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MUTATION IN THE LUMENAL PART OF THE MEMBRANE DOMAIN OF HMG-CoA REDUCTASE ALTERS ITS REGULATED DEGRADATION

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The involvement of ER lumenal domains of HMG-CoA reductase in the regulated degradation process was examined. For this purpose we studied three cell lines expressing HMG-CoA reductase molecules with introduced functional N-glycosylation sites located in the linker segments between transmembrane spans 1 and 2 (HMGal/Bins(-)), 3 and 4 (HMGal/Dins(-)) and 5 and 6 (HMGal/Fins(-)), all facing the ER lumen (Olender, E. H. and Simoni, R. D. (1992) J. Biol. Chem. 267, 4223-4235). The glycosylation insertion between spons 5 and 6 (HMGal/Fins(-)) is the only one of these mutations which eliminates regulated degradation of the enzyme. The half lives of the HMGal/Fins(-) in the presence or absence of regulatory molecules are indistinguishable. In contrast the HMGal/Bins(-) and HMGal/Dins(-) mutants show a normal pattern of regulated degradation. Tunicamycin treatment of cells expressing the HMGal/Fins(-) mutant does not significantly alter the regulation defect indicating that it is the mutation per se not the glycosylation that alters the degradation response.

These results suggest that the linker segments between transmembrane spans 5 and 6 (loop F) are involved in the process of regulated degradation of HMG-CoA reductase and that the regulated degradation process may occur on the lumenal side of the ER membrane.

3-hydroxy-3-methylglutaryl coenzyme-A reductase (HMG-CoA reductase or reductase) catalyzes conversion of HMG-CoA to mevalonate in the rate limiting and major regulatory enzyme in the cholesterol biosynthetic pathway (1). The enzyme is a 97 KDa glycoprotein (2,3) located in the endoplasmic reticulum (ER). It is composed of a cytoplasmic catalytic domain anchored, via a linker region, to a membrane embedded domain that spans the ER membrane eight times (4,5). HMG-CoA reductase is regulated by both the amount of the protein present and its activity. The amount of the reductase is controlled at transcriptional and translational levels, and by protein degradation (1). The latter is the final mechanism controling the level of the protein in the cell. Addition of the regulatory molecules, mevalonate or cholesterol, to cells accelerates the degradation rate of the enzyme. The mechanism where by these molecules regulate the degradation of HMG-

The abbreviations used are: ER, endoplasmic reticulum; CHO, Chinese hamster ovary; MEM, minimal essential medium; FCS, fetal calf serum; LPS, lipid poor serum.

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CoA reductase is not known. However, the membrane domain has been shown to be both necessary and sufficient for the regulated degradation (6-8), and the degradation process occurs in the ER (9-12).

Recent studies have shown that the degradation of various proteins occurs in the ER lumen (13-16) and is possibly mediated by specific recognition sequences in each protein (17,18). The degradation process might be prevented by steric protection from degradation by proteases within the ER lumen (19). In this study we have examined the ability of a Asn-linked oligosaccharide group facing the ER lumen to protect HMG-CoA reductase from accelerated degradation. For this purpose we utilized three different mutants, each with the sequence for the native glycosylation site inserted into a different loop of the HMG-CoA reductase membrane domain. The three mutants chosen had all been shown to be facing the ER lumen (4). One of these mutants, has lost the regulated degradation by mevalonate and cholesterol, suggesting that this loop is involved in the regulation of HMG-CoA reductase degradation and that a site necessary for the regulatory response is on the luminal side of the ER membrane.

MATERIALS AND METHODS

Materials - All materials, unless specified, were obtained from commercial sources. Lipofectin reagent, opti-MEM medium and geneticin were purchased from Gibco BRL. The anti-β-galactosidase monoclonal antibody was obtained from Promega. 25-hydroxycholesterol was purchased from Steraloids Inc.. Tran^{35}S-label metabolic labeling reagent (specific activity >1000 Ci/mMol, 37 TBq/mMol) and protein A-bacterial adsorbent were obtained from INC Biomedicals. Compactin was a generous gift of Dr. Akiro Endo (Tokyo Noko University, Tokyo, Japan). Fluorescein-di-β-D-galactopyranoside was purchased from Molecular Probes.

Cell culture - Chinese hamster ovary (CHO-K1) cells expressing the HMGal fusion protein (CHO-HMGal) (7) and CHO cells expressing the HMGal/Bins(-), HMGal/Dins(-) and HMGal/Fins(-) glycosylation mutant proteins (CHO-HMGal/Xins(-)s) (4) were grown as monolayers at 37°C in an atmosphere of 5% CO_2. The cells were maintained in minimal essential medium (MEM) supplemented with 5% fetal calf serum (FCS) and 250 μg/ml Geneticin. Elevated levels of the HMGal and the HMGal/Xins(-)s were induced by replacement of the cell medium with MEM supplemented with 5% lipid-poor serum (LPS) (20) and 1 μm compactin and 100 μm mevalonate (9).

Transfection of CHO cells - 0.5 μg of the pSV2-neo plasmid (20) and 10 μg of pSRαHMGal/Bins(-), pSRαHMGal/Dins(-) or pSRαHMGal/Fins(-) (4) were used to co-transfect subconfluent monolayers of CHO cells by the lipofectin method (21). CHO cells, 20% confluent, were grown in 60 mm tissue culture plates and washed once with 2 ml of Opti-MEM medium. The DNA was diluted into 100 μl of H_2O. To the DNA solution, 100 μl of 50% lipofectin (v/v) were added. The transfection solution was incubated for 20 minutes at room temperature, 1.8 ml of Opti-MEM were added, and the tube was mixed gently. The DNA solution was added to the cells. Twenty four hr later, the medium was replaced with 5 ml of fresh MEM supplemented with 5% FCS. The cells were harvested 2 days later and transfectants were selected in the presence of 1 mg/ml of Geneticin.

Isolation of cells expressing elevated levels of β-galactosidase activity - CHO-HMGal/Xins(-) cells expressing the highest β-galactosidase activity were sorted using an EPICS 753 flow cytometer (Coulter Electronics, Hialeah, FL) fluorescence-activated cell sorter as previously described (9). In order to set cells with high level of expression the transfected cells were sorted five times and approximately 1% of the population was isolated each time.

Assay of mevalonate accelerated reduction of β-galactosidase activity - Cells were grown in 24 well dishes for 24 hr in medium supplemented with LPS, 100 μm mevalonate and 1

μm compactin. Mevalonate, 20 mM was added in a staggered fashion so that all time points were assayed simultaneously. The β-galactosidase activity was measured as previously described (7). Specific activity is expressed in units of A_{420} nm/h/mg protein.

Pulse-Chase experiments - Palse-Chase experiments were done as previously described (9).

Tunicamycin treatment - The tunicamycin pretreatment were done as previously described (3). Briefly, the cells were preincubated with 5 μg/ml tunicamycin 3 hr before starting the assay of mevalonate accelerated loss of β-galactosidase activity.

RESULTS

Establishment of the mutant cell lines

To examine the ability of a Asn-linked oligosaccharide group projecting into the ER lumen to protect HMG-CoA reductase against accelerated degradation, we established three cell lines stably expressing mutant proteins with an artificially inserted peptide contaning the native glycosylation site. The glycosylation site was inserted into three different lumenal loops of the HMG-CoA reductase membrane domain fused with E. coli β-galactosidase (4). CHO cells were transfected with the pSRαHMGal/Bins(-), pSRαHMGal/Dins(-) and pSRαHMGal/Fins(-) constructs which encoded the HMGal/Bins(-), HMGal/Dins(-) and HMGal/Fins(-) mutants, respectively. Cells expressing high levels of β-galactosidase activity were selected in five rounds of flow cytometry sorting (9). Schematic models of the HMGal/Bins(-), HMGal/Dins(-) and HMGal/Fins(-) mutants is represents in Figure 1.

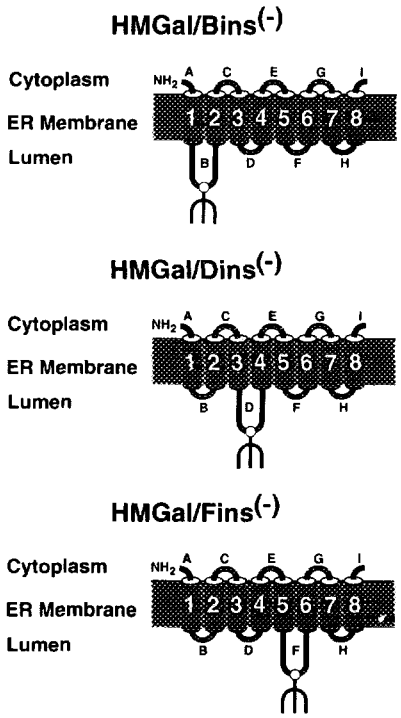

Fig. 1. **A schematic model represents the HMGal/Xins(-) glycosylation mutants.** The transmembrane spans are represented by cylinders and designated by numbers 1-8. The cytoplasmic and lumenal segments are marked by letters A-I and the glycosylation site is represented by the fork symbol.

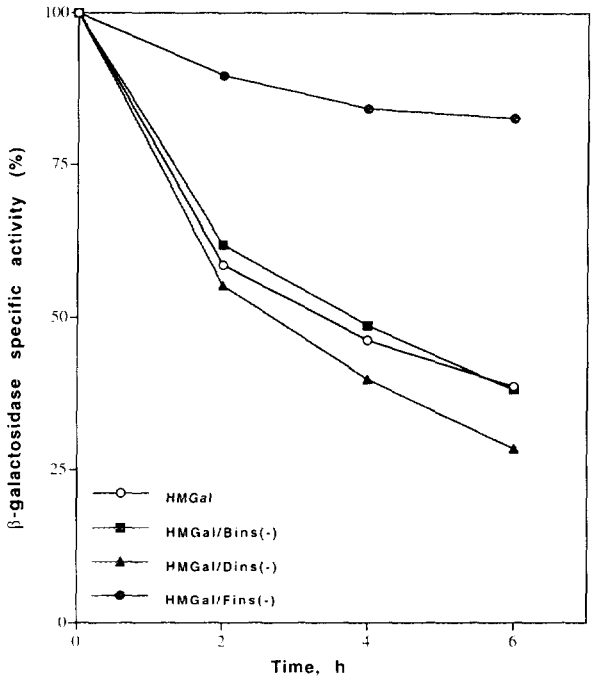

Fig. 2. **The effect of mevalonate on HMGal and HMGal/Xins(-) activity**. CHO-HMGal (open circles), CHO-HMGal/Bins(-) (closed squares), CHO-HMGal/Dins(-) (closed triangles) and CHO-HMGal/Fins(-) (closed circles) cells were grown. Mevalonate, 20 mM, was added to the wells and the β-galactosidase activity was determined as described under Materials and Methods. The 100% of activity represents the specific activity measured in cells which were not exposed to mevalonate.

Comparison of the mevalonate accelerated degradation of HMGal and HMGal/Xins(-)

In order to examine the effect of each inserted glycosylation site on the accelerated degradation of HMGal/Xins(-) mutants, the decrease in the steady state levels of β-galactosidase activity of each of the mutants was determined in the presence or absence of mevalonate, and these changes were compared to control cells (the HMGal). As shown in Figure 2 the β-galactosidase activity of the HMGal/Bins(-) and HMGal/Dins(-) mutants decreased at a similar rate as the HMGal in presence of mevalonate. After 4 h, the remaining activities were 48, 40 and 46% of the initial level, respectively. In contrast, the HMGal/Fins(-) mutant was very stable; 84% of the initial activity remained after 4 h in presence of mevalonate. The higher stability of the HMGal/Fins(-) suggested that this mutant did not undergo mevalonate accelerated degradation.

Determination of the half life of HMGal and HMGal/Fins(-)

The measurement of β-galactosidase activity reflects the stedy state balance between the rates of synthesis and the degradation of that enzyme. Therefore, to determine if the observed reduction in the rate of loss of β-galactosidase activity of the glycosylation mutant HMGal/Fins(-) was related to reduced degradation, it was nessecery to measure the half life of the mutant protein. The half life of HMGal and HMGal/Fins(-) were measured in absence or presence of mevalonate or 25-hydroxycholesterol. As shown in Figure 3 the half life of HMGal dramatically decreased upon

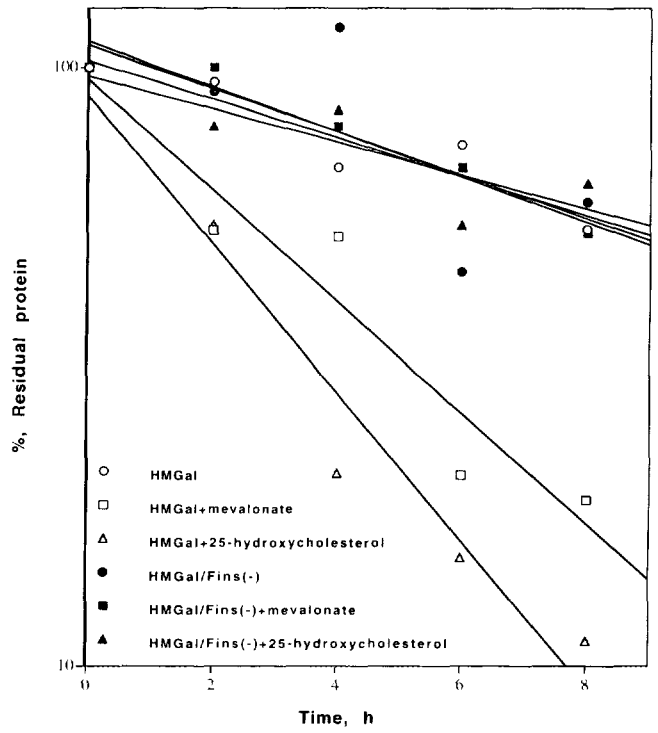

Fig 3. **Mevalonate and 25-hydroxycholesterol accelerates the degradation of HMGal but not HMGal/Fins(-).** CHO-HMGal (open symbols) and CHO-HMGal/Fins(-) (closed symbols) cells were grown in 60-mm dishes for 24 hr then pulse-labeled as described under Materials and Methods. At the end of the pulse, the cells were collected (t=0) or chased for 2-8 hr in medium supplemented with no addition (circles), or 20 mM mevalonate (squares) or 5 mM 25-hydroxycholesterol (triangles). The 100% is the amount of the respective proteins at the end of the pulse (t=0).

addition of mevalonate or 25-hydroxycholesterol. In absence of these regulatory molecules, the half life was about 9 h; in the presence of mevalonate or 25-hydroxycholesterol it decreased to 3 h and 2 h, respectively. In contrast, the half life of the HMGal/Fins(-) mutant was not affected by the presence of mevalonate and 25-hydroxycholesterol, and was about 9 h. The latter is similar to the half life of the HMGal in absence of regulatory molecules. This result clearly indicates that regulated degradation was absent in HMGal/Fins(-).

The effect of deglycosylation on the rate of degradation of HMGal and HMGal/Fins(-).

To determine if the presence of the oligosaccharide was responsible for the loss of regulated degradation in the HMGal/Fins(-) mutant, cells expressing the HMGal and HMGal/Fins(-) were treated with tunicamycin at concentration known to inhibited the glycosylation of the enzyme (3). As shown in Figure 4, tunicamycin slowed the mevalonate accelerated degradation on the HMGal (at 4 h the remaining activity was 45 and 72% in absence or presence of tunicamycin, respectively) but did not effect the mutant (the remaining activity at 4 h were 87 and 89%). This clearly indicates that the presence of an alternate oligosaccharide group on the HMGal/Fins(-) is not responsible for the decrease in the rate of mevalonate accelerated degradation of the mutant.

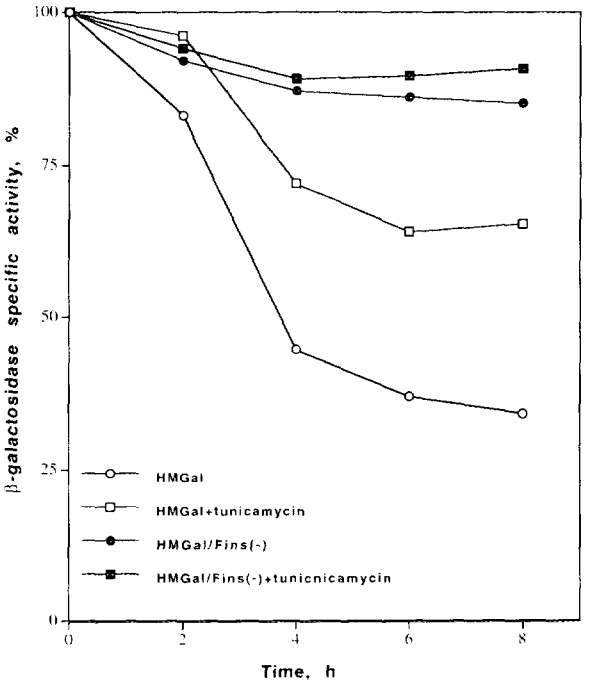

Fig. 4. **The effect of deglycosylation on the β-galactosidase activity of CHO-HMGal and CHO-HMGal/Fins(-) cells.** CHO-HMGal (open symbols) and CHO-HMGal/Fins(-) (closed symbols) cells were grown in 24-well dishes as described under Materials and Methods. Tunicamycin, 5 μg/ml (squares) was added to the wells 3 hr before addition of 20 mM mevalonate. The β-galactosidase activity was determined as described under Materials and Methods. The 100% of activity represents the specific activity measured in cells which were not exposed to mevalonate.

DISCUSSION

HMG-CoA reductase is the only integral ER membrane protein that is known to undergo regulated degradation. Several recent studies have been aimed at elucidating the mechanism underlying this process and at identifying the target(s) for degradation. It has been shown that removal or replacement of transmembrane segments can abolish the accelerated degradation (22,7,8). However, a role for residues facing into the ER lumen was not investigated. In this study, we examined the involvement of the loops of HMG-CoA reductase facing into the ER lumen in the accelerated degradation process. For this purpose, we utilized mutants in which a peptide containing the native N-glycosylation of HMG-CoA reductase was inserted into loops B, D and F, which all face the ER lumen (4).

We found that the steady state level of β-galactosidase activity of the HMGal/Fins(-) mutant declines much more slowly then the control (HMGal) and the other mutant (HMGal/Bins(-) and HMGal/Dins(-)) forms of the enzyme. This difference in enzyme activity was linked to differences in protein degradation by the demonstration that the half life of the HMGal/Fins(-) mutant was significantly slower in the presence of mevalonate or 25-hydroxycholesterol, and was similar to the half life of the control in absence of regulatory molecules. The results presented here demonstrate,

for the first time, that the native, lumenal F loop, but not loops B and D, may be a target for the accelerated degradation process.

Recent studies have shown that the interaction of glycoproteins with calnexin alters the rate of degradation (23-25). Therefore it is conceivable that the artificial insertion of N-glycosylation site in the HMGal/Fins(-) mutant might be responsible for decreased metabolite accelerated degradation. The lack of effect of tunicamycin treatment argues against this possibility and supports the hypothesis that modification in the peptide of the ER lumenal domain rather the N-glycosylation site is involved in HMGal/Fins(-) phenotype. It is interesting that the rate of degradation of the wild type is partially attenuated by tunicamycin treatment. This may suggest that at least two lumenal factors can affect the degradation process. Future studies aimed to study selective modified specific residues of the ER lumenal F loop and a possibale interaction of HMG-CoA reductase with calnexin may shed light on the complex process of regulated degradation of this enzyme.

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