identified by this method. This is sufficient structural information to identify SCD Pr as a form of Pg; more extensive structural analysis will be necessary to exclude posttranslational modifications. Retention of Pg in the ER probably does not involve lectins, because we did not detect N-linked carbohydrate in SCD Pr or Pg by N-glycosidase F digestion.
How can the generation of plasmin, a protease with broad substrate specificity, account for the highly selective degradation of SCD in microsomes? During the dissolution of clots, fibrin is specifically degraded through the formation of a ternary complex between fibrin, Pg, and tPA (26). The formation of this complex enhances the specificity of plasmin by colocalizing the activator, zymogen, and substrate and also by conformational changes that occur when tPA and Pg bind to fibrin. In the absence of fibrin, the conversion of Pg to plasmin by activators is inefficient and the specificity toward fibrin is lost. Thus, one potential mechanism for the apparent specificity of SCD cleavage in isolated microsomes is that SCD and Pg colocalize in the ER/microsomal membrane. The microenvironment of the membrane likely influences both the activation and catalytic activity of SCD Pr.

Glu-Pg is synthesized in hepatocytes as a single chain protein of 791 amino acids with 24 disulfide bridges, and a molecular weight of 92-kDa (26). Conversion of Pg to the active serine protease plasmin requires specific cleavage of the Arg562-Val563 peptide bond in the C-terminal serine protease domain. Two physiologic Pg activators have been identified: tissue-type Pg activator (tPA) and urokinase-type Pg activator (uPA). The synthesis of Pg activators by hepatocytes has not been extensively studied. In an immunohistochemical study, uPA was identified in oval cells of the liver, but not hepatocytes (27). uPA was also identified in cultured stellate liver cells by zymography (28). We identified Pg activators of Mr 35-kDa and 55-kDa in hepatic microsomal preparations by zymography (Fig. 6). Since these proteases are candidates for intracellular Pg activators, their identity and cellular origin are of interest.

Intracellular activation of Pg might not require a proteolytic Pg activator. When complexed with the Pg activator streptokinase, Pg adopts a proteolytically active conformation that does not require cleavage of the Arg562-Val563 peptide bond. Kornblatt and coworkers recently reported
that the cellular prion protein of sheep (PrP\textsuperscript{c}) forms a complex with Pg that degrades PrP\textsuperscript{c} (29). The degradation of PrP\textsuperscript{c} by Pg occurs in the absence of plasmin, but is accelerated when tPA is included in the reaction. We have recently found that extended incubation of purified microsomal SCD with excess Pg in the absence of Pg activators results in the disappearance of both proteins from SDS-PAGE gels. Whether this observation is physiologically significant is under investigation. The presence in microsomes of non-proteolytic Pg activators that stabilize a proteolytically active conformation of Pg analogous to streptokinase or PrP\textsuperscript{c} remains to be determined.

When Pg is bound to cell surfaces, its activation is markedly enhanced compared with the reaction in soluble phase (reviewed in reference 30). Two alternative mechanisms by which the activation of cell-surface Pg is enhanced have been proposed (30). The crucial element of both mechanisms is a conformational change that occurs in Glu-Pg, the native circulating form of the zymogen, in the presence of lysine analogs. In solution, Glu-Pg has a globular, ‘closed’ conformation; when bound to lysine analogs, it adopts an extended, ‘open’ conformation that is more rapidly converted to plasmin (31,32). Proteolytic conversion of Glu-Pg to Lys\textsuperscript{77}-Pg has an effect on Pg conformation and activation similar to lysine analogs (33). Thus, interaction with carboxyterminal lysyl residues on membrane surfaces may induce a conformational change in Glu-Pg associated with enhanced activation, or alternatively, binding may promote plasmic conversion of Glu-Pg to Lys\textsuperscript{77}-Pg and indirectly enhance Pg activation. It remains to be seen whether the tightly bound form of microsomal Pg is more readily activated than the soluble form.

Pg deficiency in mice causes severe thrombosis and is associated with a poorly understood wasting syndrome with a high mortality rate (34). Because loss of fibrinogen rescues mice from the pleiotropic effects of Pg deficiency, it has been suggested that the only essential
physiological role of Pg is fibrinolysis (35). More recently, Bezerra et. al. (36) reported that Pg deficiency impedes the clearance of necrotic hepatocytes after toxic liver injury, and genetically superimposed deficiency of the $\alpha$ fibrinogen chain did not correct the abnormal phenotype. Furthermore, non-fibrin substrates of plasmin have been identified in several laboratories (reviewed in reference 12 and 37), and despite exclusion of Pg and fibrinogen by the blood-brain barrier, Pg has been implicated in several natural and experimental neurodegenerative processes (38). Our results suggest that some of the plasmin substrates in these pathologic processes may be intracellular.

A number of mouse models have been described where hepatic SCD is disregulated. Up-regulation of hepatic SCD occurs in transgenic mice overexpressing sterol regulatory element-binding proteins SREBP-1a or –1c. The mRNA for SCD is elevated 7-fold in SREBP-1a mice and 3-fold in the SREBP-1c transgenic mice (39). SREBP-1a transgenic mice develop a massive fatty liver engorged with both triglycerides and cholesterol (39). Overexpression of SREBP-1c in the liver of transgenic mice produces a triglyceride-enriched fatty liver with no increase in cholesterol (39). In addition to increased levels of SCD, mice overexpressing SREBP –1a or –1c displayed up-regulated spectrum of lipogenic genes. In SREBP-1c (-/-) knockout mice, the SCD mRNA levels are low, and livers of these mice show a complete failure of the normal inductive SCD response to refeeding (40). Mice with a naturally occurring mutation in SCD (abj/abj) and mice with targeted disruption of the SCD gene (SCD1/-) have decreased plasma and hepatic levels of triglyceride and cholesteryl esters and are resistant to diet-induced obesity (41-43). Leptin deficient mice ob/- have up-regulated SCD (7-fold) and are obese (42,43). When mice deficient in hepatic SCD (abj/ abj) were intercrossed with ob/- mice, double mutant abj/abj;ob/ob mice were obtained. The double mutant mice showed a dramatic reduction in body
weight compared to littermate ob/ob controls (42,43). Analysis of food intake and energy expenditure in abj/abj;ob/ob mice showed increased oxygen consumption and consumed more food than ob/ob littermates suggesting that hepatic SCD deficiency may modulate pathways that decrease fatty acid synthesis and increase lipid oxidation (42,43). While SREBP-1a and ob/ob mice have similar up-regulated hepatic SCD these two models differ significantly, since serum lipid levels in SREBP-1a mice were largely unaltered (39). In summary, hepatic SCD is a central enzyme in the regulation of lipid metabolism. Degradation of SCD plays a key role in regulation of transient fluctuations in SCD activity that occur in response to dietary perturbations. The role of SCD degradation in model systems with sustained up-regulated hepatic SCD remains to be determined. Pg knockout mice may provide addition model to study the role of hepatic SCD in the regulation of membrane fluidity and lipid metabolism.
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The abbreviations used are: SCD, stearoyl-CoA desaturase; SCD Pr, stearoyl-CoA desaturase protease; Pg, plasminogen; ER, endoplasmic reticulum; HSWM, high salt washed microsomes; 2-D DIGE, two dimensional difference gel electrophoresis.
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FIGURE LEGENDS

Figure 1. MALDI-TOF mass spectroscopic analysis of SCD Pr and rat Pg tryptic peptides. The 90-kDa SCD Pr (A) and rat Pg (B) protein bands were excised from an SDS-PAGE gel. Peptides were generated by in-gel tryptic digestion, and concentrated by reverse phase adsorption chromatography. MALDI-TOF data were obtained for the isolated peptides as described in "Experimental Procedures." The numbers in parentheses indicate the peptides shown in Table 1. (T) indicates tryptic autolysis product.

Figure 2. Pg immunoblot and SDS-PAGE analysis of SCD protease (SCD Pr). Microsomal fractions containing SCD Pr were prepared as described in Experimental Procedures. Panel A, 6% SDS-PAGE gel stained with Coomassie blue. Lane 1, molecular weight standards with the mass marked on the left of the panel. Lane 2, high-salt washed microsomes (36 µg) (HSWM). Lane 3, TX-100 insoluble fraction of HSWM (25 µg) (TXINSOL). Lane 4, TXINSOL solubilized with CHAPS in the presence of NaCl (12 µg). Lane 5, SCD Pr preparation obtained by hydroxyapatite chromatography (3 µg). Lane 6, rat Pg (0.5 µg). Panel B, immunoblot analysis of the above samples with polyclonal anti-rat Pg.

Figure 3. SDS-PAGE, N-Glycosidase F, and 2-D DIGE analysis of SCD Pr and rat Pg. Ten percent acrylamide gels were run under non-reduced (A) and reduced (B) conditions and stained with Coomassie blue. Lane 1, molecular weight markers. Lanes 2 and 3, SCD Pr preparation obtained by Superdex 200 chromatography. Lanes 4 and 5, rat Pg at the indicated concentration. C and D, rat Pg, SCD Pr, and human Pg were treated without (-) or with (+) N-glycosidase F as described in "Experimental Procedures" and subjected to Coomassie staining (C) and anti-Pg.
immunoblot analysis (D). Only human Pg showed evidence of N-glycosilation. E, 2-D DIGE analysis of rat Pg and SCD Pr display identical mobility.

Figure 4. Rat plasmin degrades SCD and cleaves at the same sites as SCD Pr. (A) Lane 1, sample of \(^{35}\)S]SCD synthesized by in vitro transcription and translation system was incubated without additions. Lane 2, SCD incubated with 0.5µg SCD Pr. Lane 3, SCD incubated with rat Pg. Lane 4, SCD incubated with uPA activated Pg, and lane 5, with tPA activated Pg. Degradation of SCD was determined by measuring of \(^{35}\)S]SCD by SDS-PAGE followed by autoradiography as described in "Experimental Procedures." The concentration of Pg in lanes 3-5 was 0.6µM. B and C, HPLC of native SCD incubated with SCD Pr (B) and rat plasmin (C). TX100 indicates artifact peaks also observed in HPLC of blank containing detergent. Peptides were isolated as described in "Experimental Procedures" and N-terminal residues identified by sequence analysis (D). Arrow indicates cleavage site and position indicates location of the corresponding site in the SCD sequence.

Figure 5. Comparison of proteolytic activities of SCD Pr and Pg by zymography. Samples of rat Pg were pre-incubated at 37°C for 3 hr with or without supplementation. Lanes 4 to 9 contain microsomal fractions incubated at -20°C or 37°C for 16 hr. Lane 1, molecular weight standards with the mass marked on the left of the panel. Lane 2, of rat Pg (2 µg) pre-incubated without activator. Lane 3, rat Pg (2 µg) pre-incubated with urokinase-type Pg activator (uPA). Lanes 4 and 5, desaturase induced microsomes. Lanes 6 and 7, high-salt washed microsomes (HSWM). Lanes 8 and 9, TX100 insoluble fraction of HSWM. Lanes 10 and 11, TXINSOL fraction
solubilized with CHAPS/NaCl, (HAP column load). Lane 12, SCD Pr preparation obtained by hydroxyapatite chromatography.

Figure 6. Pg activator zymograms. Microsomes and SCD Pr preparations were assayed for Pg activators by electrophoresis in gels containing copolymerized casein and Pg. Panel A, Lane 1, molecular weight standards with the mass marked on the left of the panel. Lane 2, rat Pg (0.1 µg). Lane 3, desaturase induced microsomes (DsMs). Lane 4, high-salt washed microsomes (HSWM). Lane 5, TX-100 insoluble fraction of HSWM (TXINSOL). Lane 6, CHAPS/NaCl soluble fraction of TXINSOL. Lane 7, SCD Pr preparation obtained by hydroxyapatite chromatography. Lane 8, urokinase-type Pg activator (uPA). Lane 9, tissue-type Pg activator (tPA). Electrophoresis conditions were non-reducing. The gels were prepared and developed as described in "Experimental Procedures." Panel B, Aliquots of samples in (A) run on identical gels prepared without Pg.

Figure 7. Bdellin inhibits the degradation of SCD. Panel A, [^35S]SCD was incubated with SCD Pr in the absence (lane 2) and presence (lanes 3 and 4) of bdellin. Degradation was measured by the disappearance of [^35S]SCD upon SDS-PAGE analysis followed by autoradiography. Panel B, 10µl of desaturase induced microsomes (30 µg) were incubated at 37°C for the indicated time in the absence (lanes 2 and 3) and presence (lane 4) of bdellin. Samples were analyzed by SDS-PAGE and stained with Coomassie blue. The position of the SCD band is indicated on the right. Panel C, immunoblot analysis of SCD degradation in the samples shown in panel B.
Figure 8. SCD is not degraded in Pg knockout mice. Time course of mouse SCD degradation in control and Pg-/- mouse microsomes. At the indicated times, aliquots were analyzed by SDS-PAGE and visualized by Coomassie staining (A) and by immunoblotting with an antibody raised against SCD (B). C, relative amounts of SCD enzyme activity from liver microsomes of control and Pg-/- mice was obtained as described in "Experimental Procedures". Each value represents the amount of SCD activity relative to that in control mice, which is defined as 1. Inset in C, autoradiography of [14C]-stearate and its metabolite oleate separated by TLC. Equivalent amounts of protein (15 µg) from control and Pg-/- mouse liver microsomes were assayed for SCD activity.
Table 1: MALDI-MS/PSD analysis of fragmented SCD Pr and serum plasminogen yields similar peptides.

| Peptide No. | Sequence                              | m/z (Meas.) | MH+ (Calc.) |
|-------------|---------------------------------------|-------------|-------------|
| 88-97       | MRDVILFEKR(V)                         | 1306.770    | 1306.730    |
| 137-153     | YSPSTHPSEGLEANCR(N)                   | 2039.830    | 2039.877    |
| 299-306     | WSEQTPHR(H)                           | 1040.480    | 1040.491    |
| 318-325     | NLENYCR(N)                            | 1111.550    | 1111.484    |
| 493-514     | TAVTAAGTPQEEWAAQEPHSHR(I)             | 2418.990    | 2419.121    |
| 515-523     | IFTPQTNPR(A)                          | 1073.560    | 1073.574    |
| 659-672     | LVLEPNDADIALK(L)                      | 1523.890    | 1523.869    |
| 801-807     | YVNWIER(E)                            | 979.510     | 979.500     |
| 801-810     | YVNWIEREMR(N)                         | 1395.740    | 1395.684    |
| 411-426     | TPANFDPAGLEMNYCR                      | 1869.840    | 1869.826    |
| 582-600     | VVGGCVANPHSWPWQISLR                   | 2177.080    | 2177.108    |
Figure 2
Figure 3
**Figure 4**

**Panel A**

A gel blot showing the expression of SCD and its variants. The gel is loaded with samples labeled as SCD, SCD + SCD Pr, SCD + Pg, SCD + Pg + uPA, and SCD +Pg + tPA. The arrow indicates the position of [35S]SCD.

**Panel B**

A graph showing the absorbance at 280 nm (A280) over time (minutes) for the labeled SCD Pr variant. The graph includes peaks labeled 1 to 6, with peak 6 being normalized to 100.

**Panel C**

A similar graph for Rat Plasmin, with peaks labeled 1 to 9, and peak 9 normalized to 100.

**Panel D**

A table listing the cleavage sites and their positions:

| Peak # | Cleavage site | Position |
|--------|---------------|----------|
| 1      | K AAVLARIK    | 341      |
| 2      | H MLQIEISS... | 5        |
| 3      | K LVMFORRYYK  | 208      |
| 4      | K VYTLWGLI... | 97       |
| 5      | K VPLYLEED... | 37       |
| 6      | K KVPILYLEE... | 36      |
| 7      | K FSETHADP... | 162      |
| 8      | K NIOSRENI... | 278      |
| 9      | K ARLPLRI... | 129      |
Figure 7
Figure 8
A plasminogen-like protein selectively degrades stearoyl-CoA desaturase in liver microsomes
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