

Partial Deletion of Membrane-bound Domain of 3-Hydroxy-3-methylglutaryl Coenzyme A Reductase Eliminates Sterol-enhanced Degradation and Prevents Formation of Crystalloid Endoplasmic Reticulum

Hisato Jingami, Michael S. Brown, Joseph L. Goldstein, Richard G. W. Anderson, and Kenneth L. Luskey

Departments of Molecular Genetics, Internal Medicine, and Cell Biology, University of Texas Health Science Center, Southwestern Medical School, Dallas, Texas 75235

Abstract. 3-Hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase is anchored to the endoplasmic reticulum (ER) membrane by a hydrophobic NH₂-terminal domain that contains seven apparent membrane-spanning regions and a single N-linked carbohydrate chain. The catalytic domain, which includes the COOH-terminal two-thirds of the protein, extends into the cytoplasm. The enzyme is normally degraded with a rapid half-life (2 h), but when cells are depleted of cholesterol, its half-life is prolonged to 11 h. Addition of sterols accelerates degradation by fivefold. To explore the requirements for regulated degradation, we prepared expressible reductase cDNAs from which we either deleted two contiguous membrane-spanning regions (numbers 4 and 5) or abolished the single site for N-linked glycosylation. When expressed in hamster cells after transfection, both enzymes retained catalytic activity. The deletion-

bearing enzyme continued to be degraded with a rapid half-life in the presence of sterols, but it no longer was stabilized when sterols were depleted. The glycosylation-minus enzyme was degraded at a normal rate and was stabilized normally by sterol deprivation. When cells were induced to overexpress the deletion-bearing enzyme, they did not incorporate it into neatly arranged crystalloid ER tubules, as occurred with the normal and carbohydrate-minus enzymes. Rather, the deletion-bearing enzyme was incorporated into hypertrophied but disordered sheets of ER membrane. We conclude that the carbohydrate component of HMG CoA reductase is not required for proper subcellular localization or regulated degradation. In contrast, the native structure of the transmembrane component is required to form a normal crystalloid ER and to allow the enzyme to undergo regulated degradation by sterols.

THE endoplasmic reticulum (ER)¹ houses a variety of resident proteins that are degraded at heterogeneous rates. It also serves as a conduit for proteins destined for insertion into the plasma membrane or secretion into the extracellular fluid. The mechanisms that determine the rates at which resident ER proteins are degraded and the signals responsible for sorting proteins in the ER are just beginning to be understood. An experimental system to dissect these factors is provided by 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase, a resident protein of the ER that is synthesized and degraded at rates that are not only rapid, but regulated (Faust et al., 1982; Edwards et al., 1983). This enzyme produces mevalonate, a crucial intermediate in the biosynthesis of cholesterol and other isoprenoid substances (Brown and Goldstein, 1980). When cultured cells are depleted of cholesterol, they produce increased amounts of HMG CoA reductase as a result of enhanced transcription

of the gene (Luskey et al., 1983). When sterols are added to the culture medium, the rate of transcription of the mRNA declines, the mRNA level falls, and enzyme synthesis declines. The existing enzyme in the ER is rapidly degraded with a half-life of ~2 h (Brown et al., 1974; Chin et al., 1985).

The amplitude of the regulatory changes in reductase can be heightened by growing cells in the presence of compactin, a competitive inhibitor of the reductase that creates a shortage of mevalonate, thereby blocking endogenous cholesterol synthesis (Brown and Goldstein, 1980). When cells are incubated in compactin and in the absence of exogenous sterols, the rate of synthesis of reductase is accelerated, and the rate of degradation is slowed so that the half-life becomes 5–10 h (Faust et al., 1982; Chin et al., 1985; Gil et al., 1985). Under these conditions the addition of sterols markedly decreases the synthesis of reductase and speeds degradation to a half-life of <1.5 h.

When cultured cells are adapted to growth in the presence of progressively increasing concentrations of compactin,

1. *Abbreviations used in this paper:* CHO, Chinese hamster ovary; Con A, concanavalin A; ER, endoplasmic reticulum; HMG CoA, 3-hydroxy-3-methylglutaryl coenzyme A; nt, nucleotide.

they survive by producing massively elevated levels of HMG CoA reductase (Chin et al., 1982). The enzyme is synthesized initially on ribosomes attached to the nuclear envelope (Pathak et al., 1986). As the amount of enzyme increases, the nuclear envelope protrudes in a series of redundant folds or cisternae. Eventually these folds pinch off to form tubules. These tubules are packed together in extremely regular hexagonal arrays referred to as crystalloid ER (Anderson et al., 1983; Orci et al., 1984). The crystalloid ER is especially prominent in UT-1 cells, a line of Chinese hamster ovary (CHO) cells that has amplified the gene for HMG CoA reductase by 15-fold (Chin et al., 1982; Luskey et al., 1983). HMG CoA reductase accounts for 1–2% of the protein in UT-1 cells, and most of it is housed in the crystalloid ER, as determined by immunocytochemistry (Anderson et al., 1983; Orci et al., 1984). A similar, crystalloid ER develops in livers of rats that are treated with another HMG CoA reductase inhibitor (mevinolin) together with a bile acid sequestrant (Singer et al., 1984).

The membrane of the crystalloid ER appears to have very little cholesterol, as determined by its lack of staining with filipin (Orci et al., 1984). When sterols are added to UT-1 cells, the sterols are intercalated into the crystalloid ER membrane, and this is followed sequentially by the accelerated degradation of the reductase and the disappearance of the crystalloid ER membrane (Orci et al., 1984). We earlier postulated that intercalation of sterols into the ER membrane is the factor that dictates enhanced degradation of the reductase and the crystalloid ER. In addition, the sterols drastically reduce the rate of synthesis of new HMG CoA reductase and thus they also prevent the synthesis of new crystalloid ER.

HMG CoA reductase is a single-chain glycoprotein that contains two recognizable domains: a membrane-associated domain and a catalytic domain (Liscum et al., 1985). The NH₂-terminal 339 amino acids consist of alternating hydrophobic and hydrophilic sequences that are associated with the ER membrane. The amino acid sequence suggests that the enzyme spans the membrane seven times with the NH₂ terminus and the first nine amino acids in the lumen and the COOH terminus extending into the cytoplasm. Each of the putative membrane-spanning regions is encoded by a separate exon, an observation that supports the notion that each hydrophobic region is a discrete entity whose function is to span the membrane (Liscum et al., 1985). Between the sixth and seventh putative membrane-spanning regions, there is a long sequence of 93 amino acids that contains the only consensus site for N-linked glycosylation in the membranous region (residue 281) (Liscum et al., 1985). Structural studies have shown that the reductase contains a partially trimmed high mannose, endoglycosidase H-sensitive, N-linked carbohydrate in this region, presumably attached to asparagine 281 (Liscum et al., 1983a). This finding indicates that asparagine 281 is within the lumen of the ER, as predicted by the seven membrane-spanning region model. The COOH-terminal two-thirds of HMG CoA reductase (548 residues) is a hydrophilic structure that extends into the cytoplasm. This segment can be released from the membrane by proteolysis, and it retains all of the catalytic activity of the enzyme (Liscum et al., 1985).

The membranous segment of HMG CoA reductase was previously shown to be necessary for sterol-mediated en-

hanced degradation of the enzyme (Gil et al., 1985). These experiments were performed by transfection of expressible cDNAs into UT-2 cells, a mutant CHO cell line that lacks HMG CoA reductase (Mosley et al., 1983). UT-2 cells were transfected with pRed-227, a plasmid that contains a full-length cDNA copy of the HMG CoA reductase mRNA, or with a plasmid that encodes a truncated and soluble form of the reductase. The latter cDNA produces an enzyme that has the first nine hydrophilic amino acids attached directly to the COOH terminal, enzymatically active portion and therefore lacks the entire membranous region. This plasmid produced a soluble, catalytically active form of HMG CoA reductase that was degraded very slowly with a half-life >10 h. Sterols did not accelerate the degradation of this cytosolic enzyme, whereas they produced a three- to fivefold increase in the rate of degradation of the membrane-bound enzyme encoded by pRed-227 (Gil et al., 1985). Cells that expressed high levels of the soluble form of reductase did not have any hypertrophied or crystalloid ER (unpublished observations), indicating that the membrane domain of reductase is required to form this structure.

Consistent with the hypothesis of an important functional role for the membranous region of HMG CoA reductase is the finding of a striking conservation of amino acid sequence in the membrane domain of reductase from two species, hamster and human (Luskey and Stevens, 1985). Of the 186 amino acids that are thought to be embedded in the membrane, only three residues show substitutions between the two sequences. This is in contrast to another region of the protein (amino acids 340–449), in which 32 out of 110 residues show substitutions. This latter region is believed to function as a linker between the membrane-bound domain and the catalytic domain (Liscum et al., 1985).

The current studies were undertaken to determine whether the membranous domain of reductase plays a role in determining the organization of the crystalloid ER and to determine whether the native structure of the membranous portion of the protein is required for sterol-mediated enhancement of degradation of the enzyme. To answer these questions, we have prepared a mutant form of HMG CoA reductase that lacks two adjacent transmembrane segments (numbers 4 and 5), thereby distorting the structure of the membranous domain. In addition, we have assessed the role of the asparagine-linked carbohydrate chain by substituting glutamine for the normally glycosylated asparagine at position 281. These mutated forms of HMG CoA reductase were transfected into UT-2 cells to study the effects on sterol-mediated degradation and organization of the crystalloid ER.

Materials and Methods

Materials

We obtained [³⁵S]methionine and DL-3-hydroxy-3-methyl-[3-¹⁴C]glutaryl coenzyme A from New England Nuclear (Boston, MA); cholesterol and 25-hydroxycholesterol from Alltech Associates, Inc. (Deerfield, IL) and Steraloids Inc. (Wilton, NH), respectively; restriction enzymes from New England Biolabs (Beverly, MA); AMV reverse transcriptase from Life Sciences, Inc. (St. Petersburg, FL); Ham's F-12 medium and G418 sulfate from Gibco (Grand Island, NY); goat anti-rabbit IgG conjugated to FITC from Zymed (Burlingame, CA); and Immun-Blot™ Assay Kit from BioRad Laboratories (Richmond, CA). Newborn calf lipoprotein-deficient serum (*d* < 1.215) was prepared as previously described (Goldstein et al., 1983). IgG fractions of rabbit anti-HMG CoA reductase polyclonal antibody and

mouse anti-HMG CoA reductase monoclonal antibody (IgG-A9) were prepared as previously described (Liscum et al., 1983b). Compactin was kindly provided by Akira Endo (Tokyo Noko University, Tokyo, Japan).

Plasmid Constructions

Two plasmids with mutated coding regions for hamster HMG CoA reductase were constructed from plasmid pRed-227, which contains a full length cDNA for the normal HMG CoA reductase (Chin et al., 1984). Plasmid p Δ TM 4+5 encodes a protein that lacks the fourth and fifth membrane spanning regions (amino acids 115–187), and plasmid pCarbh⁻ encodes a protein in which a glutamine replaces an asparagine at position 281, the site of attachment of an N-linked carbohydrate chain in the normal enzyme (Liscum et al., 1983a, 1985).

p Δ TM 4+5

The 2.4-kb *Sall-XmaI* fragment from pRed-227 (Chin et al., 1984) was subcloned into M13mp18 (Messing, 1983). The site-specific deletion of the 219 base pairs encoding amino acids 115–187 in HMG CoA reductase was carried out as described by Zoller and Smith (1983, 1984). A mutagenic oligonucleotide of 40 nucleotides (nt) corresponding to nt 323–342 and nt 562–581 in pRed-227 was used as a primer in a 20-fold molar excess over the M13 template together with the universal primer of 17 nt, which was used in 1:1 molar ratio with template. The primer-extended products were used to transform competent *E. coli* TG-1 cells, and the resulting plaques were screened by hybridization with the ³²P-5'-end labeled mutagenic oligonucleotide. Single-stranded DNA prepared from positive plaques was subjected to DNA sequencing to confirm that the predicted sequence was present at the deletion joint. To re-insert the mutagenized fragment into the full-length cDNA, we prepared a double-stranded *Sall-XmaI* fragment by primer extension, using 5 pmol of the mutagenized M13 DNA and 50 pmol of universal primer in the presence of all four dideoxynucleotides and AMV reverse transcriptase (Maniatis et al., 1982), followed by cleavage with the restriction enzymes *Sall* and *XmaI*. The resulting 2.2-kb fragment was isolated by agarose gel electrophoresis and recloned into the *Sall* and *XmaI* sites of pRed-227 to yield p Δ TM 4+5.

pCarbh⁻

We synthesized a mutagenic oligonucleotide that corresponded to nt 832–852 of pRed-227 except that the AAT codon for Asn₂₈₁ was replaced with a CAA codon for Gln. This oligonucleotide was used as described above to mutagenize the *Sall-XmaI* fragment in the M13 clone. Positive plaques were identified, and the nucleotide replacement was confirmed by DNA sequencing. The double-stranded, mutated *Sall-XmaI* fragment was prepared by primer extension and cloned into the *Sall* and *XmaI* sites of pRed-227 to yield pCarbh⁻.

Isolation of Cells Expressing Transfected HMG CoA Reductase cDNAs

Plasmids p Δ TM 4+5 and pCarbh⁻ were separately introduced into UT-2 cells, a mutant line of CHO cells that lacks immunoreactive or functional HMG CoA reductase (Mosley et al., 1983). Briefly, on day 0, 5×10^5 UT-2 cells were seeded into each 100-mm petri dish containing 10 ml of medium A (Ham's F-12 medium containing 25 mM HEPES [pH 7.4], 100 U/ml penicillin, 100 μ g/ml streptomycin, 2 mM glutamine) supplemented with 10% fetal calf serum and 0.2 mM sodium mevalonate. On day 1, the plasmid encoding the mutated HMG CoA reductase (9.5 μ g) and pSV₃-Neo (0.5 μ g), a plasmid conferring G418 resistance (Southern and Berg, 1982), were coprecipitated with calcium phosphate and added to the medium of each dish. After 5 h the precipitate was removed, and the cells were washed twice with Dulbecco's PBS and incubated with medium A containing 10% (vol/vol) glycerol for 4 min at 37°C. The glycerol was removed, and 10 ml of medium A containing 10% fetal calf serum and 0.2 mM mevalonate were added to each dish. On day 2, selection was begun by changing to medium A containing 10% fetal calf serum and 700 μ g/ml G418, but lacking mevalonate. The cells were refed every 3–4 d with this medium until visible colonies appeared. Growing colonies were picked and adapted to growth in medium A containing 8% lipoprotein-deficient serum, 2% fetal calf serum, and increasing concentrations of compactin (0.1–30 μ M). Several clones expressing the p Δ TM 4+5-encoded reductase were analyzed by direct enzyme assay of detergent-solubilized cell extracts. The clone TR-108 was selected on the basis of its high expression of reductase activity. In this paper the

TR-108 cell line is referred to as p Δ TM 4+5-transfected cells. Similarly several clones were analyzed for the expression of the pCarbh⁻-encoded reductase. The clone TR-137 was selected and is referred to as pCarbh⁻-transfected cells.

Cell Culture

All cells were grown in monolayer cultures at 37°C in an atmosphere of 5–7% CO₂. Stock cultures of CHO cells were grown in medium A supplemented with 10% (vol/vol) fetal calf serum. Stock cultures of UT-2 cells were grown in medium A supplemented with 10% fetal calf serum and 0.2 mM mevalonate. Transfected cell lines were grown in medium A supplemented with 8% lipoprotein-deficient serum, 2% fetal calf serum, and 1–30 μ M compactin as indicated.

For experiments, cells were set up according to a standard format. On day 0, 4×10^4 CHO cells or 1×10^5 transfected cells were seeded into each 60-mm petri dish in 3 ml of medium A supplemented with 8% lipoprotein-deficient serum and 2% fetal calf serum. Unless otherwise indicated, the cells were refed medium A supplemented with 10% lipoprotein-deficient serum and the appropriate amounts of compactin on days 1 and 2.

Measurement of HMG CoA Reductase

Immunoreactive HMG CoA reductase was assayed by SDS gel electrophoresis and immunoblotting with mouse monoclonal antibody IgG-A9 (Liscum et al., 1983b) as previously described (Gil et al., 1985). The bound monoclonal antibody was visualized as described in the BioRad Immun-Blot™ Assay Kit. The rate of degradation of HMG CoA reductase was determined by labeling cell monolayers with [³⁵S]methionine followed by a chase period with medium containing 0.3 mM unlabeled methionine. At the indicated times of the chase, cells were solubilized, and the amount of ³⁵S-labeled HMG CoA reductase was measured by immunoprecipitation with a rabbit polyclonal anti-reductase antibody as previously described (Gil et al., 1985). Enzymatic activity of HMG CoA reductase was measured in detergent-solubilized cell extracts as described previously (Goldstein et al., 1983). One unit of reductase activity represents the formation of 1 nmol of [¹⁴C]mevalonate per min at 37°C. Protein concentration was measured by a modification of the procedure of Lowry et al. (1951).

Indirect Immunofluorescence

Cells were grown in 60-mm petri dishes on glass coverslips and fixed with 3% (vol/vol) paraformaldehyde in 10 mM sodium phosphate (pH 7.4) containing 2 mM MgCl₂ and 0.15 M NaCl for 20 min at room temperature. After washing with 50 mM NH₄Cl, cells were permeabilized with 0.1% (vol/vol) Triton X-100 and incubated with rabbit polyclonal anti-HMG CoA reductase IgG (0.2 mg/ml) or nonimmune IgG (0.2 mg/ml) for 1 h at 37°C. Cells were then washed and incubated with anti-rabbit IgG conjugated to FITC (50 μ g/ml) for 1 h at 37°C. Photographs were taken with a Zeiss photomicroscope III using an automatic photometer system.

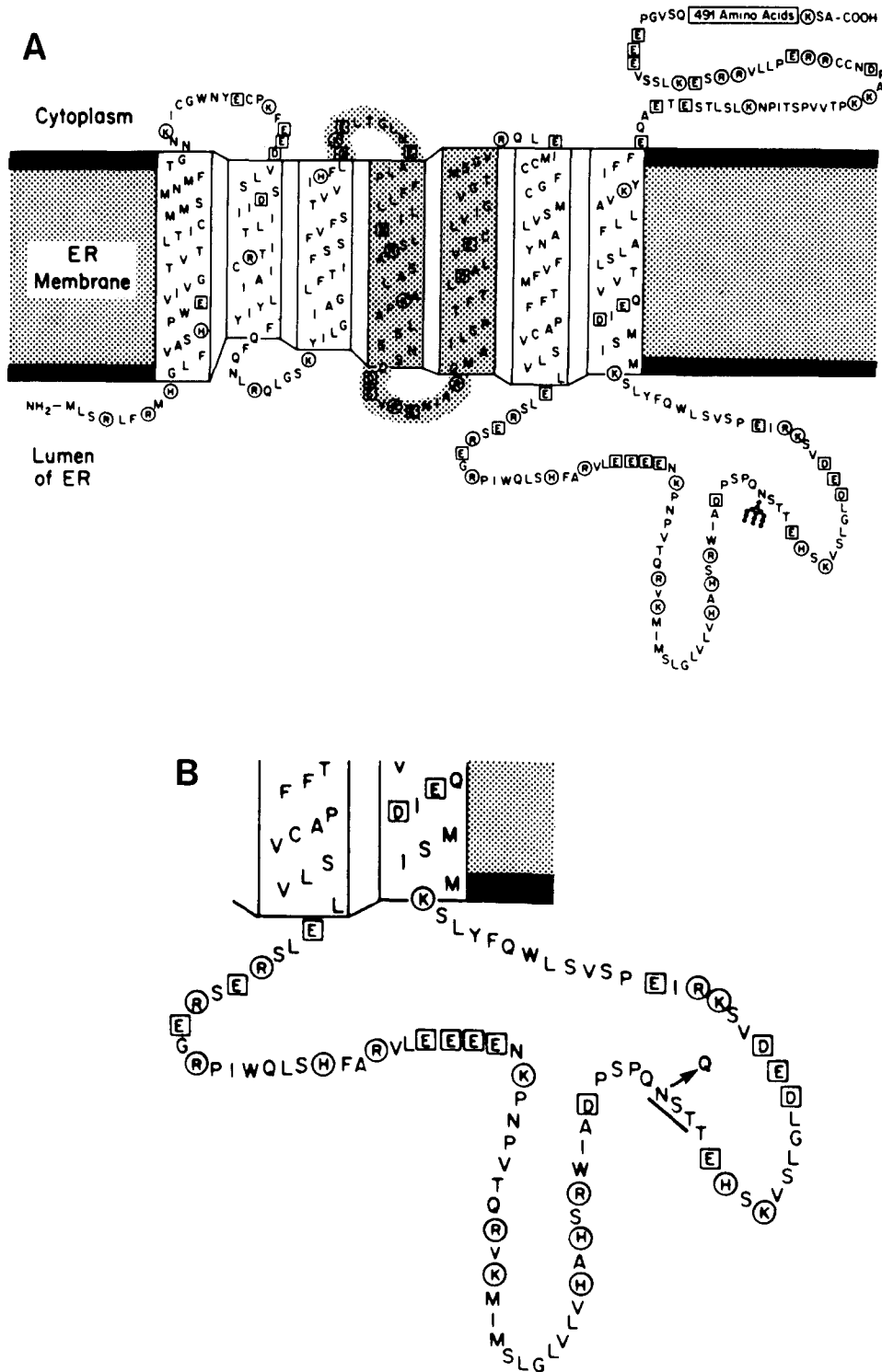
Electron Microscopy

Cells were grown in 60-mm petri dishes and fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate (pH 7.4). The samples were postfixed with 2% OsO₄ and stained en bloc with 0.5% uranyl acetate. The cells were then dehydrated and released from the surface of the plastic dish by brief treatment with propylene oxide. The cells were pelleted in propylene oxide by centrifugation at 12,000 rpm for 5 min in a microfuge and embedded in Spurr's low viscosity embedding medium (Electron Microscopy Sciences, Fort Washington, PA). All electron micrographs were taken with a JEOL 100CX electron microscope.

Results

Mutations in HMG CoA Reductase

Fig. 1 A shows the current model for the seven postulated membrane-spanning domains of hamster HMG CoA reductase. Oligonucleotide-directed mutagenesis was used to introduce two separate mutations into this region. One mutant protein, encoded by plasmid p Δ TM 4+5, lacks the 73 amino acids that constitute the putative fourth and fifth transmem-



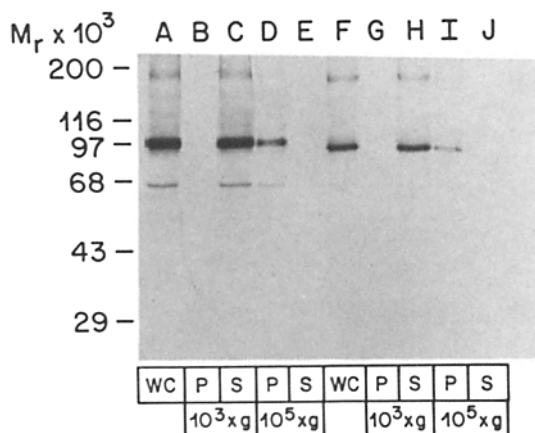


Figure 2. Localization of HMG CoA reductase after fractionation of cells transfected with pRed-227 (lanes A-E) or pΔTM 4+5 (lanes F-J). Cells were grown in 60-mm dishes by the standard procedure in medium containing 10 or 30 μM compactin for pΔTM 4+5- or pRed-227-transfected cells, respectively. On day 4, four dishes of each cell line were washed, harvested, pelleted by centrifugation, and stored at -70°C. After thawing, the pellets were incubated for 29 min at 4°C in the presence of 0.4 ml of homogenization buffer (10 mM Tris-chloride, 0.1 mM leupeptin, 5 mM sodium EGTA, 5 mM sodium EDTA, and 5 mM dithiothreitol at pH 7.4). The cells were then disrupted with 40 strokes of a Dounce homogenizer followed by 10 2-s bursts of sonification. The whole cell homogenate (WC) was adjusted to 0.1 M sodium carbonate, incubated for 3 min at 4°C, then centrifuged at 10³ g for 5 min at 4°C. The 10³ g supernate (S) was then spun at 10⁵ g for 20 min at 4°C. The 10³ g and 10⁵ g pellets (P) were each resuspended in the original volume of homogenization buffer containing 0.1 M sodium carbonate. Aliquots of equal volume of each fraction were precipitated with 10% (vol/vol) TCA. The resulting precipitate was collected by centrifugation and resuspended in buffer containing 62.5 mM Tris-chloride, 15% (wt/vol) SDS, 8 M urea, 10% (vol/vol) glycerol, and 100 mM dithiothreitol at pH 6.8. Aliquots of the solubilized extracts were subjected to SDS PAGE, transferred to nitrocellulose, and incubated with 5 μg/ml of mouse monoclonal IgG-A9. The nitrocellulose was then incubated with alkaline phosphatase-conjugated goat anti-mouse IgG. The immunoreactive bands were detected by color development using reagents provided in a BioRad Immun-Blot™ Assay Kit. An identical immunoblot was incubated with a control monoclonal antibody and processed similarly and revealed no immunoreactive bands. *M_r* standards are indicated. For the whole cell homogenates, 23 and 17 μg of protein were analyzed for pRed-227- and pΔTM 4+5-transfected cells, respectively.

promoter, which is not suppressed by sterols. Changes in reductase activity are due solely to posttranscriptional mechanisms. A plasmid encoding each of these reductase cDNAs was separately introduced into UT-2 cells, a mutant CHO cell line that lacks HMG CoA reductase and is a mevalonate auxotroph. A plasmid that confers resistance to the antibiotic G418, pSV₃-Neo, was cotransfected with the reductase cDNAs. Transfected cells were subsequently selected by their ability to grow in the presence of G418 as well as in the absence of mevalonate, a condition that requires the expression of HMG CoA reductase. After the initial selection of clones, expression of the transfected HMG CoA reductase was amplified by growing the cells in increasing concentrations of compactin. Each cell line was initially adapted to 0.1 μM compactin and then resistant cells were sequentially adapted to increasing concentrations of compactin. For ex-

periments in this paper, we used transfected cells that had been adapted to growth in from 1 to 30 μM compactin. The transfected cell line expressing the native reductase was previously called TR-36 (Chin et al., 1985; Gil et al., 1985). In this paper we refer to these cells as pRed-227-transfected cells.

Expression of HMG CoA Reductase Lacking the Fourth and Fifth Transmembrane Regions

Cells transfected with either pΔTM 4+5 or pRed-227 were homogenized and subjected to differential centrifugation and immunoblotting to determine if the HMG CoA reductase was membrane-bound. In the pRed-227-transfected cells, reductase was found in the supernatant fraction after a 10³ g centrifugation (Fig. 2, lane C). After a 10⁵ g centrifugation, the enzyme was found in the membrane pellet (lane D). The pΔTM 4+5-transfected cells contained a slightly smaller reductase molecule in the 10³ g supernatant fraction (lane H) that was also pelleted by centrifugation at 10⁵ g (lane I). The size change detected by SDS PAGE was consistent with a 73 amino acid deletion, giving a molecular weight of ~90,000.

Although the above fractionation experiment suggests that an HMG CoA reductase molecule containing only five transmembrane regions is membrane-bound, it does not indicate whether the enzyme is localized to the same membrane organelle as the native reductase. To more precisely localize the enzyme, we analyzed transfected cells by indirect immunofluorescence with an anti-reductase antibody. In pRed-227-transfected cells grown in a high concentration of compactin (30 μM), most of the immunoreactive reductase was found in large clumps that surrounded the nucleus of the cell (Fig. 3 A). Immunoreactive enzyme was also associated with the nuclear envelope. The staining of pΔTM 4+5-transfected cells was similar in that large clumps of fluorescence were found in the cytoplasm (Fig. 3 C). However, only minimal staining of the nuclear envelope was observed in these cells.

By electron microscopy the cells that expressed high levels of the transfected cDNA encoding the normal enzyme (pRed-227) contained numerous well-defined crystalloid ER profiles (Figs. 4 A and 5 A). These were indistinguishable from the crystalloid ER found in UT-1 cells (Chin et al., 1982; Orci et al., 1984; Pathak et al., 1986). In contrast, cells transfected with pΔTM 4+5, contained numerous sheets of membrane that were arranged in disorganized aggregates (Figs. 4 B and 5, B-D). We never saw any membranes that resembled the crystalloid ER. The disorganized membranes in pΔTM 4+5-transfected cells bound anti-HMG CoA reductase IgG, as determined by indirect immunoperoxidase immunocytochemistry (data not shown).

We next asked whether the activity of the deleted form of HMG CoA reductase was susceptible to suppression by sterols (Fig. 6). In normal CHO cells, reductase activity was suppressed to <10% of control levels within 8 h after the addition of cholesterol and 25-hydroxycholesterol to the culture medium. Previous studies have shown that this suppression is mediated by a decreased rate of transcription of the reductase gene as well as an enhanced rate of degradation of the reductase protein (Chin et al., 1985; Gil et al., 1985). In the pRed-227-transfected cells, reductase activity was suppressed to ~30% of control values after addition of sterols.

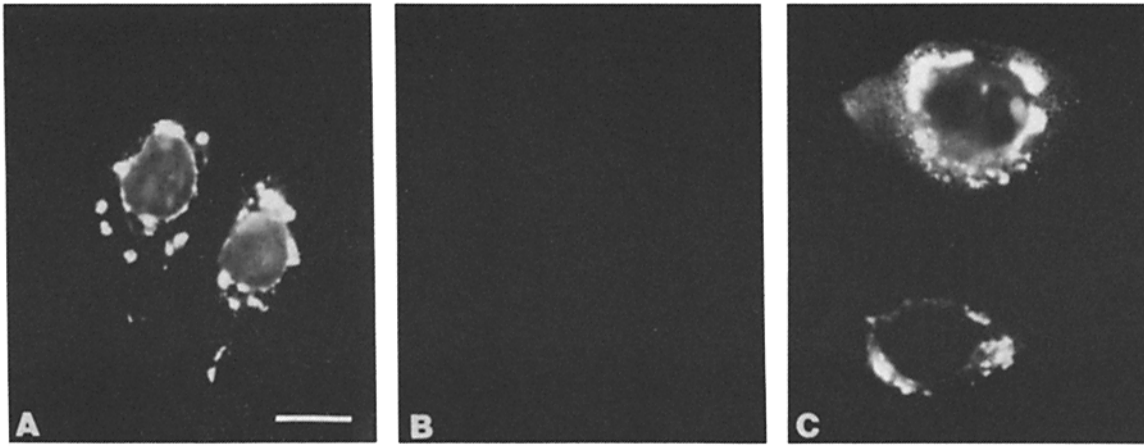


Figure 3. Indirect immunofluorescence localization of HMG CoA reductase in pRed-227-(*A* and *B*) and pΔTM 4+5-(*C*) transfected cells. Cells were grown in 60-mm dishes on coverslips by the standard procedure in medium containing 30 μ M compactin. On day 3, cells were fixed, permeabilized, and incubated with either a rabbit polyclonal anti-HMG CoA reductase IgG (*A* and *C*) or nonimmune IgG (*B*). Each set of cells was then processed for indirect immunofluorescence. Bar, 20 μ m.

Previous studies have shown that this suppression is attributable to the enhanced rate of degradation of the reductase protein (Chin et al., 1985; Gil et al., 1985). In contrast, reductase activity in cells expressing the transfected pΔTM 4+5 was unaffected by sterols. This finding suggested that

the mutated HMG CoA reductase was no longer sensitive to the sterol-mediated acceleration of degradation.

To study the degradation of the enzyme directly, we performed a series of pulse-chase experiments (Fig. 7). In cells transfected with pRed-227 and grown in the absence of

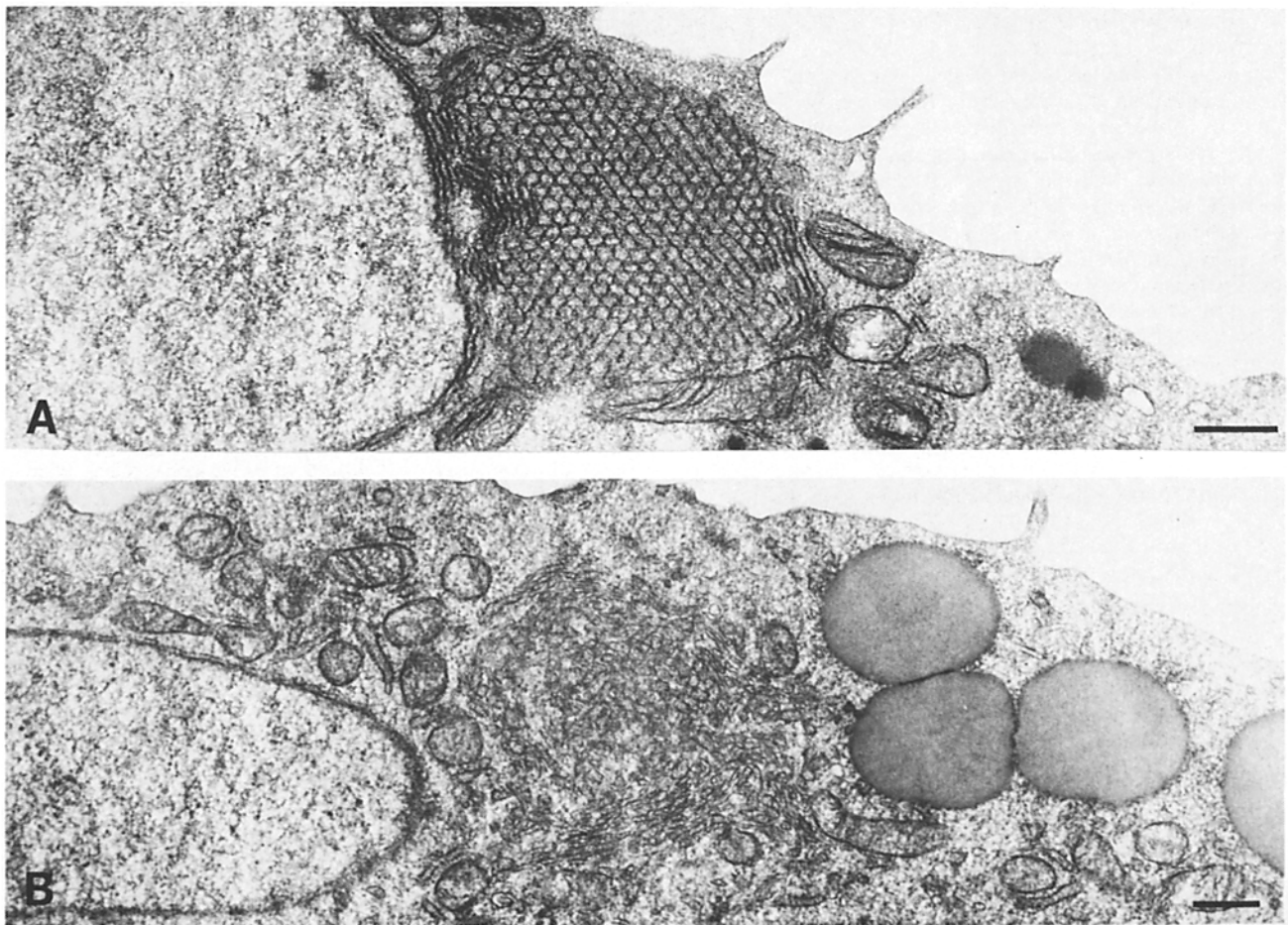


Figure 4. Electron microscopy of proliferated ER in pRed-227-(*A*) and pΔTM 4+5-(*B*) transfected cells. Cells were grown in 60-mm dishes by the standard procedure in medium containing 30 μ M compactin. On day 3, cells were harvested and processed for electron microscopy. Bar, 0.5 μ m.

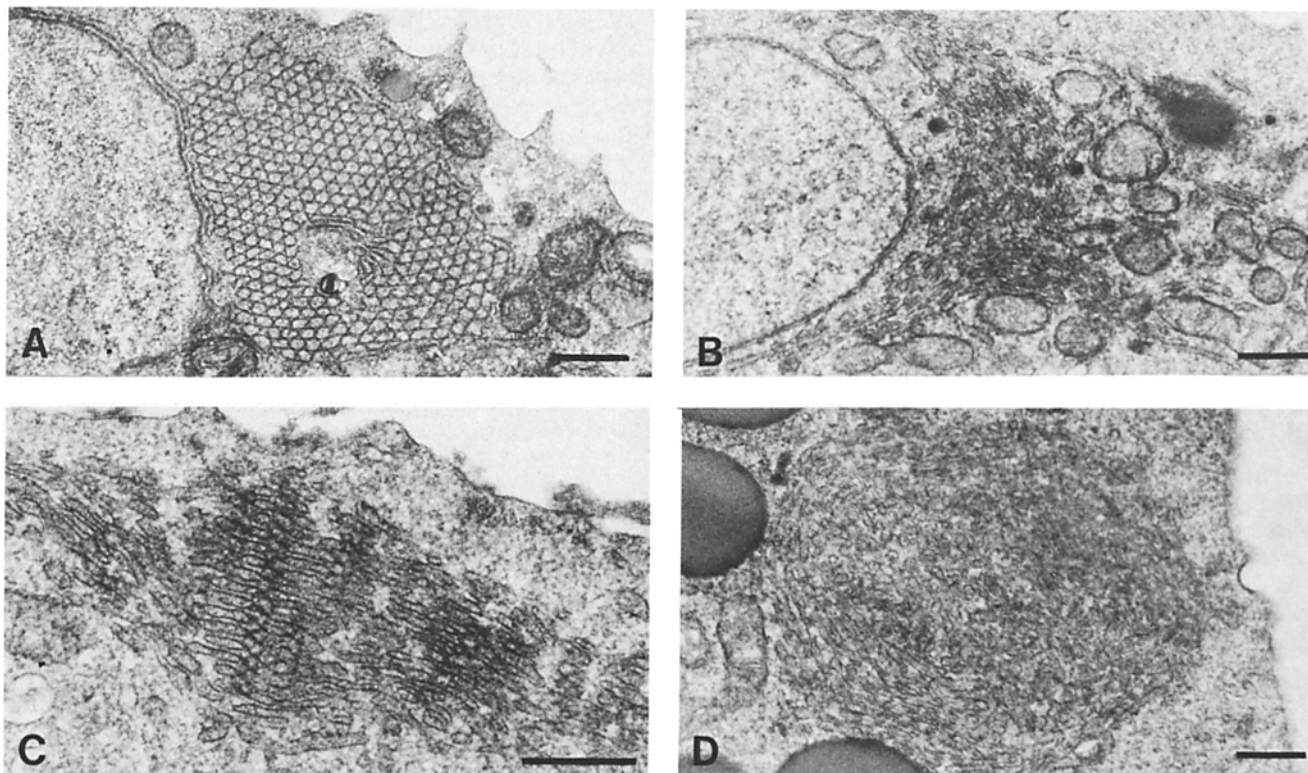


Figure 5. Different views of the proliferated ER in pRed-227-(A) and pΔTM 4+5-(B-D) transfected cells. Cells were grown and processed as described in the legend to Fig. 4. Representative views of the ER in transfected cells are shown. Bar, 0.5 μm .

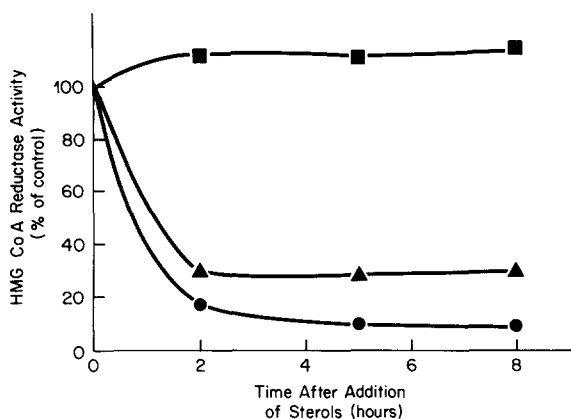


Figure 6. Sterol-mediated suppression of HMG CoA reductase activity in pΔTM 4+5-transfected cells (■), pRed-227-transfected cells (▲), and CHO cells (●). On day 0, cells were plated in 60-mm dishes by the standard procedure. Additions of cholesterol (10 $\mu\text{g/ml}$) and 25-hydroxycholesterol (1 $\mu\text{g/ml}$) in a total volume of 10 μl ethanol were made in a staggered fashion such that all the dishes were harvested at the same time on day 3 after incubation with sterols for the indicated time. pRed-227-transfected cells were grown in the presence of 30 μM compactin, and pΔTM 4+5-transfected cells were grown in the presence of 10 μM compactin. The "100% of control" values for reductase activity (units/mg protein) were as follows: 1.0 for CHO cells; 36 for pRed-227-transfected cells; and 32 for pΔTM 4+5-transfected cells. Each value represents the mean of triplicate incubations. The variation in triplicate values averaged <10%.

sterols, the [^{35}S]methionine-labeled reductase was degraded with a half-life of 5 h (Fig. 7 A). The rate of degradation was enhanced approximately threefold when sterols were present. In contrast, the enzyme encoded by pΔTM 4+5 was degraded with a half-life of ~ 2 h either in the absence or presence of sterols (Fig. 7 B). SDS PAGE of the immunoprecipitated reductase revealed that the bulk of the radioactivity was present in the full-length 97- or 90-kD molecules for the pRed-227 or pΔTM 4+5 encoded enzymes, respectively. Similar results were obtained in three separate experiments (Table I). Together, these experiments show that the reductase encoded by pΔTM 4+5 is degraded somewhat faster than the native reductase in the absence of sterols, and that there is no increased degradation when sterols are added.

Fig. 8 shows the morphology of the crystalloid ER in cells transfected with the normal reductase cDNA and then incubated in the absence (Fig. 8 A) or presence (Fig. 8 B) of sterols. The hexagonal lattice of tubules observed in the pRed-227-transfected cells was maintained 24 h after the addition of sterols at a time when enzyme activity fell 64%.

Expression of HMG CoA Reductase Lacking N-Linked Carbohydrate

UT-2 cells were transfected with pCarbh⁻, and cells expressing HMG CoA reductase were selected and amplified by growth in increasing concentrations of compactin. To determine if the enzyme produced by the transfected cDNA lacked carbohydrate, we measured the ability of the ^{35}S -labeled reductase to bind to concanavalin A (Con A) (Table II). Transfected cells were incubated with [^{35}S]methionine and then solubilized in Zwittergent. The solubilized extract

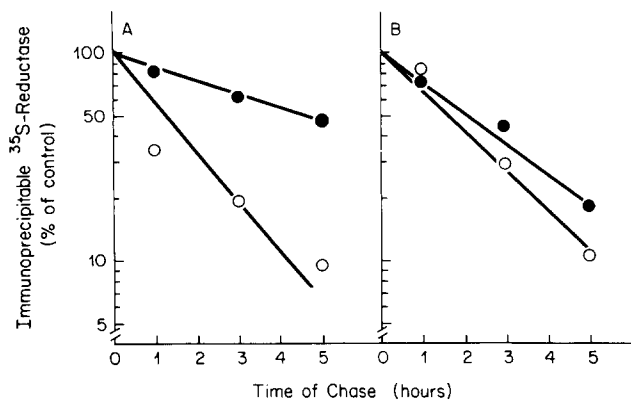


Figure 7. Effect of sterols on the turnover of ^{35}S -labeled HMG CoA reductase in pRed-227-transfected cells (A) and p Δ TM 4+5-transfected cells (B). On day 0, cells were plated in 60-mm dishes by the standard procedure and grown in the presence of 1 μM compactin. On day 2, the cells were preincubated in medium A with 10% lipoprotein-deficient serum and 40 μM compactin for 4 h. Each monolayer was then washed with PBS after which the cells were pulse-labeled with 125 $\mu\text{Ci/ml}$ of [^{35}S]methionine in methionine-free medium containing 10% lipoprotein-deficient serum and 40 μM compactin. After a 40-min pulse, the medium was changed to medium A containing 10% lipoprotein-deficient serum, 40 μM compactin, and 0.3 mM unlabeled methionine. After 20 min, each monolayer received 10 μl of ethanol containing either no sterols (\bullet) or 10 $\mu\text{g/ml}$ cholesterol plus 1 $\mu\text{g/ml}$ 25-hydroxycholesterol (\circ). At the indicated time after the addition of sterols, cells were harvested, solubilized, and processed for immunoprecipitation of HMG CoA reductase. Cells from two dishes were pooled for each point, and the amount of ^{35}S -labeled reductase was calculated by subtracting the nonimmune IgG immunoprecipitation value from the anti-reductase IgG immunoprecipitation value. The "100% of control" values for ^{35}S -reductase were 834 and 889 dpm/ μg protein for pRed-227- and p Δ TM 4+5-transfected cells, respectively. Reductase accounted for 0.43 and 0.13% of the protein synthesized from [^{35}S]methionine in pRed-227- and p Δ TM 4+5-transfected cells, respectively. Each value represents the mean of duplicate incubations. The variation in duplicate values averaged $<9\%$.

was incubated with Con A-Sepharose in the presence and absence of α -methyl-D-mannoside, which competes for specific binding to Con A. After centrifugation the amount of labeled reductase in the supernatant was determined by im-

Table I. Effect of Sterols on the Half-life of HMG CoA Reductase in pRed-227- and p Δ TM 4+5-transfected Cells

Transfected cDNA	Addition of sterols to medium	Half-life		Stimulation of degradation by sterols
		Mean	Range	
		<i>h</i>		<i>-fold</i>
pRed-227 (<i>n</i> = 3)	-	4.6	3.7-5.6	3.5
	+	1.3	1.2-1.5	
p Δ TM 4+5 (<i>n</i> = 3)	-	2.6	2.2-3.0	1.2
	+	2.2	2.0-2.5	

Transfected cell lines grown in 1-3 μM compactin were set up for experiments, preincubated with 40 μM compactin, pulse-labeled with [^{35}S]methionine, and chased for 5 h in the presence or absence of 10 $\mu\text{g/ml}$ cholesterol and 1 $\mu\text{g/ml}$ 25-hydroxycholesterol as described in the legend to Fig. 7. The amount of immunoprecipitable ^{35}S -labeled reductase was quantified at various times of chase, and these values were used to calculate the half-life as shown in Fig. 7. The values shown represent the mean and range of three separate experiments.

munoprecipitation. As shown in Exp. A of Table II, $\sim 40\%$ of the reductase encoded by pCarbh $^-$ was bound to Con A-Sepharose either in the absence or presence of α -methyl-D-mannoside. This binding was considered to be nonspecific. In contrast, 89% of the wild-type enzyme encoded by pRed-227 bound to Con A-Sepharose in the absence of α -methyl-D-mannoside, and this was reduced to the nonspecific value (38%) in the presence of α -methyl-D-mannoside. Thus, at least 50% of the wild-type enzyme, but none of the enzyme encoded by pCarbh $^-$ showed specific α -methyl-D-mannoside-sensitive binding to Con A. Attempts to confirm this observation with endoglycosidase H were inconclusive because the change in electrophoretic mobility after removal of the single high-mannose oligosaccharide chain is insufficient to give unambiguous results (Liscum et al., 1983a).

Exp. B of Table II shows that the enzyme encoded by p Δ TM 4+5 also bound to Con A in a specific fashion. 85% of this enzyme bound to Con A-Sepharose in the absence of α -methyl-D-mannoside, and this binding was reduced to the nonspecific value (41%) in the presence of α -methyl-D-mannoside. These findings indicate that the bulk of the enzyme produced by p Δ TM 4+5 must be oriented so that the linking loop between transmembrane segments 6 and 7 is in its normal position on the luminal side of the ER membrane where it is accessible for glycosylation.

To determine if the reductase encoded by pCarbh $^-$ was membrane-bound, we subjected homogenates to differential centrifugation and immunoblotting (Fig. 9). The reductase in pCarbh $^-$ -transfected cells and pRed-227-transfected cells behaved similarly, both sedimenting at a 10^5 g centrifugation (lanes D and I), but not at 10^3 g (lanes B and G).

The structure of the ER that formed upon high level expression of the glycosylation-deficient reductase was examined by electron microscopy of pCarbh $^-$ -transfected cells. A typical crystalloid ER indistinguishable from that associated with the normally glycosylated enzyme was observed (Fig. 10).

To study the regulation of the carbohydrate-deficient HMG CoA reductase, we measured the decline in enzyme activity after addition of cholesterol and 25-hydroxycholesterol (Fig. 11). The activity of reductase declined in cells transfected with pCarbh $^-$, suggesting that degradation was accelerated. To confirm this analysis, we performed a pulse-chase experiment designed to compare the effect of sterols on the degradation of HMG CoA reductase in pRed-227- and pCarbh $^-$ -transfected cells (Fig. 12). As in previous experiments (Fig. 7 A), the degradation of the pRed-227-encoded reductase was enhanced by the addition of sterols (Fig. 12 A). In pCarbh $^-$ -transfected cells, the reductase was degraded with a half-life of ~ 9 h in the absence of sterols (Fig. 12 B), and the degradation rate increased threefold upon the addition of sterols. Table III summarizes the results of three separate experiments in which the degradation of the pRed-227- and pCarbh $^-$ -encoded reductase were compared in the presence and absence of sterols. The proteins encoded by both of these plasmids were degraded at similar rates. On average, the rate of degradation of the pCarbh $^-$ -encoded reductase was enhanced about 2.5-fold in the presence of sterols.

Discussion

The current results indicate that the membranous domain of

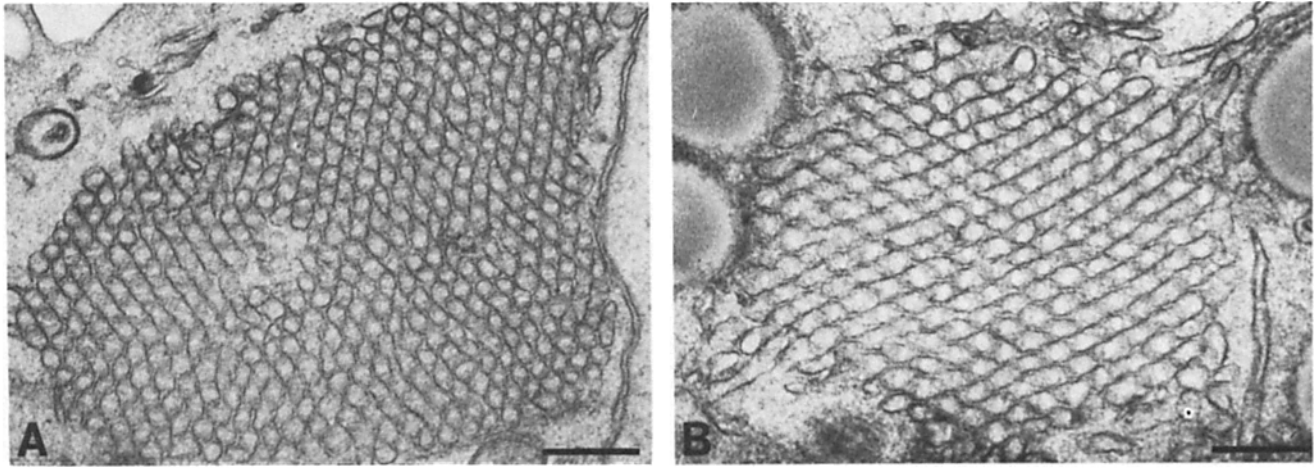


Figure 8. Electron microscopy of proliferated ER in pRed-227-transfected cells grown in the absence (A) and presence (B) of sterols. Cells were grown in 60-mm dishes by the standard procedure in medium containing 30 μ M compactin. On day 2, each dish received 10 μ l of ethanol containing either no sterols (A) or 10 μ g/ml cholesterol plus 1 μ g/ml 25-hydroxycholesterol (B). 24 h later (day 3), cells were harvested and processed for electron microscopy. Reductase activity (units/mg protein) was 34 and 12 for cells grown in the absence and presence of sterols, respectively. Bar, 0.5 μ m.

HMG CoA reductase plays an important role in determining the structure of the crystalloid ER as well as the susceptibility of HMG CoA reductase to sterol-enhanced degradation. When the sequences encoding two of the seven postulated membrane-spanning regions were deleted from the cDNA, the cells incorporated the enzyme into disordered sheets of ER rather than neatly packed membrane tubules. Moreover, degradation of the enzyme was no longer regulated by sterols.

In UT-1 cells, which express high levels of endogenous HMG CoA reductase owing to adaptation to compactin, the addition of sterols leads to a rapid degradation of HMG CoA reductase followed by a disappearance of the crystalloid ER (Orci et al., 1984). In the current studies, we found that cells transfected with a cDNA encoding the normal enzyme (pRed-227) had a fully developed crystalloid ER (Figs. 3–5).

When these cells were treated with sterols, the rate of degradation of HMG CoA reductase increased, but the crystalloid ER did not change in appearance (Fig. 8). The lack of disappearance of the crystalloid ER is likely attributable to a continued synthesis of HMG CoA reductase in the pRed-227-transfected cells. Since the expression of this cDNA is driven by the SV-40 promoter, it is not repressed by sterols (Chin et al., 1985). Thus, when reductase continues to be synthesized even in the presence of rapid degradation, a crystalloid ER can be maintained.

In cells transfected with p Δ TM 4+5, the failure to form a crystalloid ER may be attributable to the failure of the mutated HMG CoA reductase to be synthesized at its normal site. In previous studies we have found that induction of HMG CoA reductase in UT-1 cells leads to an initial appear-

Table II. Binding of Native and Mutated Reductase to Con A-Sephrose

	Total 35 S-reductase incubated with resin dpm/sample	Percent bound to Con A-Sephrose		
		- α -MeMan (a)	+ α -MeMan (b)	Specific binding (a-b)
Exp. A			%	
pCarbh ⁻	3,450	38	41	0
pRed-227	52,000	89	38	51
Exp. B				
p Δ TM 4+5	12,100	85	41	44
pRed-227	8,360	96	26	70

Cells were grown in 60-mm dishes by the standard procedure. On day 3, the monolayers were labeled at 37°C with [35 S]methionine (150 μ Ci/ml) for 90–120 min. The cells were then washed, scraped, pooled, and stored at -70°C. After thawing, each pooled pellet was solubilized in 50 mM Tris-chloride, 2% (wt/vol) Zwittergent 3-14, 0.15 M NaCl, 0.5 mM MgCl₂, 0.5 mM MnCl₂, 5 mM sodium EGTA, 10 mM dithiothreitol, 0.2 mM leupeptin, 0.2 mM phenylmethylsulfonyl fluoride, 1 mM *o*-phenanthroline at pH 7.5. The extract was incubated at 4°C for 30 min and then centrifuged at 4°C at 12,000 *g* for 5 min. The supernatant was removed and diluted fourfold with the above buffer containing no detergent. Aliquots of the solubilized cells were then incubated with Con A-Sephrose CL-4B in the absence or presence of 0.1 M α -methyl-D-mannoside (α -MeMan) for 60 min at 4°C. The resin was then removed by low speed centrifugation, and the amount of 35 S-labeled HMG CoA reductase not bound to the Con A-Sephrose was determined by immunoprecipitating the supernatant with a rabbit polyclonal anti-reductase antibody. In Exp. A, pCarbh⁻ and pRed-227-transfected cells were grown in the presence of 10 and 30 μ M compactin, respectively. Eight dishes were initially solubilized in 0.36 ml, and extracts containing 70 μ g of protein were incubated with the Con A-Sephrose. In Exp. B, both transfected cell lines were grown in 1 μ M compactin. Four dishes were initially solubilized in 0.25 ml, and extracts containing 20–25 μ g of protein were incubated with the Con A-Sephrose.

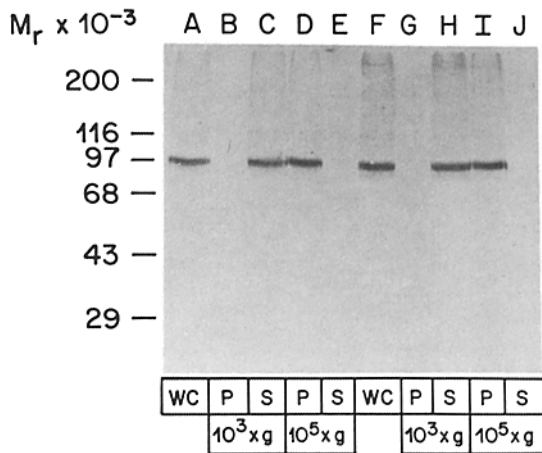


Figure 9. Localization of HMG CoA reductase after fractionation of cells transfected with pRed-227 (lanes A-E) or pCarbh⁻ (lanes F-J). Cells were plated on day 0 and cultured by the standard procedure in medium containing 1 or 10 μ M compactin for pRed-227- or pCarbh⁻-transfected cells, respectively. On day 3, cell monolayers were washed, harvested, and pooled. A whole cell homogenate was prepared and subjected to fractionation as described in the legend to Fig. 2. Aliquots of the whole cell homogenate (WC) as well as the pellets (P) and supernates (S) from the 10³ g and 10⁵ g separations were analyzed. For the whole cell homogenates, 37 and 40 μ g protein were analyzed for the pRed-227- and pCarbh⁻-transfected cells, respectively. Aliquots derived from equivalent volumes of cell extract were analyzed for the other fractions. Recovery of total protein after fractionation was >90%. The samples were subjected to SDS gel electrophoresis and immunoblotting as described in the legend to Fig. 2. An identical blot was incubated with a control monoclonal antibody, and no immunoreactive bands were detected. *M_r* standards are indicated.

ance of the enzyme on the nuclear envelope (Pathak et al., 1986). Only later does the enzyme appear in crystalloid ER, which appears to be formed as an outpouching of the nuclear envelope that develops only when large amounts of HMG

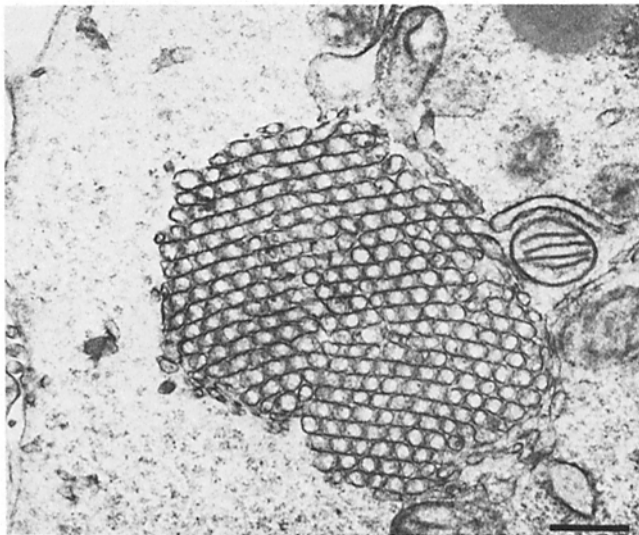


Figure 10. Electron microscopy of proliferated ER in pCarbh⁻-transfected cells. Cells were grown in 60-mm dishes by the standard procedure in medium containing 30 μ M compactin. On day 3, cells were harvested and processed for electron microscopy. Bar, 0.5 μ m.

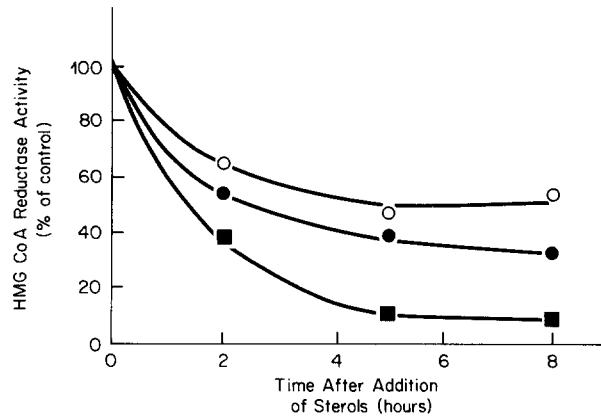


Figure 11. Sterol-mediated suppression of HMG CoA reductase activity in pCarbh⁻-transfected cells (○), pRed-227-transfected cells (●), and CHO cells (■). On day 0, the cells were plated in 60-mm dishes according to the standard procedure. pRed-227-transfected cells were grown in the presence of 1 μ M compactin and pCarbh⁻-transfected cells were grown in the presence of 10 μ M compactin. Additions of cholesterol (10 μ g/ml) and 25-hydroxycholesterol (1 μ g/ml) in a total volume of 10 μ l were made in a staggered fashion such that all the dishes were harvested at the same time on day 3 after incubation with sterols for the indicated time. The "100% of control" values for reductase activity (units/mg protein) were as follows: 0.72 for CHO cells; 7.1 for pRed-227-transfected cells; and 9.2 for pCarbh⁻-transfected cells. Each value represents the mean of triplicate incubations. The variation in triplicate values averaged <12%.

CoA reductase are present. When cells were transfected with pRed-227, prominent staining of the nuclear envelope was seen (Figs. 3 A and 4 A), and this was associated with the development of a crystalloid ER. Although the p Δ TM 4+5 construct gave rise to abundant HMG CoA reductase, much less of this enzyme was visualized on the nuclear envelope as compared with the pRed-227-transfected cells, and no crystalloid ER was seen (Figs. 3 C and 4 B). These findings raise the possibility that the p Δ TM 4+5-encoded enzyme was not synthesized on the nuclear envelope, but rather was synthesized in rough ER. This rough ER might not have the capacity to differentiate into crystalloid ER. It is possible that one role of the membrane-spanning region is to target the ribosomes synthesizing HMG CoA reductase to the nuclear envelope, and this targeting may be necessary for a crystalloid ER to be formed. We cannot exclude the alternative possibility that the disordered membrane structure is a direct effect of the altered protein structure, i.e., that the presence of the deleted enzyme itself alters the structure of the ER membrane.

The enzyme encoded by p Δ TM 4+5 was at least partially in a correct orientation relative to the two surfaces of the ER membrane. It appeared to be glycosylated normally, as evidenced by its adherence to Con A in an α -methyl-D-mannoside-sensitive fashion (Table II). This observation suggests that asparagine 281 and the linker region between membrane-spanning regions 6 and 7 were on the luminal side of the ER membrane. The COOH-terminal catalytic domain presumably faced the cytoplasm since it folded properly and was sufficiently active to allow cell growth in the presence of compactin. Inasmuch as the catalytic activity of the reductase is strongly dependent upon a sulfhydryl

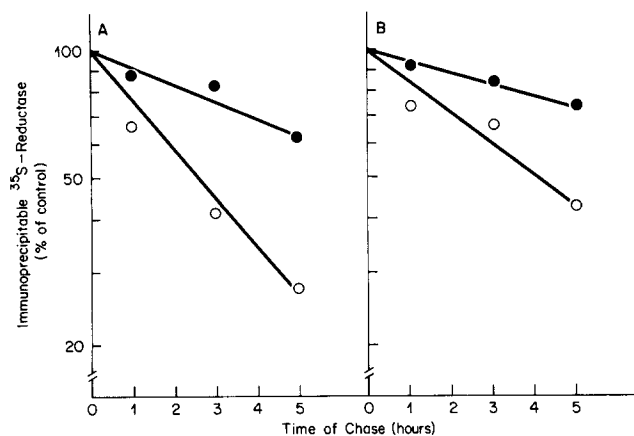


Figure 12. Effect of sterols on the turnover of ^{35}S -labeled HMG CoA reductase in pRed-227-transfected cells (A) and pCarbh⁻-transfected cells (B). Cells were plated in 60-mm dishes on day 0 by the standard procedure. pRed-227-transfected cells were grown in the presence of 1 μM compactin, and pCarbh⁻-transfected cells were grown in 10 μM compactin. On day 3, the cells were incubated in 40 μM compactin for 4 h, labeled with [^{35}S]methionine for 40 min, chased with medium containing 0.3 mM methionine in the absence (●) or presence of 10 $\mu\text{g}/\text{ml}$ cholesterol plus 1 $\mu\text{g}/\text{ml}$ 25-hydroxycholesterol (○) as described in the legend to Fig. 7. The amount of ^{35}S -labeled reductase was determined at the indicated times after the addition of sterols by subtracting the nonimmune immunoprecipitation value from the anti-reductase immunoprecipitation value. The "100% of control" value was 888 and 403 dpm/ μg for pRed-227- and pCarbh⁻-transfected cells, respectively. HMG CoA reductase represented 0.22 and 0.18% of the protein synthesized from [^{35}S]methionine in pRed-227- and pCarbh⁻-transfected cells, respectively. Each value represents the mean of duplicate incubations. The variation in duplicate values averaged <3%.

reducing environment, the active site of either the normal or the mutated enzyme cannot have resided in the lumen of the ER. Thus, the seventh membrane-spanning region of the p ΔTM 4+5-encoded enzyme must have been in its normal orientation. We do not know the orientation of the other membrane-spanning regions. A normal topology might be expected based on the earlier studies of Friedlander and Blobel (1985) with bovine opsin, a protein that also has seven membrane-spanning regions. These investigators showed that the enzyme was inserted into ER membranes in an apparently normal orientation *in vitro* even when several membrane-spanning regions were deleted.

Like normal HMG CoA reductase, the enzyme encoded by p ΔTM 4+5 had a rapid turnover rate when cells were incubated in the presence of sterols. However, unlike the case with the normal enzyme, the p ΔTM 4+5 enzyme failed to become stabilized when sterols were removed from the culture medium. These findings suggest that the intact native structure of the membrane region is necessary to stabilize the enzyme and to retain it in the ER in the absence of sterols. These data also raise the possibility that the basal unregulated rate of degradation of HMG CoA reductase is rapid and that one specific function of the membrane region is to allow stabilization when sterols are depleted.

If the current findings with HMG CoA reductase turn out to be generalizable to other ER proteins, they would suggest that resident proteins that remain in the ER would be rapidly

Table III. Effect of Sterols on the Half-life of HMG CoA Reductase in pRed-227- and pCarbh⁻-transfected cells

Transfected cDNA	Addition of sterols to medium	Half-life		Stimulation of degradation by sterols
		Mean	Range	
		<i>h</i>		<i>-fold</i>
pRed-227 (<i>n</i> = 3)	-	7.0	5.0-9.0	3.7
	+	1.9	1.5-2.2	
pCarbh ⁻ (<i>n</i> = 3)	-	9.2	7.0-10.5	2.5
	+	3.8	2.2-4.0	

Transfected cell lines grown in 1-10 μM compactin were set up for experiments, preincubated with 40 μM compactin, pulse-labeled with [^{35}S]methionine, and chased for 5 h in the presence or absence of 10 $\mu\text{g}/\text{ml}$ cholesterol and 1 $\mu\text{g}/\text{ml}$ 25-hydroxycholesterol as described in the legend to Fig. 7. The amount of immunoprecipitable ^{35}S -labeled reductase was quantified at various times of chase, and these values were used to calculate the half-life as shown in Fig. 7. The values shown represent the mean and range of three separate experiments.

exported or degraded unless they can interact with some factor that allows them to be held in place. This hypothesis could be tested by preparing cDNAs encoding mutated forms of other ER proteins that normally have much longer half-lives than HMG CoA reductase. If these long half-lives are dependent on a specific structural feature of the protein, then mutations should eliminate this protection and lead to rapid degradation. Unfortunately, there are very few ER proteins whose genes have been cloned so as to allow such a study. The major candidate for such study would be one of the cytochrome P-450 proteins whose cDNA has been isolated (Mizukami et al., 1983).

One feature of the membranous domain of HMG CoA reductase that might contribute to its stabilization is its carbohydrate component. Hamster HMG CoA reductase contains one consensus site for asparagine-linked glycosylation in the spacer between membrane-spanning regions 6 and 7 (Liscum et al., 1985). The human HMG CoA reductase contains this glycosylation site plus another glycosylation site in the same spacer segment (Luskey and Stevens, 1985). In some circumstances, the presence of N-linked sugars can be a necessary factor in the transport of proteins (such as vesicular stomatitis virus G protein) from the ER to the Golgi complex (Machamer et al., 1985). In the case of HMG CoA reductase, however, the N-linked sugars do not seem to play such a role. When asparagine 281 was replaced with a glutamine, the enzyme was degraded at a near-normal rate, and it continued to show stabilization in the absence of sterols. In addition, the morphology of the crystalline ER that housed the carbohydrate-deficient enzyme was unchanged. These studies show that glycosylation of reductase is not crucial to the structure of the ER or the localization of the enzyme in the ER and is also not essential for the sterol-regulated degradation of the enzyme.

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