The N terminus of microsomal Δ9 stearoyl-CoA desaturase contains the sequence determinant for its rapid degradation

Hassan Mziaut, George Korza, and Juris Ozols*

Department of Biochemistry, University of Connecticut Health Center, Farmington, CT 06030-3305

Communicated by Ronald W. Estabrook, University of Texas Southwestern Medical Center, Dallas, TX, June 8, 2000 (received for review February 1, 2000)

Stearoyl-CoA desaturase (SCD) is a key regulator of membrane fluidity, turns over rapidly, and represents a model for selective degradation of short-lived proteins of the endoplasmic reticulum (ER). The mechanism whereby specific ER proteins are targeted for degradation in the midst of stable proteins coexisting in the same membrane is unknown. To investigate the intracellular fate of SCD and to identify the determinants involved in the rapid turnover of SCD, we created chimeras of SCD tagged at the C terminus with the green fluorescent protein (GFP). The fusion proteins were expressed in Chinese hamster ovary cells and exhibited an ER localization. Unlike native GFP, the SCD–GFP construct was unstable and had a half life of a few hours. Truncated fusion proteins consisting of residues 27–358 and 45–358 of SCD linked to the N terminus of GFP were stable. To investigate the general applicability of the N terminus of SCD in the destabilization of proteins, we fused residues 1–33 of SCD to the N terminus of GFP. The resulting chimera was extremely short lived. To investigate the effect of membrane sidedness on the fusion protein degradation, we attached a lumenal targeting signal to the N terminus of SCD 1–33-GFP. The construct was localized to the lumen of ER and was metabolically stable, indicating that SCD degradation signal functions on the cytosolic rather than the lumenal side of the ER. These results demonstrate that the N-terminal segment of some 30 residues of SCD constitutes a motif responsible for the rapid degradation of SCD.

Metabolic instability is a property of many regulatory proteins. The structural features that distinguish short-lived proteins from metabolically stable ones remain largely unknown. The lipid composition of cellular membranes is regulated to maintain a specific membrane fluidity and perhaps the rate of endocytosis. Stearoyl CoA desaturase (SCD) is the key enzyme in this process. SCD introduces a cis-double bond in the Δ9 position of saturated fatty acids in a reaction requiring NADH, the flavoprotein NADH-reductase, cytochrome *b*5, and oxygen, resulting in the production of monounsaturated fatty acyl derivatives (1). Rat liver SCD is a single polypeptide of 358 residues that spans three cellular compartments: cytosol, endoplasmic reticulum (ER) membrane, and the ER lumen (2).

In liver microsomes, SCD can be induced more than 50-fold by the administration of a fat-free high-carbohydrate diet. Abrupt termination of the dietary regimen causes a rapid decrease of SCD activity and the protein content to a very low level (1). The levels of cytochrome *b*5 and the NADH-cytochrome *b*5 reductase are not altered by the dietary regimen. In kidney, lung, spleen, and heart, induction of SCD by dietary manipulations occurs to a lesser extent. In contrast to hepatocytes, which express only one form of SCD (SCD1), adipose tissue and neural tissue express SCD1 and SCD2 isoforms (3). The SCD1 and SCD2 genes encode proteins of 358 amino acids with greater than 88% identity but differ markedly in their promoter sequence (3). The induction and rate of degradation of the desaturases in these tissues remain to be elucidated.

Studies propose that short-lived cytosolic proteins and some proteins of ER are degraded by the ubiquitin-proteasome pathway (4–6). Evidence has been presented that proteins present in the lumen of yeast ER may undergo ubiquitin-dependent retrotranslocation through membrane channels to the cytosol before degradation by the cytosolic proteasome (4). However, the key question in the degradation of short-lived ER proteins such as the SCD is how these proteins are targeted for degradation amongst the stable proteins coexisting in the same membrane.

We previously showed that degradation of SCD in liver microsomes is highly selective, with a half life of a few hours (7, 8). In the present study, we investigated the trafficking and degradation of SCD in living cells by using the reporter protein green fluorescent protein (GFP). We have identified the amino acid sequence of SCD that is responsible for its rapid degradation. Our data show that the removal of the N terminus of SCD leads to the stabilization of the enzyme. Furthermore, fusion of the N-terminal 33 residues of SCD to GFP, which is known to be a metabolically stable protein, leads to rapid degradation of the construct. Additionally, we demonstrate that the degradation of the GFP chimera does not occur when the N-terminal segment is translocated to the lumen of ER. This is the first demonstration that the N terminus of SCD functions as a signal to the cellular degradation machinery for the rapid degradation of the protein.

Materials and Methods

Materials. Chemical products were purchased from Sigma unless otherwise noted. Chinese hamster ovary (CHO) K1 cells were from American Type Culture Collection. Cell reagents and restriction and modification enzymes were from GIBCO. The site-directed mutagenesis system was from Promega. The GFPS65T protein in pEGFP-N1 vector and an anti-GFP mouse monoclonal antibody were from CLONTECH. Anti-SCD polyclonal antibody was raised in a rabbit by injecting keyhole limpet hemocyanin-conjugated 20-residue synthetic peptide corresponding to residues 338–358 of SCD antigen (8). Alkaline phosphatase-conjugated anti-mouse and anti-rabbit IgG were from Sigma.

Construction of Plasmids. All expressions of GFP chimeras were driven by the cytomegalovirus promoter–enhancer contained in the pEGFP-N1 vector. The cDNAs encoding desaturase of various lengths were amplified by the PCR by using as a template the 1,065-bp DNA fragment encoding for rat liver SCD lacking the first two amino acids, pDs 3–358 (9).

Abbreviations: SCD, stearoyl CoA desaturase; ER, endoplasmic reticulum; GFP, green fluorescent protein; CHO, Chinese hamster ovary; LTS, lumenal target signal; ODC, ornithine decarboxylase.

*To whom reprint requests should be addressed at: Department of Biochemistry, School of Medicine of the University of Connecticut Health Center, Farmington, CT 06030-3305. E-mail: ozols@sun.uchc.edu.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

The expression vector SCD 1–358-GFP encoding the full length of rat liver desaturase and five residues PPVAT of the multiple cloning site before the first codon of GFP was constructed as follows: An upstream primer P1 (5′-GAGCGAATTCCCACCATGCCGGCCCACATGCTCCAAGAGATCTCC) was used to introduce the first two residues MP lacking in the template, a Kozak sequence, and an *Eco*RI site. The downstream primer P2 (5′-GGCGACCGGTGGCCGGCTACTCTTGTGGCTCCCATC) contained an *Age*I site. The PCR fragment was digested with *Eco*RI–*Age*I and ligated into *Eco*RI–*Age*I-digested pEGFP-N1 vector.

The expression vectors SCD 27–358-GFP, SCD 34–358-GFP, and SCD 45–358-GFP encoding fusion proteins consisting of amino acids 27–358, 34–358, and 45–358 of desaturase, respectively, the five residues of the multiple cloning site, and the GFP were constructed as follows: The primers P3 (5′-GAGCGAATTCCCACCATGCTGCAGAATGGACGAGAGAAG), P4 GAGCGAATTCCCACCATGAAGAAGGTGCCCCTCTAT) and P5 (5′-GAGCGAATTCCCACCATGAGAGAAGATATCCACGACCCC) were used to introduce a new translation codon (for the fragment SCD 27–358), a Kozak sequence, and an *Eco*RI site at the 5′ end of the fragments SCD 27–358, SCD 34–358, and SCD 45–358, respectively. The downstream primer was P2. The PCR fragments were digested with *Eco*RI–*Age*I and ligated into *Eco*RI–*Age*I-digested pEGFP-N1 vector.

The expression vectors SCD 1–27-GFP and SCD 1–33-GFP encoding fusion proteins consisting of amino acids 1–27 and 1–33 of desaturase, respectively, the five residues of the multiple cloning site, and the GFP were constructed as follows: The sequences corresponding to amino acids 1–27 and 1–33 of desaturase were amplified by PCR with P1 as upstream primer and P5 (5′-GGCGACCGGTGGCAGGTTTCCGGAAGGAGG-3′) and P6 (5′-GGCGACCGGTGGCTTCTCTCGTCCATTCTGCAG-3′) as downstream primers for fragments 1–27 and 1–33, respectively. The PCR fragments were digested with *Eco*RI–*Age*I and ligated into *Eco*RI–*Age*I-digested pEGFP-N1 vector.

Lumenal target signal (LTS)-SCD 1–33-GFP and LTS (Δ16–21)-SCD 1–33-GFP encoding a fusion protein consisting of the rabbit esterase 3 LTS, (MGVKTVLLLIVGVLGAYYVYTPLPDNIEEPWRLL) and the LTS-lacking residues 16–21 (MGVKTVLLLIVGVLGPLPDNIEEPWRLL), respectively, the five amino acids of the multiple cloning site, and the GFP were constructed as follows: Primers P7 (5′-CTTCTGGATCCAATGCCGGCCCACATGCTC) that incorporated a *Bam*HI site at 5′ end and P6 that incorporated an *Age*I site at the 3′ end of the SCD 1–33 fragment. The SCD 1–33 fragment was cleaved with *Bam*HI–*Age*I and then ligated into *Bam*HI–*Age*I-digested LTS-GFP and LTS (Δ16–21)-GFP expression vectors (10). The construct SCD 1–33(K33/A)-GFP was made from SCD 1–33-GFP by using the Promega Gene Editor *in vitro* site-directed mutagenesis system. The integrity of the constructs was confirmed by DNA sequencing.

Cell Culture and Transient Transfection. CHO K1 cells were cultured in F-12 medium supplemented with 10% heat-inactivated FBS and 10 μg/ml gentamycin. The cells were seeded at approximately 50% confluency onto 75-cm^2 flasks at 37°C under 5% CO_2. After 24 h, when the cells became 60–90% confluent, they were transfected by using the Lipofectamine reagent according to the manufacturer's instructions. The cells were then incubated at 37°C under 5% CO_2 for 48 h.

Confocal Microscope Analysis. Twenty-four hours after transfection in 25-cm^2 flasks, CHO cells were plated out onto coverslips (18 × 18 mm) at about 60–70% confluency. After 24 h, the cells were cultured without (0 h) or with cycloheximide at final concentration of 10 μg/ml for 2 h and 4 h before paraformaldehyde fixation. The coverslips were mounted in Mowiol 4.88 and examined under a Zeiss LSM 410 confocal microscope.

Flow Cytometry. The cells were treated 48 h after transfection without (0 h) or with cycloheximide at final concentration of 10 μg/ml for 2 h and 4 h. The cells were then washed twice in PBS before being removed by scraping and pelleted in PBS, then resuspended in PBS with a final concentration of 10^6 cells/ml. A population of 10,000 cells was then analyzed for GFP fluorescence intensity by using a FACS caliber flow cytometer (Becton Dickinson). GFP was excited at 488 nm, and emission was detected by using a 500 long-pass edge filter.

Subcellular Fractionation and Immunoblot Analysis. The subcellular fractionation was performed as described previously (11). For immunoblot analysis, disruption of cells was done in SDS lysis buffer, and proteins were separated by SDS/PAGE and transferred onto Immobilon-P membranes. The proteins were detected either by using a monoclonal antibody against GFP or a polyclonal antibody against desaturase and visualized with alkaline phosphatase-conjugated secondary antibodies.

Results

To study the biosynthesis, trafficking, and degradation of SCD *in vivo*, we used GFP as a reporter protein. We constructed a cDNA fusion that joins SCD to GFP. This SCD-GFP construct was transiently expressed in CHO cells. Forty-eight hours after transfection, the cells were treated with 10 μg/ml of cycloheximide for 0, 2, and 4 h, and the change in fluorescence intensity of the transfected cells was examined by fluorescence microscopy. The fluorescence intensity of the fusion protein in the cells rapidly declined as cycloheximide treatment was extended, indicating that the fusion protein is unstable (Fig. 1*A*). In contrast, no significant change in the fluorescence intensity in the GFP-transfected cells (Fig. 1*A*) was observed. To determine the half life of the SCD fusion protein, we used flow cytometry to measure the change in fluorescence of cells expressing the SCD-GFP construct. As seen in Fig. 1*B*, after 4-h treatment, approximately half of the cells had lost their fluorescence. Cells transfected with GFP alone did not exhibit apparent fluorescence change after the cycloheximide treatment. Next, we constructed SCD-GFP mutants lacking some 40 residues from the N terminus of SCD (construct SCD 45–358-GFP). Surprisingly, mutants with 44 N-terminal residues deleted were found to be stable (Fig. 2*A*). Fusion proteins lacking the 26 N-terminal residues were also stable but to a lesser extent (Fig. 2*A*). Flow cytometric analysis was used to confirm the degradation rates of the N-deleted fusion proteins more accurately. As seen in Fig. 2*B*, the fluorescence intensity of the cells transfected with truncated SCD-GFP derivatives after cycloheximide treatment was greater than that of cells transfected with intact SCD-GFP. To determine whether the N terminus of SCD can destabilize GFP, we synthesized SCD 1–33-GFP construct. On examination of cells expressing SCD 1–33-GFP, we found that the degradation of this mutant was the most rapid amongst all of the other SCD-GFP fusion proteins (Fig. 3).

To examine the correlation between the half lives of the SCD-GFP constructs and their fluorescence, GFP- and SCD-GFP-transfected cells were used for flow cytometry and also for immunoblot analysis. Polyclonal antibody against the C terminus of SCD as well as monoclonal antibody against GFP were used as reagents. As shown in Fig. 4, both antibodies revealed that the predicted size for the SCD-GFP constructs coincided with the observed size. After cycloheximide treatment of the cells, immunoblot analysis of the cell lysates showed an absence of the SCD-GFP protein. The most pronounced destabilization was again observed with the SCD 1–358-GFP and SCD 1–33-GFP mutants (Fig. 4). The fusion proteins could be clearly identified

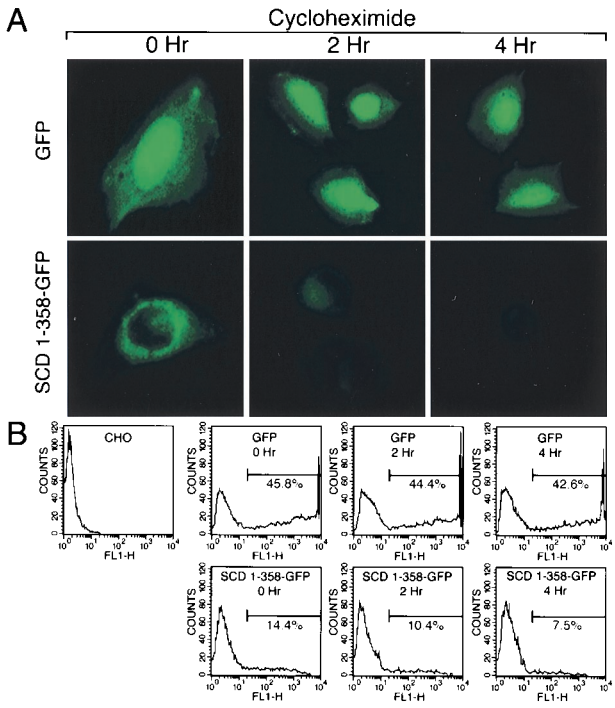

Fig. 1. Visualization of SCD degradation by expression of the SCD-GFP construct. (*A*) CHO cells were transfected with vector expressing the GFP alone or SCD 1–358-GFP construct. After 48 h, the transfected cells were treated with cycloheximide 10 μg/ml for 0, 2, and 4 h and their fluorescence determined. (*B*) Flow cytometric analysis of the fluorescence stabilities of GFP and SCD 1–358-GFP constructs. CHO cells were transfected with the constructs and after 48 h, cells were treated with cycloheximide for 0, 2, and 4 h. The treated cells were collected, and 10,000 cells were subjected to FACS analysis. The percentage of cells deemed fluorescent is indicated in the figure.

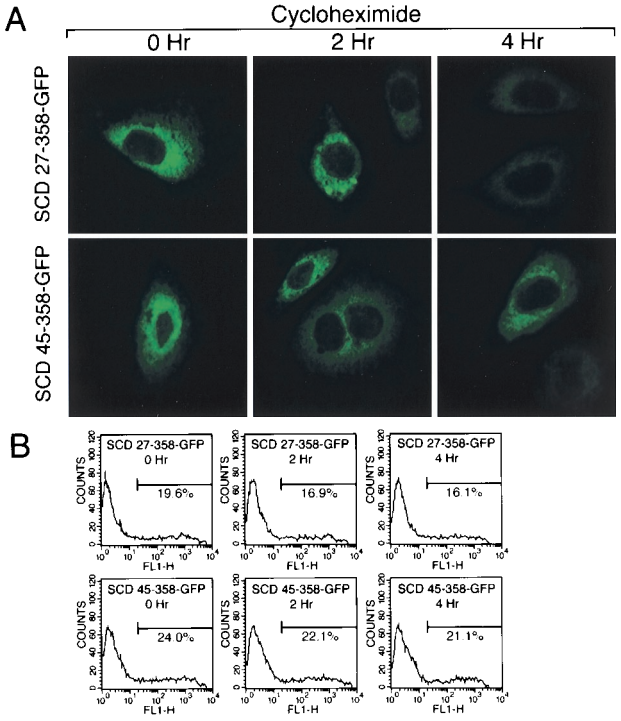

Fig. 2. Deletion of the N terminus of SCD inhibits the degradation of SCD-GFP constructs. (*A*) Fluorescence of SCD 27–358-GFP and SCD 45–358-GFP after cycloheximide treatment. The constructs localized to the ER remained fluorescent without relocalization to other cellular compartments. (*B*) A flow cytometric analysis of the fluorescence stabilities of SCD-GFP constructs with deleted N termini. After 48 h, the transfected cells were treated with cycloheximide for 0, 2, and 4 h. The treated cells were collected, and 10,000 cells were subjected to FACS analysis. The percentage of cells deemed fluorescent is indicated.

by Western blot analysis of cells expressing constructs SCD 27–358-GFP or SCD 45–358-GFP after cycloheximide treatment (Fig. 4). To further dissect the covalent structure requirements of the degradation signal, we deleted six residues from the C terminus of the signal. Surprisingly, cells expressing the SCD 1–27-GFP construct displayed a fusion protein that was more stable than the SCD 1–33-GFP construct (Figs. 3*A* and 4*C*). To confirm that the SCD 27–358-GFP construct does not actually represent the translation of the internal methionine at position 34 or that endoproteolysis was occurring, the chimera SCD 34–358-GFP was constructed. High-resolution comparative immunoblot analysis of these two constructs clearly showed their different mobilities and thus their appropriate initiation of translation (Fig. 4*D*). Furthermore, no evidence of endoproteolysis could be observed. Because truncation of residues 28–33 (Q N G R E K) stabilizes the chimera, we investigated the role of the single lysyl residue, which is conserved among the known SCD sequences of different species. Lysine-33 was changed to alanine, forming the construct SCD 1–33(K33/A)-GFP. This mutation clearly resulted in a fluorescently stable construct (Fig. 5 *A* and *B*). Interestingly, the immunoblot revealed not only a band corresponding to the predicted mass of this construct but also lower molecular weight bands reactive toward the GFP antibody, indicating that partial degradation was confined to the N-terminal segment and not to the GFP moiety (Fig. 5*C*). By comparison, the immunoblots of the other chimeras and of the control GFP expressed in cells indicated no significant partial degradation. It was also observed that the confocal fluorescent pattern of SCD 1–33(K33/A)-GFP was similar to GFP staining, whereas immunoblot analysis showed that the proteins were differently processed. To investigate whether this processing is because of differential compartmentation, subcellular fractionation was performed on cells expressing SCD1–33(K33/A)-GFP. This chimera was localized in both soluble and membrane fractions (Fig. 5*D*), contrary to native GFP, which is found only in the soluble fraction (10).

We previously reported that a specific amino acid sequence (LTS) permits targeting of single-spanning membrane proteins to the lumen of ER (10, 12). To determine whether translocation of the N terminus of SCD to the lumen of ER is also associated with the destabilization of the fusion protein, we attached LTS to N-terminal residues (1 to 33) of SCD. In the experiment of Fig. 6, CHO cells were transfected with LTS-SCD 1–33-GFP construct and examined by fluorescence microscopy before and after treatment with cycloheximide. The fluorescence intensity of the fusion protein in the cells was not altered as the cycloheximide treatment was extended, indicating that the fusion protein was stable. Immunoblot analysis of LTS-SCD 1–33-GFP before and after cycloheximide treatment confirmed that the fusion protein was stable when expressed on the lumenal side of the membrane (Fig. 6*C*). To distinguish the effect of nonspecific blocking of the N terminus from the effect of fusion of LTS to the degradation signal, we created chimera with altered LTS at the N terminus. Previous studies showed that construct LTS (Δ16–21)-GFP was localized to cytosol and failed to target the fusion protein to the lumen of ER (10). The appended construct, LTS (Δ16–21)-SCD1–33-GFP, was relatively unstable (Fig. 6).

Discussion

These studies represent analysis of how a particular ER membrane protein such as SCD is selected for rapid degradation

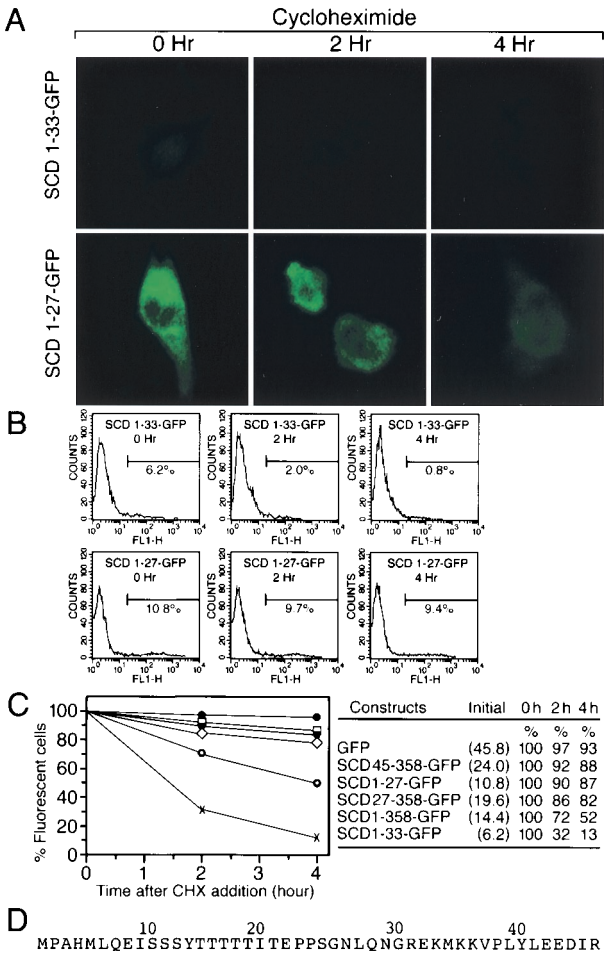

Fig. 3. The N terminus of SCD contains a signal that targets the construct for rapid degradation. (*A Upper*) Fluorescence of SCD 1–33-GFP before and after cycloheximide treatment. The construct rapidly disappears from the cytosol. *Lower* shows the increased stability of C-terminal deletion construct SCD 1–27-GFP. (*B*) A flow cytometric analysis of the fluorescence stabilities of SCD 1–33-GFP and SCD 1–27-GFP. The percentage of cells deemed fluorescent is indicated. (*C*) Comparison of the degradation rates of SCD-GFP constructs. The percentage of fluorescent cells after the indicated time of cycloheximide treatment was plotted for each of the expressed constructs. Solid circles, GFP; open squares, SCD 45–358-GFP; solid squares, SCD 1–27-GFP; diamonds, SCD 27–358-GFP; open circles, SCD 1–358-GFP; and crosses, SCD 1–33-GFP. The table shows fluorescent cells for each time point as a percentage of initial fluorescence. (*D*) Primary structure of N terminus of rat liver microsomal SCD.

amongst many stable proteins. Three major pathways of protein degradation are known to regulate many cellular proteins: lysosomal cathepsins, calcium-dependent cytosolic calpains, and the proteasome complex. Which system is responsible for the degradation of short-lived membrane proteins is presently controversial. The signals for the selection of membrane proteins to the various degradation pathways are poorly understood.

Recently, we reported that SCD is selectively degraded *in vitro* when rat liver microsomes induced for SCD are incubated at 37°C. The degradation of SCD *in vitro* was not inhibited by lysosomal or proteasome inhibitors (7). More recently, by using purified SCD and detergent-solubilized microsomes, we demonstrated that the microsomal protease that degrades SCD is highly selective and resists removal by procedures that solubilize peripheral and lumenal ER proteins (8). The protease system responsible for SCD degradation remains to be identified.

The present study was designed to address the trafficking of SCD expressed in CHO cells and to probe how SCD is specifically recognized by the protease system as a protein destined for rapid turnover. We used GFP as the reporter protein because of its intense fluorescence, nontoxicity, and ease of detection in

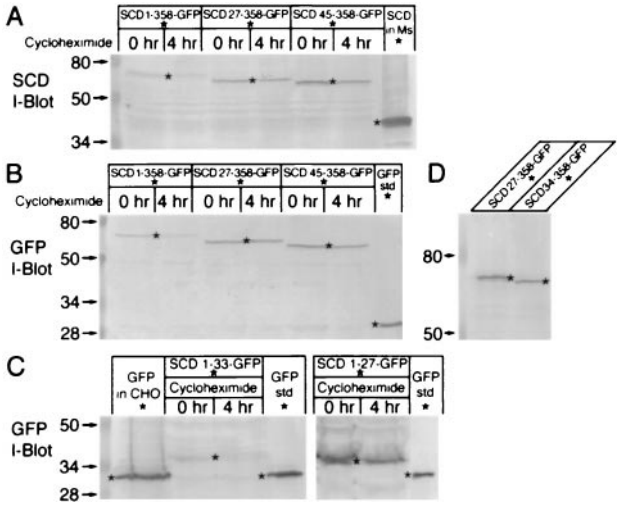

Fig. 4. Immunoblot analysis of the degradation of SCD-GFP constructs and appropriate translation initiation of SCD 27–358-GFP. GFP and derivatives of SCD-GFP were expressed in CHO cells and treated with cycloheximide. The cell lysates were subjected to SDS/PAGE and transferred onto Immobilon-P membrane. (*A*) Immunoblot of SCD-GFP fusion proteins with a polyclonal antibody against SCD. (*B*) Immunoblot with monoclonal antibody against GFP. (*C*) Immunoblot analysis of cells expressing GFP, SCD 1–33-GFP, and SCD 1–27-GFP with monoclonal antibody against GFP. (*D*) Resolution of SCD 27–358-GFP and SCD 34–358-GFP after 15% SDS/PAGE and immunoblot with monoclonal antibody against GFP.

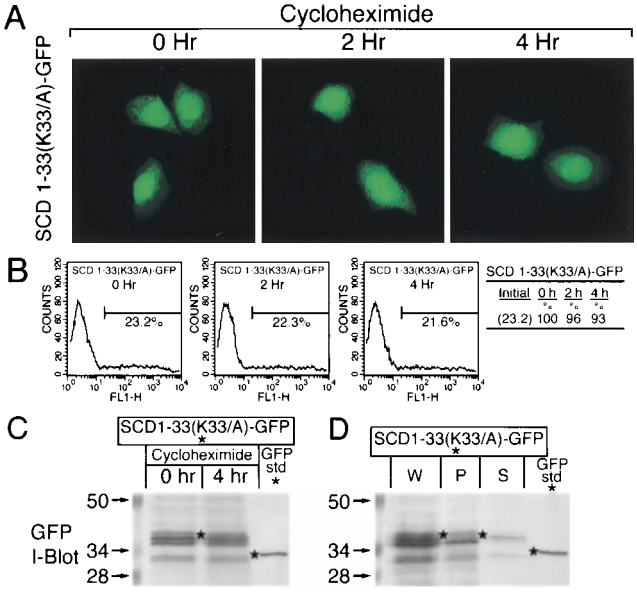

Fig. 5. Mutation of lysine to alanine at position 33 [SCD 1–33(K33/A)-GFP] leads to a more stable chimera. (*A*) Fluorescence of SCD 1–33(K33/A)-GFP before and after cycloheximide treatment. (*B*) A flow cytometric analysis of the fluorescence stability of the mutated construct. The percentage of cells deemed fluorescent is indicated. The table shows fluorescent cells for each time point as a percentage of initial fluorescence. (*C*) Immunoblot analysis of cells expressing SCD 1–33(K33/A)-GFP with monoclonal antibody against GFP after treatment with cycloheximide. (*D*) Immunoblot analysis of the mutant in cellular subfractions. The whole cell extract (W), pellet fraction (P), and soluble fraction (S) are shown.

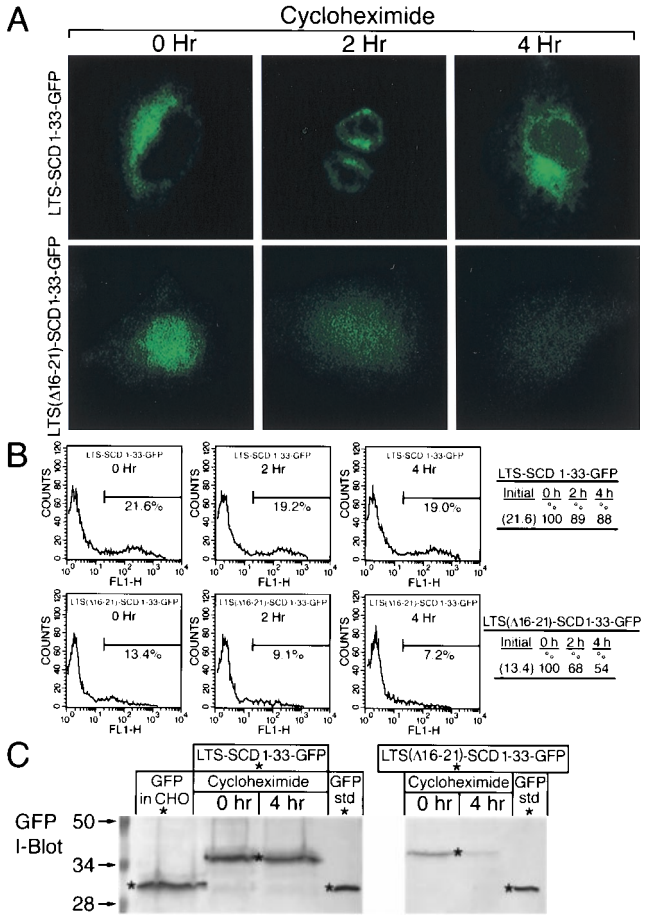

Fig. 6. The degradation signal has specific ER sidedness. The stability of SCD-GFP derivatives fused with the ER LTS. (*A*) Fluorescence of LTS-SCD 1–33-GFP and LTS(Δ16–21)-SCD 1–33-GFP after cycloheximide treatment. LTS denotes luminal targeting sequence, N-terminal sequence, residues 1–33 of microsomal esterase 3 (10, 12). (*B*) Flow cytometric analysis of the fluorescence stability of the above constructs. The percentage of cells deemed fluorescent is indicated. The table shows fluorescent cells for each time point as a percentage of initial fluorescence. (*C*) Immunoblot analysis of the above fusion proteins with monoclonal antibody against GFP.

cells (13). By fusing the N terminus of the reporter protein GFP to the C terminus of SCD, we were able to monitor the trafficking and degradation of SCD expressed in living cells. We used the CHO cells to express the SCD-GFP constructs because mutants of this cell line (M-1 and M-19) have been identified that require unsaturated fatty acids for growth, implying a decreased expression of SCD (14–16). Hence, cell lines M-1 and M-19 may serve as additional tools for a study to correlate SCD expression and degradation.

As seen in Fig. 1, fluorescence of the SCD-GFP fusion protein displayed reticular pattern staining typical of ER, consistent with previous findings that the SCD is localized to ER and the nuclear membrane (7). The SCD-GFP fusion protein, however, was unstable on expression in CHO cells. When protein synthesis was inhibited by the addition of cycloheximide to cells expressing SCD-GFP, the amount of SCD-GFP rapidly declined. Analysis by flow cytometry of CHO cells expressing the fusion protein indicated that the half life of SCD-GFP is approximately 4 h (Fig. 3*C*). This value is close to the observed half life of rat liver SCD (17). In contrast, cells expressing GFP alone showed no significant change in fluorescence intensity after cycloheximide treatment. Surprisingly, deletion of 26 N-terminal residues of SCD led to the stabilization of the GFP fusion protein (SCD 27–358-GFP). The rationale for deletion of the N terminus of SCD was that truncation of the first 26 residues, previously documented by us, affected neither enzyme activity nor membrane insertion (9).

Removal of an additional 18 N-terminal residues, as in construct SCD 45–358-GFP, led to further stabilization of the construct (Figs. 2 *A* and *B* and 3*C*). Because all three SCD-GFP constructs displayed the anticipated ER localization, it is unlikely that the observed stabilization in the N-deleted mutants was because of altered subcellular localization. Next, we tested whether the N terminus of SCD has the ability to induce GFP degradation *in vivo*. Unanticipated, the construct SCD 1–33-GFP was the most unstable of all the GFP fusion proteins examined (Fig. 3). Remarkably, truncation of residues Q N G R E K from the C terminus of the degradation signal, as in construct SCD 1–27-GFP, led to a more stable fusion protein than SCD 1–33-GFP (Fig. 3). Therefore, information downstream of residue 27 is required for the degradation signal to function. Except for the second and the two C-terminal residues of this region, the deleted sequence Q N G R E K is not conserved among rat, mouse, and human SCD sequences. The sequence of this deletion mutant included the elimination of the single lysyl residue. Before degradation, many proteins require ubiquitin modification, which is catalyzed by specific enzymes requiring lysyl residues for ubiquitin attachment (18, 19). The presence of the lysyl residue at position 33 raised the possibility of ubiquitin involvement in the degradation pathway. Site-directed mutagenesis of this lysine to an alanine yielded a chimera [SCD1–33(K33/A)-GFP] that did display a marked increase in fluorescence stability (Fig. 5 *A* and *B*). However, immunoblot analysis revealed the presence of more than one band with faster electrophoretic mobility, indicating that indeed partial degradation of this N-terminal moiety is occurring with the resultant intermediates having GFP as a stable final product. That this profile was not observed for the other chimeras would indicate that the degradation pathway for this mutant is unique compared with the other chimeras. Together with the subcellular fractionation finding of this mutant being in both membranous and soluble fractions, this result implies that the mutation is inhibitory to the degradation system or leads to altered subcellular localization of the mutant. Whether the ubiquitin pathway plays a role in the degradation of the N terminus of SCD remains to be determined. It is unlikely that residues Q N G R E K may function as a degradation signal alone, because the N G R E K sequence frequently occurs in many unrelated proteins. At the same time, it is clear that residues Q N G R E K are involved in recognition by the targeting machinery; however, additional residues are required for the proper presentation of the degradation signal, as shown by the stability of the SCD 27–358-GFP construct. To confirm that the fusion proteins were expressed as designed, the results of Figs. 1–3 were confirmed by immunoblot analysis with antibodies against both SCD and GFP. As presented in Fig. 4, both antibodies revealed that the observed M_r for the SCD-GFP (≈66-kDa) constructs coincided with the predicted mass of the fusion protein.

Clearly these results indicate the presence of a sequence motif of 33 residues in the N terminus of the SCD molecule that constitutes a signal for its rapid degradation. Although these findings represent a clear advancement in identifying and defining the signal for the SCD degradation, many aspects of this process remain to be elucidated. Presently we do not know how the N-terminal signal recruits proteases to the SCD. SCD mRNA is selectively translated on free cytoplasmic polysomes into a polypeptide which, on SDS/PAGE analysis, is identical to the mass of the isolated preparation and therefore does not have a signal peptide (2). Thus it is unlikely that the translocation channel is involved in the insertion of SCD, which would argue

against retrograde transport of SCD via the translocation channel to the cellular proteolysis machinery.

In an earlier study, we identified and characterized a sequence motif (LTS) that is necessary and sufficient to accomplish the trafficking of proteins across the ER with lumenal projection of the C-terminal domain (10, 12). Therefore, it was of interest to determine whether trafficking of the N terminus of SCD to the lumen of ER alters its rapid degradation. Expression of the construct LTS-SCD 1–33-GFP resulted in a stable fusion protein in the presence of cycloheximide (Fig. 6). These results imply the possible existence of a receptor that recognizes the signal on the cytosolic side of the membrane that does not extend into the lumen of ER. To exclude that addition of the LTS to the N terminus is not caused by extension of the N terminus with nonessential residues, we constructed a fusion protein with nonfunctional LTS. Because construct LTS (Δ16–21)-SCD 1–33-GFP was relatively unstable (Fig. 6), we conclude that the degradation signal is position independent.

The covalent structure of the N terminus of SCD is shown in Fig. 3*D*. The most remarkable feature of this sequence is an extended stretch of hydroxylated amino acids. The first 10 residues are comprised of uncharged residues, except for the single histidyl residue at position 4 and glutamyl residue at position 8. This segment is followed by some 10 residues of hydroxylated amino acids, terminating with glutamyl residue at position 21. Of interest is the presence of casein kinase II phosphorylation site T I T E at residues 18 to 21 (20). Phosphorylation and dephosphorylation have been implicated as signals for protein degradation. For example, the degradation of cyclin and IκBα requires an upstream phosphorylation event, and the phosphorylation of c-Jun targets degradation of c-Fos in AP-1 complexes (21, 22). Presently, we do not know whether phosphorylation of SCD occurs before its degradation.

The existence of a sequence motif that confers rapid degradation for a short-lived cytosolic protein has been described for ornithine decarboxylase (ODC), reviewed in ref. 23. The C terminus of mammalian ODC, residues 423 to 461, is essential for its rapid degradation, which is initiated by a protein denoted as antizyme (24). The active ODC is a homodimer and is in equilibrium with the monomer, which is enzymatically inactive. The antizyme is a 26-kDa protein that reversibly binds to the monomer of ODC. The binding of antizyme to the monomer of ODC targets the protein for degradation (24). The C-terminal region of ODC is essential for the antizyme-dependent degradation of ODC. Although there is no detectable sequence similarity between the N terminus of SCD and residues 423 to 449 of ODC, both sequences are enriched with Pro, Glu, Ser, and Thr, the PEST sequences (23). It has been suggested that sequences rich in PEST residues may function as degradation signals in proteins (25, 26). However, it has been shown that the degradation rate is not proportional to the number of PEST residues (27). Whether an antizyme protein equivalent exists for the SCD and the possible existence of the proteasome analog in the ER remain to be determined.

The work herein demonstrates that the N terminus of SCD constitutes a signal for its rapid degradation. Conceivably, the N terminus interacts with an unknown receptor on the cytosolic side of ER that targets the membrane protein degradation system to the SCD. The subject for future studies will involve the biochemical characterization of the peptide with respect to the secondary and tertiary structure, in addition to identification of the postulated recognition receptor for the SCD.

This work was supported by Grant R01 GM-26351 from The National Institutes of Health (to J.O.).

1. Strittmatter, P., Spatz, L., Corcoran, D., Rogers, M. J., Setlow, B. & Redline, R. (1974) *Proc. Natl. Acad. Sci. USA* **71**, 4565–4569.
2. Thiede, M. A., Ozols, J. & Strittmatter, P. (1986) *J. Biol. Chem.* **261,** 13230–13235.
3. Kaestner, K. H., Ntambi, J. M., Kelly, T. J. & Lane, M. D. (1989) *J. Biol. Chem.* **264,** 14755–14761.
4. Hilt, W. & Wolf, D. H. (1996) *Trends Biochem. Sci.* **21,** 96–102.
5. Hochstrasser, M. (1996) *Cell* **84,** 813–815.
6. Matlack, K. E. S., Mothes, W. & Rapoport, T. A. (1998) *Cell* **92,** 381–390.
7. Ozols, J. (1997) *Mol. Biol. Cell* **8,** 2281–2290.
8. Heinemann, F. S. & Ozols, J. (1998) *Mol. Biol. Cell* **9,** 3445–3453.
9. Strittmatter, P., Thiede, M. A., Hackett, C. S. & Ozols, J. (1988) *J. Biol. Chem.* **263,** 2532–2535.
10. Mziaut, H., Korza, G., Hand, A. R., Gerard, C. & Ozols, J. (1999) *J. Biol. Chem.* **274,** 14122–14129.
11. Mori, M., Morita, T., Ikeda, F., Amaya, Y., Tatibana, M. & Cohen, P. P. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 6056–6060.
12. Ozols, J. (1998) *Biochemistry* **37,** 10336–10344.
13. Chalfie, M., Euskirchen, G., Ward, W. W. & Prasher, D. C. (1994) *Science* **263,** 802–805.
14. Hasan, M. T. & Chang, T. Y. (1994) *Somatic Cell Mol. Genet.* **20,** 481–491.
15. Hasan, M. T., Chang, C. C. Y. & Chang, T. Y. (1994) *Somatic Cell Mol. Genet.* **20,** 183–194.
16. Brown, M. S. & Goldstein, J. L. (1997) *Cell* **89,** 331–340.
17. Omura, T., Siekevitz, P. & Palack, G. E. (1967) *J. Biol. Chem.* **242,** 2389–2396.
18. Goldberg, A. L. & Rock, K. L. (1992) *Nature (London)* **357,** 375–379.
19. Hershko, A. & Ciechanover, A. (1992) *Annu. Rev. Biochem.* **61,** 761–807.
20. Bairoch, A., Bucher, P. & Hofmann, K. (1997) *Nucleic Acids Res.* **25,** 217–221.
21. Tsurumi, C., Ishida, N., Tamura, T., Kakizuka, A., Nishida, E., Okumura, E., Kishimoto, T., Inagaki, M., Okazaki, K. & Sagata, N. (1995) *Mol. Cell Biol.* **15,** 5682–5687.
22. Papavassiliou, A. G., Treier, M., Chavrier, C. & Bohmann, D. (1992) *Science* **258,** 1941–1944.
23. Hayashi, S., Murakami, Y. & Matsufuji, S. (1996) *Trends Biochem. Sci.* **21,** 27–30.
24. Li, X. & Coffino, P. (1993) *Mol. Cell. Biol.* **13,** 2377–2383.
25. Rogers, S. R., Wells, R. & Rechsteiner, M. (1986) *Science* **234,** 364–368.
26. Ghoda, L., Van Daalen Wetters, T., Macrase, M., Ascherman, D. & Coffino, P. (1989) *Science* **243,** 1493–1495.
27. Li, X., Zhao, X., Fang, Y., Jiang, X., Duong, T., Fan, C., Huang, C. & Kain, S. (1998) *J. Biol. Chem.* **273,** 34970–34975.