Sterol regulatory element-binding proteins (SREBPs) are synthesized as membrane-bound precursors and processed to generate transcriptionally active forms. The active SREBPs translocate to the nucleus, induce the expression of responsive genes, and are degraded very rapidly. Treatment with proteasome inhibitors elevates the amount of the endogenous nuclear SREBPs, but not the precursors in HeLa cells. Nuclear forms of human SREBP-1a (amino acids 1–487) and SREBP-2 (amino acids 1–481), which are transiently expressed in stable Chinese hamster ovary cell lines (CHO-487 and -481), are also stabilized by proteasome inhibitors, suggesting that the nuclear SREBPs are likely to be substrates for the proteasome-dependent proteolysis. The stabilized nuclear SREBPs actively induce the expression of responsive genes including hydroxymethylglutaryl (HMG)-CoA synthase, fatty acid synthase, and the low density lipoprotein receptor. The rapid turnover of nuclear SREBP-1a is not affected by the intracellular sterol levels, and the half-life is estimated to be ~3 h. The nuclear SREBPs are found conjugated with a polyubiquitin chain. When this conjugation is inhibited by overexpression of mutant ubiquitin that is defective in polyubiquitination, the nuclear SREBPs are partly stabilized and induce the expression of the responsive gene, suggesting that the ubiquitin-conjugated SREBPs are substrates for the proteasome. Taken together, these results demonstrate that the ubiquitin-proteasome system degrades SREBPs and that this system controls the expression of SREBP-responsive genes.

Sterol regulatory element-binding proteins (SREBPs) are involved in the transcriptional regulation of genes encoding the low density lipoprotein (LDL) receptor as well as key enzymes of cholesterol and triglyceride biosynthesis (1). These transcription factors belong to a large class of transcription factors containing a basic helix-loop-helix leucine zipper (bHLH-Zip) motif. The SREBP family comprises three subtypes: SREBP-1a and SREBP-1c, which are generated by alternative splicing, and SREBP-2. Human SREBP-1a contains 1147 amino acids and is 47% identical to human SREBP-2, which contains 1141 amino acids (2, 3). SREBP-1c, which in most tissues is the form predominantly expressed, now appears to be a strong candidate as a general mediator of the action of insulin on the regulation of metabolism via effects on gene expression (4, 5). In contrast, SREBP-2, the expression of which is controlled by sterols, is thought to be deeply involved in the regulation of cholesterol metabolism (6).

Unlike other members of the bHLH-Zip transcription factors, the SREBPs are synthesized as membrane-bound precursors on the endoplasmic reticulum (ER) and activated by a two-step proteolytic process (7–9). These proteins contain an N-terminal transcriptional activation domain with a bHLH-Zip motif and a C-terminal regulatory domain separated by two transmembrane helices. The C-terminal regulatory domain associates with SREBP cleavage-activating protein, an ER membrane protein with eight membrane-spanning segments (10). The C-terminal domain of SREBP cleavage-activating protein contains five copies of the WD repeat, a protein/protein interaction motif, forming a complex with SREBPs that is required for SREBP cleavage by Site-1 protease (S1P), the active form of which is located in a post-ER compartment (11). The complex is localized in the ER as long as intracellular cholesterol levels are high, thereby stabilizing the SREBP precursors. In cells depleted of cholesterol, ER-derived membrane vesicles containing this complex move to the Golgi where a sequential cleavage of the SREBPs by S1P and Site-2 protease (S2P) occurs, releasing the active forms (12).

Once the active SREBPs are released into the cytoplasm, they are actively transported into the nucleus in an importin β-dependent manner (13). We have previously shown that the HLH-Zip domain contains a novel type of nuclear localization signal, which binds directly to importin β. When the active form of SREBP-1 or -2 is transiently expressed in certain cultured cells, the synthesized SREBP is not detected in the cytoplasmic fraction but rather in the nucleus, suggesting that the transport is very rapid and efficient. It has been further demonstrated that dimerization of the SREBPs through the leucine zipper motif is a prerequisite process for nuclear import mediated by importin β (14).

In general, most transcription factors are extremely unstable. For example, c-Jun, c-Fos, STAT1, p53, c-Myc, and E2F-1, are all unstable proteins, with in vivo half-lives ranging from a few minutes to a few hours (15–20). Recently, it has been
shown that the ubiquitin-proteasome pathway mediates degradation of various transcription factors, being a major route for intracellular degradation of both short-lived regulatory proteins and abnormal proteins (21, 22). Ubiquitin-mediated proteolysis is a process that covalently tags with polyubiquitin chains and signals for substrate destruction by the 26 S proteasome (23, 24). This is accomplished in three sequential steps and is catalyzed by the successive actions of E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme), and E3 (ubiquitin ligase). Ubiquitin, a 76-amino acid protein, is initially activated by E1, and then activated ubiquitin is transferred to E2, whose principal function is to shuttle ubiquitin to E3. E3 binds to the substrate and catalyzes the covalent tagging of ubiquitin to the substrate. Once the first ubiquitin molecule is transferred to a lysine residue within the substrate, a polyubiquitin chain is generated via a series of isopeptide bonds between a lysine residue of the tagging ubiquitin and the C-terminal glycine of the next added ubiquitin molecule. Repeated rounds of ubiquitination result in a polyubiquitinated substrate, which is degraded rapidly by the 26 S proteasome in an ATP-dependent reaction.

A number of studies have shown that nuclear export is required for the ubiquitin-dependent degradation of certain nuclear proteins including p27Kip1, cyclin D1, p53, and IκBα as well as the aryl hydrocarbon receptor (25–29). These results make it clear that ubiquitin-proteasome degradation occurs in the cytoplasm. On the other hand, certain other transcription factors such as Sp1 and MyoD are reported to be degraded by the nuclear ubiquitin-proteasome system (30, 31). The reason for these alternative pathways for the degradation of nuclear proteins remains to be elucidated.

In the present study, we demonstrate that the ubiquitin-proteasome system plays a crucial role in the control of cellular lipid metabolism.

Experimental Procedures

Materials—Lactacystin and E-64-d were purchased from Peptide Institute, Inc. (Osaka, Japan). Protease inhibitors and lipoprotein-deficient serum (LPDS) were from Sigma. N-Acetyl-Leu-Leu-norleucinal (ALLN) was purchased from Nakalai Tesque Inc. (Kyoto, Japan).

Construction of Plasmids—The pME-His vector was generated by inserting synthetic oligonucleotides encoding an MRGSH/H subsequence into the EcoRI and XhoI sites of pME-18S. To generate plasmids for expression of human SREBP1a-(1-487), an expression vector with a NcoI site of pOPRSV1/MCS (Stratagene) was inserted at the 3′ end of GFP. Similarly, an expression vector with an XhoI site of pOPRSVSREBP2, as described previously (32), CHO-Lac that expresses the lac repressor, CHO-487 for human SREBP-2-(1-481), and CHO-481 were cultured with Dulbecco’s modified Eagle’s medium containing 100 units/ml penicillin and 100 μg/ml streptomycin (medium A) plus 10% fetal bovine serum (FBS) at 37 °C under 5% CO2 atmosphere. On day 1 the medium was removed, and the cells were then washed with phosphate-buffered saline and refed with medium containing 5% LPDS supplemented either with 1 μg/ml 25-hydroxycholesterol plus 10 μg/ml cholesterol or a 50 μM concentration of an HMG-CoA reductase inhibitor (pravastatin) plus 50 μM sodium mevalonate. For the last 5 h the cells depleted of sterols were treated with various inhibitors, and nuclear extracts were prepared as described (8). To prepare the membrane fractions, HeLa cells were cultured with medium A plus 10% FBS for 2 days. For the last 5 h the cells were treated with 100 μM ALLN and/or 1 μg/ml 25-hydroxycholesterol, and membrane fractions were prepared as described (8).

CHO-481 cells expressing human SREBP-2-(1-481) with a Lac- Switch Inducible Mammalian Expression System (Stratagene) were used to overexpress CHO-Lac cells with an expression vector encoding pOPRSVSREBP, as described previously (32). CHO-Lac that expresses the lac repressor, CHO-487 for human SREBP-2-(1-481), and CHO-481 were cultured with Dulbecco’s modified Eagle’s medium/ Ham’s F12 medium containing 100 units/ml penicillin and 100 μg/ml streptomycin (medium B) plus 10% FBS. Monolayers of these stable cells (70–80% confluent) were refed with medium B supplemented with 10% fetal bovine serum, 1 μg/ml 25-hydroxycholesterol, 10 μg/ml cholesterol, and 1 or 3 mM IPTG. The cells were cultured for 19 h, and the indicated inhibitors were added for the last 5 h after which nuclear extracts were prepared.

To analyze the stability of SREBP1a, S2P-deficient CHO cells, M19 (33), were set up (1.5 × 105 cells/100-mm dish) with medium B containing 1% FBS on day 0. On day 1, the cells were transfected with 4.9 μg of pME-His-SREBP1a-(1-481) using LipofectAMINE (Invitrogen) according to the manufacturer’s protocol. After 24 h of incubation, the transfected cells were trypsinized and seeded in five 6-well plates. On day 3, protein synthesis was inhibited by 30 μg/ml cycloheximide (Sigma) at time 0, and the cell lysates were prepared at the time indicated to analyze the fate of SREBP-1a.

Construction of SREBP-1a-(2-487) and CHO-487 were set up with medium A supplemented with 10% FBS on day 0. On day 1, the cells were transfected with the DEAE-dextran method for a total 6 μg of plasmids including 4.8 μg of pME-His-Ub and 1.2 μg of either pSREBP1a-(1-487)/see Ref. 34, an expression vector with SREBP-1a-(1-487), or an empty vector, pME-18S. On day 3, the cells were treated with 50 μM ALLN for 12 h and then lysed with the binding buffer containing 10 mM Tris/HCl (pH 7.4), 0.1% Triton X-100, 0.1% SDS, and 2 mM EDTA. After centrifugation at 13,000 × g for 10 min, the supernatant was incubated with an anti-SREBP-1 antibody (RS005) at 4 °C for 3 h and incubated further with 20 μl of a 50% slurry of protein A-Sepharose beads (Sigma) at 4 °C for 2 h. The beads were collected, washed five times with binding buffer, and boiled for 5 min with SDS sample buffer, and the recovered samples were subjected to SDS-PAGE followed by Western blotting with an anti-RGS Hs, antibody or an anti-SREBP-1 antibody.

Effect of Overexpression of Mutant Ubiquitin—HeLa cells (5.0 × 105 cells/60-mm or 2.0 × 105 cells/25-mm dish) were set up on day 0 in medium A supplemented with 10% FBS. On day 1, the cells were transfected with various vectors using X-tremeGENE Q2 Transfection Reagent (Roche Molecular Biochemicals). After transfection, the cells were refed with medium A containing 5% LPDS, 50 μM pravastatin, and sodium mevalonate. After 48 h of culture, Northern blotting, Western blotting and luciferase assays were performed as described previously (6, 34). For Northern blotting analysis, the cells (60-mm dishes) were transfected with the indicated amount of pME-His-mutUb as described above. A 300-bp probe fragment from human HMG-CoA synthase, an 80-bp probe fragment from the human LDL receptor, and an 100-bp probe fragment from human fatty acid synthase were used as probes. For the luciferase assay, the cells (35-mm dishes) were transfected with 0.2 μg of pHMG-S, a reporter plasmid containing 0.5 kilobases of the human HMG-CoA synthase promoter (35), 0.01 μg of pRL-CMV (Promega), and the indicated amount of pME-His-mutUb. To determine the luciferase activities, a Dual-Luciferase Reporter System (Promega) was used.
Sterols ( ). Aliquots of the membrane fraction protein (90 ng/lane) were observed under a fluorescence microscope (Olympus).

During the last 5 h, the cells were incubated with 100 μM ALLN and/or 25-hydroxycholesterol (25-OH C). Aliquots of the membrane fraction protein (90 μg/lane) were subjected to SDS-PAGE and Western blotting using an anti-SREBP-1 or -2 antibody.

Intracellular Localization of GFP-SREBP1α—COS1 cells were transfected by a DEAE-dextran method with either pME-GFP-SREBP1α-(2–487) or pFPAl (36), an expression plasmid for GFP-Pap1 fusion protein. After 24 h of incubation, the cells were treated with 5 ng/ml LMB or LMB plus 100 μM ALLN for 2 h. The cellular distribution of GFP-fused proteins was observed under a fluorescence microscope (Olympus).

RESULTS

Proteasome Inhibitors Increase the Amount of the Nuclear Form of SREBPs—Previous studies demonstrated that the nuclear SREBPs are rapidly degraded and that a calpain inhibitor, ALLN, can block this degradation (7, 37). In this study we examined whether another cysteine protease inhibitor, E-64-d, or a proteasome-specific inhibitor, lactacystin, could also inhibit the degradation of endogenous SREBPs in HeLa cells. In addition, we examined the effect of LMB, an inhibitor of CRM1-dependent nuclear export (38), on degradation to determine if nuclear export is involved in SREBP turnover. HeLa cells were cultured under sterol-depleted conditions for 2 days to increase the nuclear SREBPs. During the last 5 h, the cells were incubated with either one or two of the indicated inhibitors, and the nuclear extracts were subjected to immunoblot analysis for SREBP-1 and -2. In response to sterol depletion, the amounts of nuclear SREBP-1 and -2 were increased, and the addition of ALLN or lactacystin induced a still further increase (Fig. 1A), suggesting that the proteasome is involved in the turnover of SREBPs. Neither E-64-d nor LMB treatment changed the rate of turnover. A combination of ALLN and LMB did not enhance the effect of ALLN. These results reveal that the proteasome system, which can be inhibited by either ALLN or lactacystin, degrades the SREBPs and that the CRM1-dependent nuclear export pathway may be only a minor contributor to SREBP turnover.

The membrane fractions were also prepared from the cells incubated with ALLN and/or 25-hydroxycholesterol for the final 5 h to figure out whether the precursors are substrates for the proteasome. Fig. 1B shows that the precursor SREBP levels are not affected by ALLN in the presence or absence of sterols, suggesting that major substrates for the proteasome are likely to be the nuclear SREBPs but not the precursor proteins.

Proteasome Inhibitors Prolong the Half-life of Nuclear SREBPs—Although Fig. 1 shows that the proteasome might be involved in the turnover of the nuclear SREBPs, we cannot rule out the possibility that these inhibitors might slightly stabilize the precursor SREBPs on the ER membrane, resulting in an increase in nuclear forms. To further confirm the direct effects of proteasome inhibitors on the nuclear SREBPs, we assessed the fate of an exogenous nuclear form of SREBPs in CHO cell lines transiently expressing either a human nuclear form of SREBP-1 (amino acids 1–487, CHO-487) or SREBP-2 (amino acids 1–481, CHO-481). These cells constitutively express the lac repressor, thereby blocking the expression of exogenous SREBPs driven by the lac operator-containing promoter. The cells were cultured for 19 h with a medium containing sterols that eliminates the endogenous nuclear SREBPs and IPTG to induce the expression of human nuclear SREBPs. Preliminary Northern blot analysis for HMG-CoA synthase demonstrates that comparable levels of the SREBPs are expressed in stable cells by 1 mM IPTG treatment for CHO-487 and 3 mM for CHO-481. The effect of inhibitors added for the last 5 h on the rate of transiently expressed SREBPs was examined by Western blot analysis. As shown in Fig. 2, the nuclear forms of SREBPs were not detected in CHO-Lac cells expressing only the lac repressor. The exogenously expressed nuclear forms of both SREBP-1 and SREBP-2 were stabilized by the proteasome inhibitors, ALLN and lactacystin, but not by E-64-d or LMB, which is consistent with the results shown in Fig. 1. These results strongly support the notion that the nuclear forms of SREBPs, which are released from the precursor proteins, are the substrates for the proteasome-dependent protein degradation.

LMB Blocks the Nuclear Export of Proteins Containing the Nuclear Export Signal but Does Not Alter the Localization of
To ensure that LMB was active in the inhibition of nuclear export, we carried out a series of transfection experiments using COS1 cells. A fusion protein, GFP-Pap1, which is a fission yeast AP-1-like transcription factor, was normally localized in the cytoplasm but accumulated in the nucleus when CRM1 was inactivated with LMB (Fig. 3), suggesting that LMB was active in the experiment. GFP-SREBP1a-(2–487) localized in the nucleus in the presence or absence of LMB or ALLN. SREBP-1 localization was not altered by ALLN treatment for 24 h (data not shown).

Proteasome Inhibitors Induce the Expression of SREBP-responsive Genes—To investigate the transcriptional activity of SREBPs stabilized by proteasome inhibitors, we carried out Northern blot analyses for SREBP-responsive genes such as HMG-CoA synthase, fatty acid synthase, and the LDL receptor. Cholesterol depletion significantly induced the mRNA level of these genes in HeLa cells, and proteasome inhibitor treatment further induced the level 2- to 2.5-fold, suggesting that the nuclear SREBPs stabilized by proteasome inhibitors are active as transcription factors in the nucleus (Fig. 4). The induction of mRNA was not observed by treatment with E-64-d or LMB (data not shown).

Turnover of Nuclear SREBP-1 under Sterol-loaded or Sterol-depleted Conditions—Our next question was whether the turnover of the nuclear SREBPs is controlled by sterols. It is possible that the degradation system might be working only when cells are depleted of sterols and that proteasome-dependent degradation does not occur in the absence of the nuclear SREBPs when cells are sterol-loaded. To address this question, we utilized a mutant CHO cell line, named M19, which lacks S2P (39), resulting in the absence of the nuclear SREBPs. The cells were transfected with an expression plasmid coding a nuclear form of human SREBP-1a (amino acids 2–487), pME-His-SREBP1a-(2–487), and turnover of exogenous nuclear SREBP-1 was examined. In these cells one can determine the half-life of exogenous nuclear SREBP-1 under either sterol-depleted or sterol-loaded conditions in the absence of the endogenous nuclear SREBPs. In the presence of an translation inhibitor, cycloheximide, the amount of SREBP-1 decreased in a time-dependent manner, and sterols had no effect on the turnover rate (Fig. 5). The intensity of the bands corresponding to β-actin in each lane is almost equal and is used for correction for loading differences. The calculated half-life of human nuclear SREBP-1a is approximately 3 h under either the sterol-depleted or sterol-loaded condition. Similar results were obtained when the cells were transfected with an expression plasmid coding His-tagged human nuclear SREBP-2 (amino acids 2–481) (data not shown).

Nuclear SREBP-1 Is Polyubiquitinated—To determine if the nuclear SREBPs are polyubiquitinated as a substrate for ubiquitin-proteasome degradation, we analyzed the conjugation of ubiquitin to nuclear SREBP-1. COS1 cells were transfected

![Fig. 3. Effect of LMB on the localization of transiently expressed nuclear form SREBP-1. COS1 cells were transfected with 2 μg of pME-GFP-SREBP1a-(2–487) (SREBP-1) or pFPA1 (Pap1) by a DEAE-dextran method. After a 24-h incubation, the cells were treated with the indicated inhibitors for 2 h. Localization of the fusion proteins was observed by fluorescence microscopy. LMB, 5 ng/ml LMB; LMB+ALLN, 5 ng/ml LMB plus 100 μM ALLN; −, control.](image)

![Fig. 4. Proteasome inhibitors induce the expression of SREBP-responsive genes. HeLa cells were cultured under sterol-depleted or sterol-loaded conditions and treated with the indicated inhibitors as described in the legend to Fig. 1. Total RNA (10 μg/lane) was subjected to electrophoresis and blot hybridization with the indicated 32P-labeled probe. The resulting bands were quantified by exposure of the filters to a FluorImage Analyzer with Image Gauge (FUJI FILM), and the results were normalized to the signal generated from 36B4 mRNA. In four separate experiments the same relative mRNA levels were obtained.](image)
Ubiquitin-Proteasome System Degrades SREBPs

with pSREBP1-(1–487) plus an expression plasmid coding ubiquitin, pME-His-Ub, and synthesized nuclear SREBP-1 was immunoprecipitated with a polyclonal antibody against human SREBP-1. The immunoprecipitates were subjected to SDS-PAGE, and Western blot analysis was carried out using a monoclonal antibody against the His tag. Ubiquitin-SREBP conjugate ladders were observed when the cells expressed both SREBP-1 and ubiquitin (Fig. 6, lane 3), whereas these ladders were not detected under other conditions (lanes 1, 2, and 4). The recovery of SREBP-1 in the immunoprecipitates was confirmed by Western blot analysis using an anti-SREBP-1 antibody (lanes 2, 3, and 5 in the lower panel in Fig. 6). Nonspecific bands of around 62 kDa are observed in all lanes and are marked by an asterisk. The same results were obtained when COS1 cells were transfected with an expression plasmid for SREBP-2-(1–481) (data not shown). These results indicate that the nuclear forms of SREBPs are polyubiquitinated.

The Degradation of SREBPs Is Ubiquitin-dependent—To verify that SREBP degradation is ubiquitin-dependent, we examined whether an overexpression of mutant ubiquitin is able to block it, bringing about an increase in the amounts of the nuclear SREBPs and mRNA of one of the responsive genes, HMG-CoA synthase. Fig. 7A shows that mutant ubiquitin, defective in its ability to form polyubiquitin chains, increased the amount of HMG-CoA synthase mRNA in a dose-dependent manner. At the highest concentration of mutant ubiquitin DNA (3 μg) both the nuclear SREBP-1 and -2 increased slightly (Fig. 7B). The same results were further observed when luciferase assays were performed using a reporter gene containing the promoter region of the human HMG-CoA synthase gene. Overexpression of mutant ubiquitin also enhances the luciferase activities in a dose-dependent manner (Fig. 8). These results show that blockage of polyubiquitination stabilizes the nuclear SREBPs, resulting in an up-regulation of the expression of the HMG-CoA synthase gene and that the conjugation of a polyubiquitin chain to the SREBPs may be what triggers their degradation.

DISCUSSION

The results presented in the current study are the first direct demonstration that the ubiquitin-proteasome pathway is functionally pivotal in the degradation of the SREBPs. It is likely that the major substrates for this degradation are not the precursors of SREBPs but the proteolytically activated SREBPs containing the bHLH-Zip domain. It has been reported that most of the various kinds of nuclear proteins including transcription factors are degraded through the ubiquitin-proteasome pathway (15–20). It is therefore likely that most transcription factors are labile and turnover rapidly after regulating the expression of target genes in the nucleus and that the SREBPs are an example of how these factors are subjected to this degradative regulatory pathway.

It has been reported that HMG-CoA reductase, a key enzyme for cholesterol biosynthesis, is degraded in a sterol-dependent manner and that the ubiquitin-proteasome system is involved (40–42). Another example of sterol-dependent proteolysis is the processing of SREBPs on the ER-Golgi membrane. These facts prompted us to examine whether the fate of nuclear SREBPs is also controlled by sterols. There would seem to be two reasonable hypotheses. The nuclear SREBPs, which are proteolytically generated in response to the intracellular sterol depletion, might be relatively stable in the nucleus, and hence able to induce the transcription of their responsive genes. On the other hand, the nuclear SREBPs might be rapidly degraded and hence lose their transcriptional activities when cells become loaded with sterols. In the current study we transfected M19 cells, defective in generation of the nuclear SREBPs because of the lack of S2P, with an expression plasmid for the
nuclear SREBP-1a to determine the turnover rate of the exogenous SREBP-1a under either sterol-loaded or sterol-depleted conditions. The results we obtained show that the turnover rate of SREBPs is not affected by the intracellular sterol levels (Fig. 4), suggesting that the degradation system might be a constitutive process for most transcription factors rather than one that is sterol-mediated.

Amino acid sequence analysis reveals that both of the active forms of SREBP-1a and -2 contain one or more PEST sequences, sequences that are possessed by many short-lived proteins and that have been reported to be motifs for rapid degradation. The N-terminal transactivation domain of the SREBPs, which is rich in acidic amino acid residues, corresponds to a PEST sequence. In addition, only the active form of SREBP-1 has two more PEST sequences (amino acids 418–446 and 446–468) in the C-terminal region. It is not yet clear how these motifs are functionally related to ubiquitin-proteasome pathway degradation. The observation that the active SREBPs are polyubiquitinated suggests that regions containing one or more lysine residues conjugated with a polyubiquitin chain might be important for degradation. However, no lysine residues are conserved between SREBP-1a and -2 in the N-terminal or C-terminal PEST sequences. There are more than 10 conserved lysine residues located in, or close to, the bHLH-Zip motif in the SREBPs, and we are now investigating which, if any, of these lysine residues is ubiquitinated as well as the biological significance of the PEST sequences for SREBP degradation.

Several studies have shown that nuclear export of the substrates is required for the ubiquitin-dependent degradation of nuclear proteins (25–29). We, therefore, hypothesized that the SREBPs might be proteolyzed in the cytoplasm after nuclear export. When CRM1-dependent nuclear export was inhibited by treatment with LMB, the localization of a control protein bearing a nuclear export signal, Pap1, was affected, but neither the degradation nor localization of the SREBPs was changed (Fig. 3). These results raise the possibility that there is a CRM1-independent nuclear export of SREBPs that participates in degradation or that the degradation may occur in the nucleus. When COS1 cells were transfected with GFP-SREBP-1a and its fate followed in the presence of ALLN, the nucleous, not the cytoplasm, was stained even 24 h after treatment (data not shown). This would support the hypothesis that the degradation of SREBPs may not require their nuclear export but rather occurs in the nucleus. The validity of this hypothesis will be the subject of future studies.

Ubiquitin is covalently attached in an isopeptide bond via its ε-amino group to the ε-amine group of specific lysine(s) in the substrate protein. It is thought that a polyubiquitin chain is formed mainly with lysine residue 48 in ubiquitin itself. To eliminate the attachment of ubiquitin to other lysine residues in a ubiquitin molecule, we introduced seven point mutations into ubiquitin (lysine to arginine substitutions). We observed that this mutant ubiquitin does not produce any high molecular weight conjugates observed in COS1 cells expressing both SREBP-1 and wild-type ubiquitin (Fig. 6 and data not shown). Overexpression of mutant ubiquitin in HeLa cells modestly increases the mRNA level for HMG-CoA synthase (Fig. 7A) and the nuclear SREBPs (Fig. 7B), suggesting that polyubiquitin conjugation to the active SREBPs leads the substrates to degradation. Because the transfection efficiency of the expression plasmid for mutant ubiquitin does not attain 100%, and since the endogenous ubiquitin in the transfected HeLa cells is likely too abundant to be overcome by an exogenous mutant form, the 1.5-fold increase in the mRNA level (significantly less than the 2.5-fold increase observed in the cells treated with the proteasome inhibitors) might be in line with what should be expected. The luciferase assay with a reporter gene bearing the human HMG-CoA synthase promoter also reveals that the partial
block of polyubiquitination by mutant ubiquitin may facilitate the activity of the active SREBPs (Fig. 8). It is notable that the SREBPs stabilized by proteasome inhibitors or mutant ubiquitin are able to stimulate the transcription of their responsive genes. We have not yet identified which SREBP form exerts a block of polyubiquitination by mutant ubiquitin may facilitate the regulation of SREBPs through such modifications, including DNA binding activity (45). Further investigation into the regulation of SREBPs through such modifications will be necessary to unravel the complex workings of the ubiquitin-proteasome degradation pathway.

Acknowledgments—We thank Dr. T. Y. Chang for the M19 cells and Dr. Kevin Boru for review of the manuscript.

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