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Degradation of Hepatic Stearyl CoA Δ^9-Desaturase

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Δ^9-Desaturase is a key enzyme in the synthesis of desaturated fatty acyl-CoAs. Desaturase is an integral membrane protein induced in the endoplasmic reticulum by dietary manipulations and then rapidly degraded. The proteolytic machinery that specifically degrades desaturase and other short-lived proteins in the endoplasmic reticulum has not been identified. As the first step in identifying cellular factors involved in the degradation of desaturase, liver subcellular fractions of rats that had undergone induction of this enzyme were examined. In livers from induced animals, desaturase was present in the microsomal, nuclear (P-1), and subcellular fractions (P-2). Incubation of desaturase containing fractions at physiological pH and temperature led to the complete disappearance of the enzyme. Washing microsomes with a buffer containing high salt decreased desaturase degradation activity. N-terminal sequence analysis of desaturase freshly isolated from the P-1 fraction without incubation indicated the absence of three residues from the N terminus, but the mobility of this desaturase preparation on SDS-PAGE was identical to the microsomal desaturase, which contains a masked N terminus under similar purification procedures. Addition of concentrated cytosol or the high-salt wash fraction did not enhance the desaturase degradation in the washed microsomes. Extensive degradation of desaturase in the high-salt washed microsomes could be restored by supplementation of the membranes with the lipid and protein components essential for the reconstituted desaturase catalytic activity. Lysosomotrophic agents leupeptin and pepstatin A were ineffective in inhibiting desaturase degradation. The calpain inhibitor, *N*-acetyl-leucyl-leucyl-methional, or the proteosome inhibitor, *Streptomyces* metabolite, lactacystin, did not inhibit the degradation of desaturase in the microsomal or the P-1 and P-2 fractions. These results show that the selective degradation of desaturase is likely to be independent of the lysosomal and the proteosome systems. The reconstitution of complete degradation of desaturase in the high-salt–washed microsomes by the components essential for its catalytic activity reflects that the degradation of this enzyme may depend on a specific orientation of desaturase and intramembranous interactions between desaturase and the responsible protease.

INTRODUCTION

The formation of monounsaturated fatty acids is catalyzed by Δ^9 desaturase (EC 1.14.99.5) in a reaction requiring acyl-CoA, NADH, NADH-reductase, cytochrome b_5, phospholipid, and oxygen (Strittmatter *et al.*, 1974). The desaturase, a 40-kDa intrinsic membrane protein, can be induced more than 50-fold in the endoplasmic reticulum by the administration of fat-free diet, insulin, or certain carbohydrate metabolites to animals (Oshino and Sato, 1972). When the dietary regimen is stopped, the desaturase activity decreases to undetectable levels with a half-life of a few hours (Oshino and Sato, 1972). The proteolytic system responsible for the rapid and selective degradation of desaturase is unknown. Eukaryotic cells contain multiple proteolytic systems including the lysosomal proteases, ATP-ubiquitin–dependent and ubiquitin-independent ATP-proteosome pathway (Hershko and Ciechanover, 1992; Inoue and Simoni, 1992). Some of these proteolytic events presumably occur on the cytoplasmic side (Gardner *et al.*, 1993), whereas others occur within the lumen of endoplasmic reticulum

(Wikstrom and Lodish, 1992). Several substrate-related peptidyl aldehydes such as *N*-acetyl-leucyl-leucyl norleucinal and *N*-acetyl-leucyl-leucyl-methional (ALLM) have been identified to inhibit the calpain, and the ubiquitin-proteosome–dependent proteolytic pathways (Jensen *et al*., 1995). The *Streptomyces* metabolite, lactacystin, is a specific inhibitor of the proteosome (Fenteany *et al*., 1995). It covalently modifies the highly conserved N-terminal threonine of the mammalian proteosome subunit X, a close homologue of the LMP7 proteosome subunit encoded by the major histocompatibility complex (Fenteany *et al*., 1995). Notwithstanding, membrane proteins are selectively degraded. A number of short-lived membrane proteins, incorrectly synthesized proteins, and partially oligomerized complexes are rapidly degraded in the endoplasmic reticulum, but it is not understood how this degradation is achieved and regulated (Klausner and Sitia, 1990; Bonifacino and Klausner, 1994).

We previously derived the cDNA sequence of the desaturase (Thiede *et al*., 1986) and constructed an expression vector for the production of active desaturase in *Escherichia coli* (Strittmatter *et al*., 1988). DNA constructs expressing desaturase in mice (Kaestner *et al*., 1989), *Saccharomyces cerevisiae* (Stukey *et al*., 1990), and a soluble form of desaturase in plants (Thompson *et al*., 1991; Shanklin and Somerville, 1991) have also been reported. A form of desaturase up-regulated in response to cold has been described in fish (Tiku *et al*., 1996).

To study the degradation of this membrane protein, desaturase was induced in rat liver membranes and isolated, and its degradation in subcellular fractions was investigated. In the present study, I report that selective degradation of desaturase can be readily monitored in microsomes and that complete degradation of this enzyme may be partially altered by a high-salt wash of the microsomes. Moreover, the inhibitors of known proteolytic systems such as lysosomes, cathepsins, or the nonlysosomal 26S proteosome complex failed to inhibit the specific degradation of desaturase. These findings should permit the design of experiments to identify the specific desaturase-degrading activity among the many previously characterized cellular proteolytic systems.

MATERIALS AND METHODS

Detergents, enzyme substrates, cofactors, and chromatographic media were obtained from Sigma (St. Louis, MO). Cytochrome b_5 and cytochrome b_5 reductase were prepared from rabbit liver microsomes as described previously (Ozols, 1974, 1989; Strittmatter *et al*., 1993). ALLM, pepstatin, leupeptin, and phenylmethylsulfonyl fluoride (PMSF) were from Sigma. Porcine erythrocyte calpain was obtained from Calbiochem (San Diego, CA). Lactacystin was obtained from Professor E.J. Corey, Department of Chemistry, Harvard University (Boston, MA).

Preparation of Desaturase Containing Subcellular Fractions

Male Sprague Dawley rats weighing 200–250 g were fasted for 48 h, fed regular diet for 48 h, fasted for second 48-h period, and refed for 20 h with Nutritional Biochemical (Cleveland, OH) "Fat Free" test diet on a schedule that permitted the animals to be killed at the beginning of a day. Control animals were refed regular diet. Subcellular fractionation of livers from induced and control animals was performed according to published procedure (Ozols, 1990). The perfused livers were homogenized in a buffer containing 0.25 M sucrose, 10 mM Tris-acetate, pH 8.1, 1 mM EDTA (6 ml/g of liver) in a glass homogenizer. Pellet P-1 was obtained by centrifugation of the homogenate at 800 × *g* for 10 min. The resulting supernatant was then spun at 10,000 × *g* for 35 min yielding pellet P-2. Centrifugation of the P-2 supernatant at 130,000 × *g* for 1.5 h gave pellet P-3 and the supernatant (cytosol fraction). Pelleted microsomes (P-3) were suspended in 20 volumes of 0.1 M sodium pyrophosphate, pH 7.4, and recentrifuged at 130,000 × *g*. High-salt washed microsomes were prepared by suspending the pellet in 20 volumes of buffer containing 0.1 M Tris-acetate, 0.5 M NaCl, 10 mM EDTA and sedimenting at 130,000 × *g* for 1 h to obtain high-salt washed microsomes and the high-salt supernatant. The nuclear pellet was refractionated by the method of Fleisher and Krevina (1974). Concentration of the high-salt and cytosol fractions was accomplished on a Centricon-30 concentrator (Amicon, Danvers, MA). Subcellular fractions were stored at −70°C until use. Protein concentration in the samples was determined using the Coomassie dye binding reagent (Pierce, Rockford, IL), using bovine serum albumin as a standard.

Triton X-114 Fractionation of Pellet P-2

Precondensed 4% Triton X-114 in Tris-buffered saline, pH 7.5 (Oxford Glycosystems, Abingdon Oxon, United Kingdom) was added to 120 μg of P-2 to a final concentration of 0.8%. The reaction mixture was spun at 100,000 × *g* for 15 min at 4°C. The supernatant was layered over a cushion of 0.25 M sucrose, and the centrifuge tube was incubated for 5 min at 37°C. Centrifugation of the reaction mixture at 12,000 × *g* for 5 min at 37°C yielded detergent-containing lower phase and detergent-depleted aqueous upper phase.

Isolation of Desaturase

Desaturase from the P-1 and P-2 fractions was purified in the presence of sodium deoxycholate and Triton X-100 as described previously for the purification of microsomal desaturase (Strittmatter *et al*., 1974, 1988). Desaturase in the P-1 or P-2 fractions for sequencing purposes was subjected to 12% SDS-PAGE and electroblotted onto the Immobilon-P membrane. Sequence analysis on the electroblotted material was performed on an Applied Biosystems (Foster City, CA) model 470A sequenator on line with a model 120A phenylhydantoin analyzer.

Reconstitution of the Desaturase System

The reconstitution and desaturase assay was performed as described previously (Strittmatter *et al*., 1988). Typically, 20 μl of 600 μM cytochrome b_5, 6 μl of 100 μM cytochrome b_5 reductase, and 20–40 μl of 30 mM egg phosphatidylcholine were added to 115 μl of desaturase sample in 2% Triton X-100 and 0.4% sodium deoxycholate. After incubation for 1.5 h at 4°C, enzyme activity was measured at 25°C by the rate of NADH oxidation in the presence and absence of stearyl-CoA.

Preparation of Antibody against Rat Liver Microsomal Desaturase

To a solution of desaturase, 20 volumes of cold acetone containing 0.2% (y/y) HCl were added. After several hours at −20°C, the

protein was collected by low-speed centrifugation. About 0.5 mg of desaturase protein was mixed with 0.5 ml of Freund's complete adjuvant and injected into rabbits. Rabbit immunoglobulin G (IgG) was prepared from serum by precipitation with ammonium sulfate as described (Hardy, 1986). IgG fractions were diluted with an equal volume of 0.1 M potassium phosphate, pH 7, and the IgG was partially purified by affinity chromatography using a column of Protein A Sepharose CL-4B equilibrated in 0.1 M potassium phosphate, pH 7, and stored in 50% glycerol at −70°C.

Immunoblotting

Complete degradation of desaturase in microsomes and nuclear fractions was determined by Western Blotting (Toubin *et al*., 1979). After incubation at 37°C the samples were subjected to SDS-PAGE using 12- or 10% acrylamide gel under reducing conditions in duplicate. One gel was stained with Coomassie blue, and an identical gel was electrotransferred to an Immobilon-P transfer membrane. The membrane was reacted with rabbit antidesaturase antibody, which was then complexed with antirabbit IgG-alkaline phosphatase (Sigma product A-3687). Immunoreactive desaturase bands were visualized using phosphatase substrate system detection kit (Kirkegaard & Perry Laboratories, Gaithersburg, MD). Immunoblots were quantified by densitometry using a Kodak DCS 200 digital camera with the Image software (Center for Biomedical Imaging Technology).

Assay of Desaturase Degradation

The reaction mixtures contained 10–15 μg of protein in 50 μl of 50 mM Tris-acetate, pH 7.9, and 50 mM KCl. ATP, ATP-Mg^{2+}, and various protease inhibitors were added where indicated in the figure legend. Cytosol or high-salt fractions were concentrated on a Centricon-30 membrane (Amicon) and added to the reaction samples where indicated. The protease inhibitors were dissolved in dimethylsulfoxide and added at the concentration indicated in the figure legend. Final dimethylsulfoxide concentrations in the incubation samples were 1.5% or less. Model reactions for the protease inhibitors in microsomes were as follows: leupeptin, pepstatin, and PMSF at the concentration indicated in the figure legend was used to inhibit pepsin, papain, or endoproteases Lys-C and Asp-N. Porcine erythrocyte calpain was used as the substrate for the ALLM inhibitor. The samples were incubated 4–18 h at 37°C. Control samples were prepared at 4°C and stored at −20°C. Desaturase degradation in high-salt washed microsomes was restored by supplementation of the reaction mixture with 2 μl of 30 mM egg lecithin liposomes, 2 μl of 50 μM cytochrome b_5 reductase, 2 μl of 300 μM cytochrome b_5, and 5 μl of stearyl-CoA. Before gel electrophoresis of the digests, 75–150 μl of the loading buffer, containing 60 mM Tris-acetate, pH 6.8, 3% SDS, 5% β-mercaptoethanol, 10% glycerol, and 0.25% bromophenol blue were added. Ten-microliter aliquots of the reaction mixtures were subjected to electrophoresis in duplicate for Coomassie blue staining and immunoblot analysis.

RESULTS

Localization of Desaturase in Microsomes

As the first step in examining the desaturase degradation of desaturase, the enzyme was induced in liver by feeding fasted rats a fat-free, high-carbohydrate diet. As seen in SDS-PAGE Coomassie blue and immunoblot analysis (Figure 1, lanes 2 and 5), a 37-kDa band reacting with desaturase antibody is evident in microsomes from rat livers that had undergone dietary manipulation. In contrast, this band was not detected in liver microsomes from control animals (Figure 1, lane 3). Purified liver microsomal desaturase migrates on SDS-PAGE as a 37-kDa band, which is lower than the predicted molecular size of 41-kDa (Thiede *et al*., 1986). The cytosolic fraction obtained from desaturase-induced livers is shown in Figure 1, lane 6, revealing the absence of desaturase in the cytosol fraction.

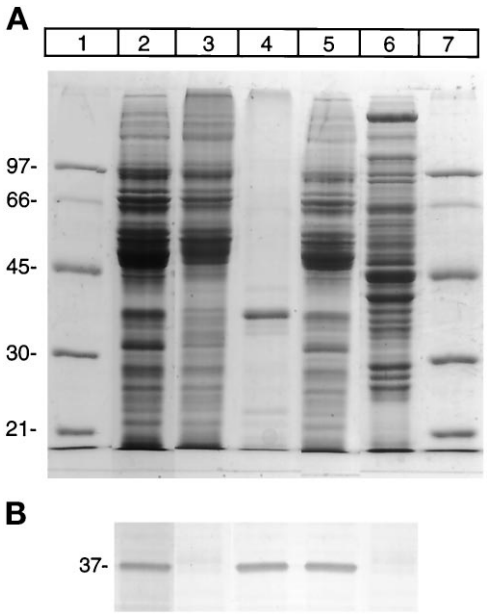

Figure 1. Identification of desaturase in liver microsomes from rats that have undergone dietary alteration. (A) SDS-PAGE, Coomassie blue-stained gel. Lanes 1 and 7 denote molecular weight standards with the mass marked on the left of the panel. Lane 2, pyrophosphate-washed and lane 5, high-salt–washed microsomes from desaturase induced animals. Lane 3 corresponds to microsomes from control animals; lane 4, purified desaturase; lane 6, postmicrosomal supernatant from desaturase-induced liver. (B) Immunoblot analysis of desaturase in the above fractions. Samples of identical amounts to those shown in Figure 1A were electrophoresed on SDS-PAGE, transferred onto polyvinylidene difluoride Immobilon-P membrane, and incubated with antibody against desaturase, followed by incubation with secondary antibody (anti-rabbit IgG-alkaline phosphatase). The bands were visualized using phosphatase substrate detection kit as described in MATERIALS AND METHODS.

Association of Desaturase with the Particulate Cellular Fractions

Differential centrifugation of the liver homogenate showed that desaturase is present in the subcellular fractions P-1 and P-2 as well as microsomes. Desaturase activity measurements in the P-1 and P-2 fractions indicated the presence of substantial amounts of the enzyme (Table 1). The total activity of desaturase in these fractions was estimated to be about 25% that of the microsomes. Figure 2 shows the immunoblot analysis of the desaturase in the P-1 fraction. When subcellular fractionation was repeated on the P-1 and P-2

Table 1. Desaturase activities of hepatic subcellular fractions from animals induced for the enzyme

Fraction	Specific Activity (nmoles/min/mg)	%
Microsomes	2.5 to 2.8	100
P-1	0.3 to 0.5	12
P-2	0.2 to 0.8	15
Microsomes (high-salt washed)	2.5 to 2.8	100
Cytosol	ND[a]	
High-salt wash	ND[a]	

Cell fractionation and desaturase assay were performed as described in MATERIALS AND METHODS.

[a] ND, not detected.

fractions, the desaturase was again present in both the low- and the high-speed sedimenting pellet, but was absent in all of the high-speed supernatants. Partitioning of proteins in Triton X-114 phase has been used to resolve membrane proteins from the soluble proteins (Barrett, 1981). Triton X-114 is a nonionic detergent that forms small micelles at low temperature and large micelles above the cloud point temperature. Phase separation of P-2 fraction proteins in Triton X-114 is shown in Figure 2, lanes 5 and 6. Most of the desaturase partitioned in the detergent phase, although some was present in the aqueous supernatant phase.

Structure of Desaturase in the P-1 and P-2 Material

To define the relationship between the microsomal and the desaturase present in the nuclear fraction, isolation, SDS-PAGE, and the N-terminal sequence

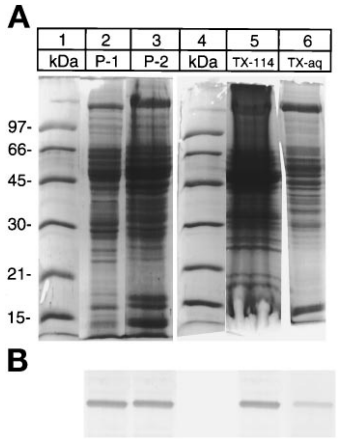

Figure 2. Association of desaturase with the components of subcellular fractions. Lane 2, P-1 (800 × *g*); lane 3, P-2 (10,000 × *g*) fractions. Lanes 5 and 6, Triton X-114 extract of P-2 fraction: lane 5, detergent-rich phase; lane 6, detergent-depleted phase. (A) Coomassie Blue-stained gel. (B) The above gel immunoblotted with antidesaturase antibody as in Figure 1B.

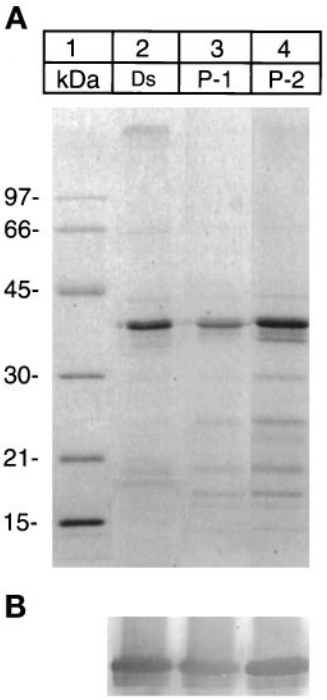

Figure 3. SDS-PAGE of desaturase preparations isolated from microsomes, P-1, and P-2 fractions. (A) Coomassie Blue-stained gel: lane 2, desaturase (Ds) isolated from microsomes; lane 3, desaturase isolated from P-1 fraction; and lane 4, from P-2 fraction. (B) Immunoblot of the above gel with the antidesaturase antibody as in Figure 1B.

analysis of the two desaturase preparations were performed. The SDS-PAGE of the two preparations were indistinguishable (Figure 3). The N terminus of the enzyme from microsomes was blocked, but sequence analysis of the desaturase in the nuclear fractions P-1 and P-2 indicated the N terminus to be open and identical to residues 3–10 of the microsomal enzyme (Table 2).

Selective Degradation of Desaturase Occurs in Microsomes and in P-1, P-2 Fractions

Incubation of microsomes at 37°C led to the disappearance of the desaturase as evidenced by the Coo-

Table 2. N-terminal sequence analysis of desaturase in microsomes and fractions P-1/P-2

Cycle/ position	Expected from cDNA	Microsomal	Fraction P1/P2	
			Cycle	Residue (pmol)
1	Met	Blocked		
2	Pro			
3	Ala			
4	His		1 His	95
5	Met		2 Met	300
6	Leu		3 Leu	230
7	Gln		4 Gln	250
8	Glu		5 Glu	250
9	Ile		6 Ile	200
10	Ser		7 Ser	50
11	Ser		8 Ser	60

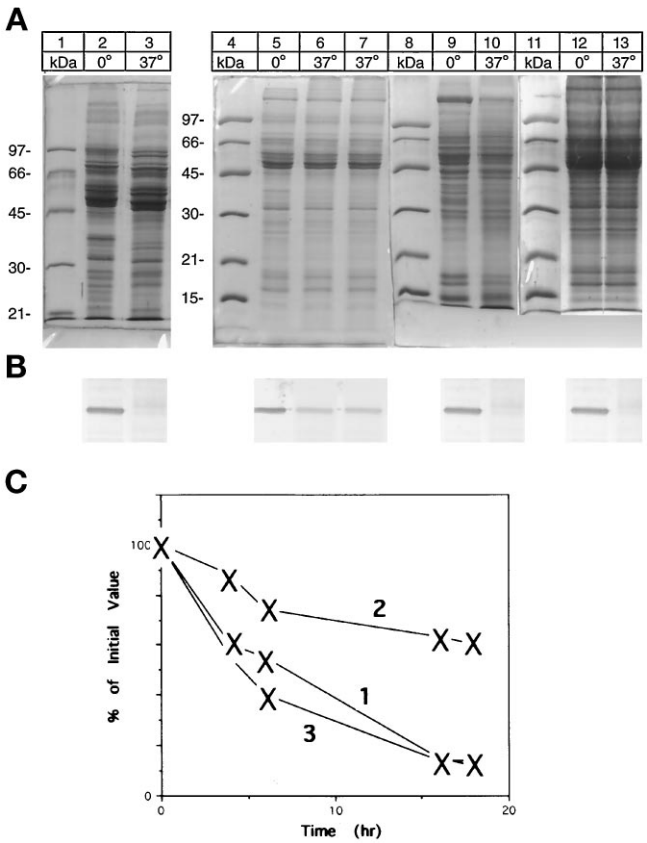

Figure 4. Selective degradation of desaturase in subcellular fractions. Aliquots of the various subcellular fractions (10–15 μg) were incubated overnight at 0 and 37°C in 50 μl of 50 mM Tris-acetate, pH 7.9 containing 50 mM KCl. Before SDS-PAGE, 75 μl of gel loading buffer were added, and a 10-μl aliquot was loaded to each lane of duplicate gels. (A) Coomassie Blue-stained gels; (B) Western Blot of the above gels with antidesaturase antibody. Lanes 2 and 3, pyrophosphate-washed microsomes; lanes 5 and 6, high-salt–washed microsomes; lane 7, high-salt–washed microsomes supplemented with ATP (2 mM) and Mg^{2+} (5 mM). Lanes 9 and 10, P-1 fraction incubated at 0 and 37°C; lanes 12 and 13, high-salt–washed microsomes supplemented with P-1 fraction and incubated at 0 and 37°C. (C) Estimated time course of the degradation of the desaturase. Aliquots of the various subcellular fractions were incubated at 37°C and control fractions at −20°C for the indicated time and interacted with desaturase antibody. Curve 1, desaturase-induced microsomes. Curve 2, high-salt–washed microsomes or high-salt–washed microsomes supplemented with ATP. Curve 3, high-salt–washed microsomes supplemented with P-1 fraction. The amount of desaturase was determined by densitometry and is expressed as a percentage of the amount present at the start of the experiment, which is set to 100%.

massie blue staining and by the immunoblot (Figure 4, lanes 2 and 3). Incubation of high-salt–washed microsomes at 37°C led to an incomplete degradation of the desaturase band upon the SDS-PAGE, confirmed on Western blot (Figure 4, lanes 5 and 6). A major pathway for the intracellular degradation of proteins is an ATP-dependent reaction (Hershko and Ciechanover, 1992). Addition of ATP and Mg^{2+} (5 mM) to the incubation reaction did not enhance the desaturase degradation in high-salt–washed microsomes (Figure 4, lane 7). The degradation of desaturase in the P-1 or P-2 fractions was complete without supplementation, but fractions solubilized with Triton X-114 failed to be degraded under the standard incubation conditions.

To further explore the incomplete desaturase degradation in the high-salt–washed microsomes, an aliquot of P-1 material was added to the salt-washed microsomal preparation before the incubation. As seen in Figure 4, lanes 12 and 13, when the quantity of desaturase in Figure 4, lane 5, was coincubated with the amount of P-1 fraction represented by lane 9, a complete desaturase degradation was observed. By comparison, the cytosolic fraction was unable to enhance the degradation in high-salt–washed microsomes, even at levels of 10 fold concentration (Figure 5, lanes 4 and 5). Addition of a 10-fold concentrated high-salt wash supernatant also failed to affect the desaturase degradation (Figure 5, lanes 6 and 7). Complete degradation of desaturase in the high-salt–washed microsomes could be restored by the addition of liposomes, cytochrome b_5, and its reductase, components essential for the desaturase reaction (Figure 5, lanes 9, 10, and 11). The time course of the degradation of the desaturase under various conditions is presented in Figures 4C and 5C. The time course of desaturase degradation in high-salt–washed microsomes supplemented with P-1 fraction is similar to that observed in intact microsomes (Figure 4C, curves 1 and 3). In contrast, in high-salt– washed microsomes only some 30–40% of the desaturase is degraded (Figure 4C, curve 2). Supplementation of the high-salt–washed microsomes with cytosol or the high-salt wash fraction does not increase the extent of the degradation, (Figure 5C, curve 1). Whereas addition of the components of the desaturase system to the high-salt–washed microsomes yields a degradation rate similar to that observed with the intact microsomes (Figure 5C, curve 2 and Figure 4C, curve 1).

Selective Degradation of Desaturase Is Not Inhibited by Lysosomal or Calpain Inhibitors

To test whether lysosomal enzymes are involved in the desaturase degradation, several lysosomal protease inhibitors were examined. No inhibition of desaturase degradation was observed with chlorquine, leupeptin, pepstatin, ALLM, or PMSF. Leupeptin and pepstatin inhibits hepatic cathepsin B and cathepsin D, respectively (Barrett, 1971). ALLM is a synthetic peptide inhibiting the activity of cathepsins B and L and calpains (Rock *et al.*, 1994). Figure 6 shows the lack of inhibition of microsomal desaturase degradation by leupeptin and pepstatin. A similar lack of inhibition of desaturase degradation was also observed in our unpublished results when the high-salt–washed micro-

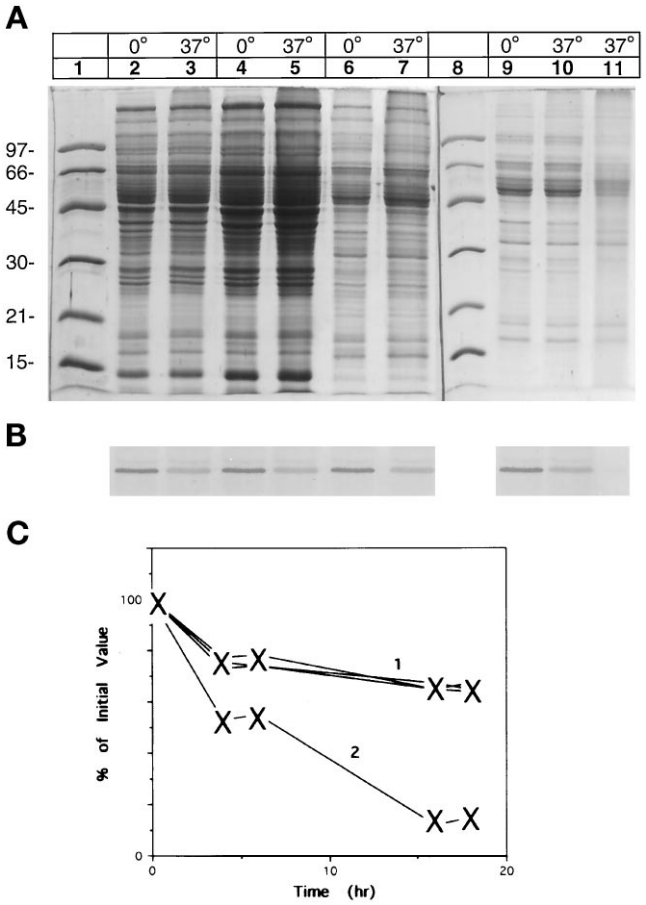

Figure 5. Proteolysis of desaturase in the high-salt–washed microsomes cannot be reconstituted by the addition of cytosol or high-salt wash supernatant but can be restored by supplementation with lipids and cytochrome b_5 reductase. Lanes 2 and 3, high-salt–washed microsomes (5 μg) were supplemented with an aliquot of cytosol (10 μg). Lanes 4 and 5, high-salt–washed microsomes supplemented with an aliquot of concentrated cytosol (20 μg) or concentrated high-salt wash (7 μg), lanes 6 and 7. Lane 9, high-salt–washed microsomes; lane 10, high-salt–washed microsomes with added lipids; lane 11, high-salt–washed microsomes with added lipids and cytochrome b_5 reductase. Samples of the incubation mixtures were run on duplicate gels. (A) Coomassie Blue-stained gel. (B) Immunoblot of the above gel with antidesaturase antibody. (C) Estimated time course of the degradation of the desaturase. Aliquots of the various fractions were incubated and quantitated as described in the legend of Figure 4C. Curve 1, high-salt–washed microsomes supplemented with cytosol, or aliquot containing high-salt wash. Curve 2, high-salt–washed microsomes with added components essential for the reconstitution of the desaturase reaction. The amount of desaturase was determined by densitometry and is expressed as a percentage of the amount present at the start of the experiment, which is set to 100%.

somes were incubated with the above inhibitors. To explore whether lysosomal proteases were responsible for desaturase degradation in the nuclear associated membranes, the above inhibitors were incubated with P-1 or P-2 fraction. As seen in Figure 7, pepstatin (180

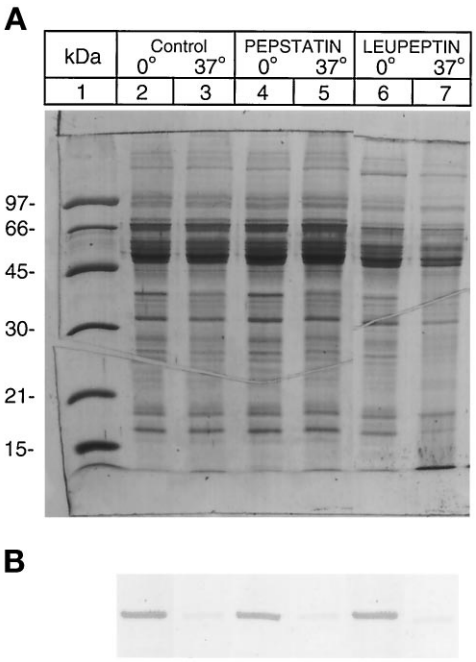

Figure 6. Microsomal degradation of desaturase is not inhibited by pepstatin or leupeptin. Experimental conditions were as in Figure 4 except that pepstatin (240 μM) was present in reaction mixtures represented by lanes 4 and 5 and leupeptin (400 μM) by lanes 6 and 7. (A) Coomassie Blue-stained gel. (B) Immunoblot of the above gel with antidesaturase antibody as in Figure 1B.

μg/ml), leupeptin (200 μg/ml), ALLM (170 μg/ml), or PMSF (1 mM) also failed to block the degradation of desaturase in the P-2 fraction.

Lactacystin Does Not Block the Degradation of Desaturase in Microsomes or P-1, P-2 Fractions

Currently, the degradation of rapid turnover of cellular proteins is thought to involve the proteosome system, which is the major proteolytic activity in both the cytosol and the nucleus (Tanaka *et al*., 1986). Very recently, lactacystin, a *Streptomyces* metabolite, was demonstrated to be a highly specific inhibitor of multiple proteosome activities (Fenteany *et al*, 1995). In view of the profound effect of lactacystin on protein processing, the influence of this reagent on the desaturase degradation in microsomes and the nuclear fractions was examined. The results of such an experiment on the desaturase degradation in microsomes and P-2 fractions are shown in Figure 8, lanes 2, 4, 6, 7, and 8. Clearly, lactacystin caused no inhibition of the desaturase degradation.

DISCUSSION

It is now recognized that the endoplasmic reticulum is an important site for intracellular protein breakdown

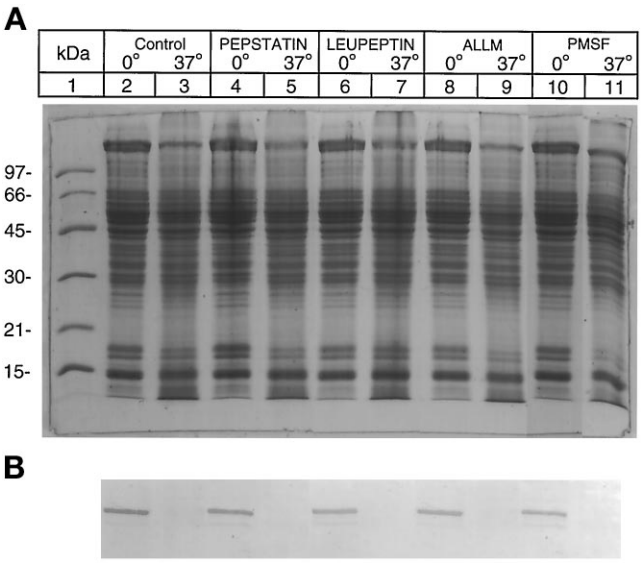

Figure 7. Degradation of desaturase in P-2 fraction is not blocked by inhibitors of lysosomal enzymes or serine proteases. Experimental conditions were as in Figure 4, except for supplementation with the protease inhibitors. Lanes 4 and 5 represent incubation mixtures containing 240 μM pepstatin; lanes 5 and 7, 400 μM leupeptin; lanes 8 and 9, 420 μM ALLM; and lanes 10 and 11, supplemented with 1 mM PMSF. Control incubation mixtures contained 1.5% dimethylsulfoxide. (A) Coomassie Blue-stained gel. (B) Immunoblot of the above gel with desaturase antibody.

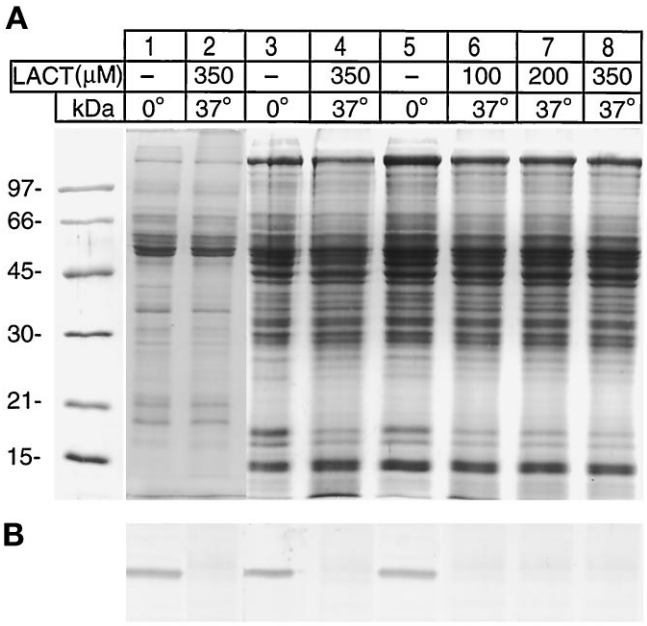

Figure 8. Proteosome inhibitor lactacystin does not inhibit desaturase degradation. Experimental conditions were as in Figure 4 except for addition of lactacystin in the indicated sample lanes. The incubation temperature and the final concentration of lactacystin in the samples are shown at the top of the panel. Lanes 1 and 2 represent incubation mixtures containing 10–15 μg of microsomes. Lanes 3 and 4, microsomes and P-2 fraction; lanes 5–8, samples containing 10–15 μg of P-2 fraction. Before the SDS-PAGE, 125 μl of the loading buffer containing SDS, β-mercaptoethanol, glycerol, and tracking dye were added, and 10-μl samples were run on duplicate gels. (A) Coomassie Blue-stained gel. (B) Immunoblot of the above gel with desaturase antibody.

(for review, see Klausner and Sitia, 1990; Bonifacino and Klausner, 1994). Short-lived proteins, unassembled components of oligomeric complexes such as the T-cell receptor subunits (Wileman *et al*., 1993), and asialoglycoprotein receptors (Wikstrom and Lodish, 1993) are degraded in the endoplasmic reticulum. Apolipoprotein B-like proteins (Furukawa *et al*., 1992) and the protein product of the cystic fibrosis-associated gene (CFTR) are degraded in the endoplasmic reticulum (Jensen *et al*., 1995; Ward *et al*., 1995). Although the list of proteins degraded in this organelle tends to grow, the proteolytic machinery of endoplasmic reticulum is poorly understood, and the responsible enzymes have not been identified.

Previously, we describe the isolation, cDNA sequence, and bacterial expression of rat liver desaturase (Thiede *et al*., 1986; Strittmatter *et al*., 1988). The results described in the current report provide a glimpse of the proteolytic processing of this short-lived membrane protein. As seen in Figure 1, fasting and refeeding a fat-free, high-carbohydrate diet induced high levels of desaturase in the liver microsomal membranes. When the dietary regimen was stopped, desaturase levels rapidly decreased to levels not detectable by immunoblots (Figure 1, lane 3). An in vivo half-life of about 2 h has been estimated for the desaturase (Oshino and Sato, 1972).

Surprisingly, a considerable amount of desaturase was present in the subcellular fractions P-1 and P-2, in addition to being present in the microsomes (Table 1). The postmicrosomal supernatant or the high-salt wash of the microsomes did not contain desaturase (Figure 1, lane 6). While the P-1 fraction consisted essentially of nuclear material, P-2 subcellular fraction was heterogeneous organelle preparation. The outer nuclear membrane of hepatocytes is continuous with the endoplasmic reticulum, implying that some of the desaturase-containing membranes may traffick to the nuclear membrane. The amount of desaturase associated with P-1 and P-2 subcellular material was significant (Table 1) and unanticipated.

To determine the relationship between the microsomal and putative nuclear enzyme present in the P-1, P-2 subcellular fractions, desaturase from the latter fractions was purified for sequence analysis. N-terminal sequence analysis of the P-1, P-2 preparations showed an absence of three residues present in the microsomal protein, whereas the microsomal enzyme has a blocked N terminus (Table 2). Thus, the desaturase present in the nuclear fraction does not represent

microsomal enzyme contamination, but appears to represent a specifically processed form of the enzyme. The relationship between the N-terminal processing and the nuclear localization remains to be elucidated, as discussed below.

Incubation of the microsomal membranes at 37°C resulted in the complete degradation of the desaturase, whereas in microsomes washed with a high-salt buffer, the degradation was incomplete as seen in Figure 4. The complete desaturase degradation, however, could not be restored by the addition of concentrated cytosol or high-salt wash fractions to the microsomes. Complete degradation of desaturase in the high-salt–washed microsomes could be restored by the addition of lipids, cytochrome b_5 and its reductase, which constitutes functional desaturase activity. In these experiments, omission of lipid or any of the protein components limited proteolysis. It appears that in high-salt–washed microsomes, only 30–40% of the desaturase is degraded (Figure 4C). Although the possible effects of the salt wash on the desaturase degradation could be the result of many factors, one explanation may involve the formation of high-salt wash–induced conformations in the desaturase population that are resistant to the protease action. The data in Figure 5C suggest that supplementation of salt-washed microsomes with the lipid, cytochrome b5 reductase, and cytochrome b5 renders the resistant form of the desaturase to further proteolysis. The formation of insoluble desaturase aggregates have been observed during centrifugation on glycerol gradient detergent-solubilized microsomal preparations in the presence of high salt. Some of these forms retain enzymatic activity whereas others do not. Notwithstanding, the degradation reconstitution experiments imply that the procedure used to reconstitute an enzymatically active desaturase system may also yield to protein conformations that are susceptible to the proteolysis of the enzyme.

Desaturase in P-1 or P-2 fractions was degraded completely. Premixing of the salt-washed microsomes with the P-1, P-2 fraction also resulted in a complete degradation of the desaturase. Desaturase antigen bands of lower molecular mass than the desaturase could not be detected in the degradation mixtures, although the desaturase antibody can recognize the bacterial synthesis product lacking some 30 residues from the N terminus, corresponding to a decrease of 3000–5000 Da (Strittmatter *et al*., 1988).

Hepatic lysosomal or endosomal proteases or their precursors are ubiquitous enzymes, and their presence in microsomes and in P-1 and P-2 fractions would not be surprising, since proteases such as the procathepsin B and L may exist in the microsomal membranes as latent precursors. To determine whether lysosomal proteases are involved in the desaturase degradation, several types of protease inhibitors were examined. Leupeptin and pepstatin, inhibitors of lysosomal and endosomal proteases, had no effect on the microsomal or the P-1, P-2 desaturase degradation (Figures 6 and 7). The cysteine protease inhibitor ALLM and the serine protease inhibitor PSMF were also ineffective in blocking the desaturase degradation (Figure 7). ALLM has been shown to inhibit the regulated degradation of microsomal HMG-CoA reductase (Inoue *et al*., 1991), and a serine protease has been implicated in the rapid degradation of unassembled Ig light chains in endoplasmic reticulum (Gardner *et al*., 1993). One nonlysosomal pathway present in the cytoplasm and nuclear components that mediates rapid elimination of proteins is the proteosome pathway. Multiple types of evidence suggest that the proteosome plays a key role in the processing of antigens for the major histocompatibility complex class I presentation (Chiechanover, 1994) and is involved in generating the active forms of molecules such as the production of the 50-kDa subunit of the transcription factor NF-κB from the 105-kDa precursor (Palombella *et al*., 1994). The proteosome is also thought to be responsible for the degradation of the HMG-CoA reductase (McGee *et al*., 1996) and of the cystic fibrosis gene product (CFTR) in the endoplasmic reticulum (Rock *et al*., 1994; Ward *et al*., 1995). The proteosome is a 26S (2000-kDa) complex, containing the 20S proteosome as a key proteolytic component (Rechsteiner *et al*., 1993; Jentsch and Schlenker, 1995; Lowe *et al*., 1995). The 20S (700 kDa) complex consists of seven different α-subunits and seven unrelated β-subunits with masses ranging from 24 to 32 kDa comprising about 1% of the protein in mammalian cells (Jentsch and Schlenker, 1995). None of the individual subunits of the proteosome have proteolytic activity or show relationship to any known proteases. Recently, a highly specific, irreversible inhibitor of the proteosome, a *Streptomyces* metabolite–lactacystin has been identified (Fenteany *et al*., 1995). Lactacystin modifies covalently the highly conserved N-terminal threonine of the mammalian proteosome subunit X, a close homologue of the LMP7 proteosome subunit encoded by the major histocompatibility complex (Fenteany *et al*., 1995). Lactacystin has not been found to inhibit any other known protease (Fenteany *et al*., 1995). In view of such a remarkable housekeeping proteolytic function of the proteosome, it was of interest to determine whether the proteosome is involved in the degradation of desaturase. Lactacystin had no effect on the microsomal desaturase degradation (Figure 8). The experiment of Figure 8 also shows that lactacystin (100–350 μM) also failed to inhibit the desaturase degradation in the P-1 and P-2 subcellular fractions.

The studies reported here show that degradation of desaturase occurs in several subcellular fractions isolated by differential centrifugation. The degradation of desaturase, however, was insensitive to the lysosomal and proteosome inhibitors. If the lysosomal proteases or proteosome do not play a significant role in the

desaturase degradation, what alternatives do we have to explain the desaturase degradation? Proteolytic activities such as the ER-60 protease have been detected in detergent-solubilized microsomal preparations (Otsu *et al*., 1995). The proteolytic activity of ER-60, however, is inhibited by leupeptin and ALLM (Otsu *et al*., 1995).

The observation that desaturase is present and readily degraded in subcellular fractions other than the microsomes implies that degradation of native desaturase may also involve targeting of the enzyme to compartments containing specific proteolytic machinery, which constitute a sorting pathway or the reverse process of protein targeting to the membranes. Of interest is that the amino acid sequence of desaturase has two segments that contain a potential nuclear localization sequence (NLS). In residues 33–36, Lys-Met-Lys-Lys and Arg-Lys-Lys-Val-Ser-Lys, residues 335–340 constitute potential consensus sequences for the import of proteins to the nucleus. Import of proteins to the nuclear pore complex is specified by short stretches of amino acids known as the NLSs (see review in Melchior and Gerace, 1995; Gorlich and Mattaj, 1996). Site-directed mutagenesis of desaturase in the two putative NLS segments should clarify the significance of this finding. Are posttranslation modifications involved in this process? Structure analysis of desaturase in the P-1 and P-2 fractions showed that it lacked three residues at the N terminus (Figure 2 and Table 2). The cDNA sequence predicts a Met-Pro-Ala sequence at the N terminus of the microsomal desaturase (Strittmatter *et al*., 1988). The N terminus of the enzyme present in microsomes is blocked, and the nature of the blocking group remains to be determined. The N-terminal–blocking groups of the two upstream essential components of the desaturase pathway, cytochrome b_5 and its reductase, are an acetyl and myristoyl residue, respectively (Ozols *et al*., 1984; Ozols, 1989). The presence of a myristoylated residue at the N terminus of desaturase is unlikely because of the absence of a consensus Gly residue in the proximity of its N terminus. The removal of an N-blocked terminus and ProAla segment from the native desaturase is of interest because we are not aware of any reports on hepatic aminopeptidases capable of cleaving residues from N-acetylated proteins. The hepatic acylpeptide hydrolase (E.C.3.4.19.1) acts only on N-acetylated peptides that are shorter than 10 to 15 residues (Tsunasawa *et al*., 1983). Cathepsins that function as aminopeptidases act only on proteins with a free N terminus.

The complete degradation of desaturase in microsomes can be inhibited by a high-salt wash of the microsomes. This inhibition cannot be restored by the addition of the proteins present in the high-salt wash fraction. The partial degradation of desaturase in high-salt–washed microsomes, however, could be restored by the addition of the components essential for the in vitro catalytic activity of the desaturase. This finding implies that desaturase degradation system may necessitate a specific membrane protein assembly, similar to that observed in reconstitution of the desaturase catalytic activity in vitro. In summary, degradation of the microsomal membrane desaturase was demonstrated in this study. This specific degradation may involve several degradation pathways including removal of the N-terminal residues and the targeting of the modified desaturase to cellular components such as the nuclear material. The possibility that a short-lived protein can be degraded according to different pathways, however, would be unprecedented. Whether the removal of the N-terminal residues from the desaturase results in the formation of a specific determinant that acts as a mediator for the observed trafficking remains to be investigated.

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REFERENCES

Barrett, A.J. (1981). Cystatin, the egg white inhibitor of cysteine proteinases. Methods Enzymol. *80*, 771–778.

Bonifacino, J.S., and Klausner, R.D. (1994). Degradation of proteins retained in the endoplasmic reticulum. In Cellular Proteolytic Systems. ed. A.J. Ciechanover and A.L. Schwartz, New York, Wiley-Liss.

Chiechanover, (1994). The ubiquitin-proteasome proteolytic pathway. Cell *79*, 13–21.

Fenteany, G., Standaert, R.F., Lane, W.S., Choi, S., Corey, E.J., and Schreiber, S.L. (1995). Inhibition of proteasome activities and subunit-specific amino-terminal threonine modification by lactacystin. Science *268*, 726–731.

Fleischer, S., and Kervina, M. (1974). Subcellular fractionation of rat liver. Methods Enzymol. *31*, 6–41.

Furukawa, S., Sakata, N., Ginsbergh, N., and Dixon, J.L. (1992). Studies of the sites of intracellular degradation of apolipoprotein B in Hep G2 cells. J. Biol. Chem. *267*, 22630–22638.

Gardner, A.M., Aviel, S., and Argon, Y. (1993). Rapid degradation of an unassembled immunoglobulin light chain is mediated by a serine protease and occurs in a pre-Golgi compartment. J. Biol. Chem. *268*, 25940–25947.

Gorlich, D., and Mattaj, I.W. (1996). Nucleocytoplasmic transport. Science *271*, 1513–1518.

Hardy, R.R. (1986). Purification and characterization of monoclonal antibodies. In: Handbook of Experimental Immunology, vol. 1: Immunochemistry, ed. D.M. Weir, Oxford, UK: Blackwell Scientific, 13.1–13.13.

Hershko, A., and Ciechanover, A. (1992). The ubiquitin system for protein degradation. Annu. Rev. Biochem. *61*, 761–807.

Inoue, S., and Simoni, R.D. (1992). 3-Hydroxy-3-methylglutaryl-coenzyme A reductase and T cell receptor alpha subunit are differ-

entially degraded in the endoplasmic reticulum. J. Biol. Chem. *267*, 9080–9086.

Inoue, S., Bar-Nun, S., Roitelman, J., and Simoni, R.D. (1991). Inhibition of degradation of 3-hydroxy-3-methylglutaryl-coenzyme A reductase in vivo by cysteine protease inhibitors. J. Biol. Chem. *266*, 13311–13317.

Jensen, T.J., Loo, M.A., Pind, S., Williams, D.B., Goldberg, A.L., and Riordan, J.R. (1995). Multiple proteolytic systems, including the proteasome, contribute to CFTR processing. Cell *83*, 129–135.

Jentsch, S., and Schlenker, S. (1995). Selective protein degradation: a journey's end within the proteasome. Cell *82*, 881–884.

Kaestner, K.H., Ntambi, J.M., Kelly, T.J., Jr., and Lane, M.D. (1989). Differentiation-induced gene expression in 3T3–L1 preadipocytes. A second differentially expressed gene encoding stearyl-CoA desaturase. J. Biol. Chem. *264*, 14755–14761.

Klausner, D.R., and Sitia, R. (1990). Protein degradation in the endoplasmic reticulum. Cell *61*, 611–614.

Lowe, J., Stock, D., Jap, B., Swickl, P., Baumeister, W., and Huber, R. (1995). Crystal structure of the 20S proteasome from the archaeon T. acidophilum at 3.4 A resolution. Science *268*, 533–539.

McGee, T.P., Cheng, H.H., Kumagai, H., Omura, S., and Simoni, R.D. (1996). Degradation of 3-hydroxy-3-methylglutaryl-CoA reductase in endoplasmic reticulum membranes is accelerated as a result of increased susceptibility to proteolysis. J. Biol. Chem. *271*, 25630–25638.

Melchior, F., and Gerace, L. (1995). Mechanisms of nuclear protein import. Curr. Biol. *7*, 310–318.

Oshino, N., and Sato, R. (1972). The dietary control of the microsomal stearyl CoA desaturation enzyme system in rat liver. Arch. Biochem. Biophys. *149*, 369–377.

Otsu, M., Urade, R., Kito, M., Omura, F., and Kikuchi, M. (1995). A possible role of ER-60 protease in the degradation of misfolded proteins in the endoplasmic reticulum. J. Biol. Chem. *270*, 14958–14961.

Ozols, J. (1974). Cytochrome b_5 from microsomal membranes of equine, bovine, and porcine livers. Isolation and properties of preparations containing the membranous segment. Biochemistry *13*, 426–434.

Ozols, J. (1989). Structure of cytochrome b_5 and its topology in the microsomal membrane. Biochim. Biophys. Acta *997*, 121–130.

Ozols, J. (1990). Subcellular fractionation of rat liver. Methods Enzymol. *182*, 225–235.

Ozols, J., Carr, S.A., and Strittmatter, P. (1984). Identification of the NH2-terminal blocking group of NADH-cytochrome b_5 reductase as myristic acid and the complete amino acid sequence of the membrane-binding domain. J. Biol. Chem. *259*, 13349–13354.

Palombella, V.J., Rando, A.L., Goldberg, A.L., and Maniatis, T. (1994). The ubiquitin-proteasome pathway is required for processing the NF-kappa B1 precursor protein and the activation of NF-kappa B. Cell *78*, 773–785.

Rechsteiner, M., Hoffmann, L., and Dubiel, W. (1993). The multicatalytic and 26 S proteases. J. Biol. Chem. *268*, 6065–6068.

Rock, L.K., Gramm, C., Rothstein, L., Clark, K., Stein, R., Dick, L., Hwang, D., and Goldberg, A.L. (1994). Inhibitors of the proteasome block the degradation of most cell proteins and the generation of peptides presented on MHC class I molecules. Cell *78*, 761–771.

Shanklin, J., and Somerville, C. (1991). Stearoyl-acyl-carrier-protein desaturase from higher plants is structurally unrelated to the animal and fungal homologs. Proc. Natl. Acad. Sci. USA *88*, 2510–2514.

Strittmatter, P., Kittler, J.M., Coghill, J.E., and Ozols, J. (1993). Interaction of non-myristoylated NADH-cytochrome b_5 reductase with cytochrome b_5-dimyristoylphosphatidylcholine vesicles. J. Biol. Chem. *268*, 23168–13171.

Strittmatter, P., Spatz, L., Corcoran, D., Rogers, M.J., Setlow, B., and Redline, R. (1974). Purification and properties of rat liver microsomal stearyl coenzyme A desaturase. Proc. Natl. Acad. Sci. USA *71*, 4565–4569.

Strittmatter, P., Thiede, M.A., Hackett, C.S., and Ozols, J. (1988). Bacterial synthesis of active rat stearyl-CoA desaturase lacking the 26-residue amino-terminal amino acid sequence. J. Biol. Chem. *263*, 2532–2535.

Stukey, J.E., McDonough, V.M., and Martin, C.E. (1990). The OLE1 gene of *Saccharomyces cerevisiae* encodes the delta 9 fatty acid desaturase and can be functionally replaced by the rat stearoyl-CoA desaturase gene. J. Biol. Chem. *265*, 20144–20149.

Tanaka, K., Ii, K., Ichihara, A., Waxman, L., and Goldberg, A.L. (1986). A high molecular weight protease in the cytosol of rat liver. I. Purification, enzymological properties, and tissue distribution. J. Biol. Chem. *261*, 15197–15203.

Thiede, M.A., Ozols, J., and Strittmatter, P. (1986). Construction and sequence of cDNA for rat liver stearyl coenzyme A desaturase. J. Biol. Chem. *261*, 13230–13235.

Thompson, G.A., Scherer, D.E., Foxall-Van Aken, S., Kenny, J.W., Young, H.L., Shintani, D.K., Kridl, J.C., and Knauf, V.C. (1991). Primary structures of the precursor and mature forms of stearoyl-acyl carrier protein desaturase from safflower embryos and requirement of ferredoxin for enzyme activity. Proc. Natl. Acad. Sci. USA *88*, 2578–2582.

Tiku, P.E., Gracey, A.Y., Macartney, A.I., Beynon, R.J., and Cossins, A.R. (1996). Cold-induced expression of delta 9-desaturase in carp by transcriptional and posttranscriptional mechanisms. Science *271*, 815–818.

Toubin, H., Staehelin, T., and Gordon, J. (1979). Electrophoretic transfer of proteins from polylacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA *76*, 4350–4354.

Tsunasawa, S., Imanaka, T., and Nakazawa, T. (1983). Apparent dipeptidyl peptidase activities of acylamino acid-releasing enzymes. J. Biochem. *93*, 1217–1220.

Ward, C.L., Omura, S., and Kopito, R.R. (1995). Degradation of CFTR by the ubiquitin-proteasome pathway. Cell *83*, 121–127.

Wikstrom, L., and Lodish, H.F. (1992). Endoplasmic reticulum degradation of a subunit of the asialoglycoprotein receptor in vitro. Vesicular transport from endoplasmic reticulum is unnecessary. J. Biol. Chem. *267*, 5–8.

Wikstrom, L., and Lodish, H.F. (1993). Unfolded H2b asialoglycoprotein receptor subunit polypeptides are selectively degraded within the endoplasmic reticulum. J. Biol. Chem. *268*, 14412–14416.

Wileman, T., Kane, L.P., Young, J., Carson, G.R., and Terhorst, C. (1993). Associations between subunit ectodomains promote T cell antigen receptor assembly and protect against degradation in the ER. J. Cell Biol. *122*, 67–78.