Immunological Evidence for Eight Spans in the Membrane Domain of 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase: Implications for Enzyme Degradation in the Endoplasmic Reticulum

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Abstract. We have raised two monospecific antibodies against synthetic peptides derived from the membrane domain of the ER glycoprotein 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the rate limiting enzyme in the cholesterol biosynthetic pathway. This domain, which was proposed to span the ER membrane seven times (Liscum, L., J. Finer-Moore, R. M. Stroud, K. L. Luskey, M. S. Brown, and J. L. Goldstein. 1985. *J. Biol. Chem.* 260:522–538), plays a critical role in the regulated degradation of the enzyme in the ER in response to sterols. The antibodies stain the ER of cells and immunoprecipitate HMG-CoA reductase and HMGal, a chimeric protein composed of the membrane domain of the reductase fused to *Escherichia coli* β-galactosidase, the degradation of which is also accelerated by sterols. We show that the sequence Arg^{224} through Leu^{242} of HMG-CoA reductase (peptide G) faces the cytoplasm both in cultured cells and in rat liver, whereas the sequence Thr^{284} through Glu^{302} (peptide H) faces the lumen of the ER. This indicates that a sequence between peptide G and peptide H spans the membrane of the ER. Moreover, by epitope tagging with peptide H, we show that the loop segment connecting membrane spans 3 and 4 is sequestered in the lumen of the ER. These results demonstrate that the membrane domain of HMG-CoA reductase spans the ER eight times and are inconsistent with the seven membrane spans topological model. The approximate boundaries of the proposed additional transmembrane segment are between Lys^{248} and Asp^{276}. Replacement of this 7th span in HMGal with the first transmembrane helix of bacteriorhodopsin abolishes the sterol-enhanced degradation of the protein, indicating its role in the regulated turnover of HMG-CoA reductase within the endoplasmic reticulum.

3-HYDROXY-3-METHYLGLUTARYL coenzyme A (HMG-CoA)[1] reductase catalyzes the conversion of HMG-CoA to mevalonate (Durr and Rudney, 1960), the precursor for a wide variety of metabolites that play central roles in cellular functions. These products of the mevalonate pathway include sterols, ubiquinone, dolichols, isopentenyladenine, heme A and the isoprenyl moieties of proteins (for a recent review see Goldstein and Brown, 1990). In mammalian cells, HMG-CoA reductase is the major regulatory enzyme in this pathway and is subject to complex metabolic control ensuring an adequate supply of intermediates and products of this pathway. This is achieved by regulation of transcription of the HMG-CoA reductase gene (Liscum et al., 1983*a*; Osborne et al., 1985), regulation of translation of its mRNA (Tanaka et al., 1983; Peffley and Sinensky, 1985; Nakanishi et al., 1988), controlled degradation of the reductase protein (Sinensky and Logel, 1983; Edwards et al., 1983; Gil et al., 1985; Chin et al., 1985), and modulation of enzyme activity (Hardie et al., 1989; Roitelman and Shechter, 1984).

Through cloning of its cDNA, the primary structure of HMG-CoA reductase from many eukaryotes, including yeast (Basson et al., 1988), plants (Learned and Fink, 1989; Caelles et al., 1989), fly (Gertler et al., 1988), schistosomes (Rajkovic et al., 1989), sea urchin (Woodward et al., 1988), frog (Chen and Shapiro, 1990), rodents (Chin et al., 1984; Skalnik and Simoni, 1985), and humans (Luskey and Stevens, 1985) has been deduced. Mammalian HMG-CoA reductase is a 97-kD membrane-bound "high mannose" glycoprotein of the ER (Liscum et al., 1983*b*; Brown and Simoni, 1984). The enzyme is co-translationally inserted into the ER membrane in a signal recognition particle-dependent manner (Brown and Simoni, 1984), and it lacks a cleavable signal sequence (Brown and Simoni, 1984; Chin et al., 1984). Under certain conditions, the enzyme appears to function as a homodimer (Edwards et al., 1985; Ness et al., 1985; Roitelman and Shechter, 1986). The catalytic site,

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1. *Abbreviations used in this paper*: HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; PPD, purified protein derivative; SLO, streptolysin O.

contained within the COOH-terminal two-thirds of the polypeptide chain, faces the cytoplasm (Liscum et al., 1983*b*, 1985), where the soluble substrates are found. Not surprisingly, the catalytic domain of the enzyme displays a high degree of homology among species.

The NH_2-terminal one-third of eukaryotic HMG-CoA reductase, which lacks any catalytic activity is, however, also homologous among many species (with the exception of yeast, plants, and schistosomes). This hydrophobic domain of the protein, which bears the carbohydrate moiety, anchors the reductase in the ER membrane (Liscum et al., 1983*b*, Chin et al., 1984; Brown and Simoni, 1984; Liscum et al., 1985). Moreover, the membrane domain of HMG-CoA reductase, when fused to *Escherichia coli* β-galactosidase, is necessary and sufficient to confer ER localization onto the chimeric HMGal protein (Skalnik et al., 1988; Chun et al., 1990).

HMG-CoA reductase belongs to a class of proteins which are degraded in a pre-Golgi compartment, probably within the ER, and includes individual subunits of oligomeric complexes or abnormal proteins (reviewed by Bonifacino and Lippincott-Schwartz, 1991). This novel process appears to constitute a cellular mechanism for "quality control" of newly synthesized proteins which are destined to exit the ER (Klausner and Sitia, 1990). For most proteins, the ER degradation is rapid and does not appear to be regulated. The degradation of HMG-CoA reductase, which exhibits some of the characteristics of the ER degradative pathway (Chun et al., 1990; Inoue et al., 1991), is unique in that the reductase is a normal resident ER protein and that the rate with which its degradation proceeds is dictated by the cellular needs for sterols and/or other metabolites of the mevalonate pathway.

Although much has been learned about the general architecture of HMG-CoA reductase, the secondary and tertiary structures of the protein remain unknown. Therefore, studying the structure of HMG-CoA reductase is of prime importance to gain insight into the structural basis underlying its function. It has been demonstrated that the membrane-bound domain of HMG-CoA reductase is necessary and sufficient for the regulated degradation of the enzyme (Gil et al., 1985; Skalnik et al., 1988; Chun et al., 1990). Based on limited proteolysis and hydropathy plot analyses of its deduced primary structure, Liscum et al. proposed a topological model in which the NH_2-terminal 340 amino acids of HMG-CoA reductase span the ER membrane seven times (Liscum et al., 1985). We have recently provided evidence indicating that HMG-CoA reductase spans the ER membrane eight times. This was based on experiments in which the insertion of mutant versions of HMG-CoA reductase into ER vesicles was studied in vitro and on the in vivo glycosylation pattern of mutants of HMGal with engineered glycosylation sites (Olender and Simoni, 1992). In this report we provide immunological evidence for the additional membrane-spanning segment. Moreover, we show that replacing this segment with the first transmembrane span of bacteriorhodopsin eliminates the sterol-accelerated degradation of HMG-CoA reductase.

Materials and Methods

Materials

Peptides were custom-synthesized by Multiple Peptide System (San Diego, CA.) Tuberculin Purified Protein Derivative (PPD) was purchased from Statens Seruminstitut (Copenhagen, Denmark). Complete and incomplete Freund's adjuvant and rhodamine-conjugated goat anti-mouse IgG were obtained from Cappel Organon Teknika Corp. (West Chester, PA). IgG purification kit was from Pierce Chemical Co. (Rockford, IL). Immunoglobulin detection kit was purchased from HyClone (Logan, UT). Goat anti-rabbit IgG conjugated to 5-nm colloidal gold was from Janssen Pharmaceutica (Piscataway, NJ). Glutaraldehyde, mevalonate, digitonin, cholestyramine, and FITC-labeled goat anti-rabbit IgG were from Sigma Chemical Co. (St. Louis, MO). Cholesterol was from Boehringer-Mannheim Biochemicals (Indianapolis, IN). 25-hydroxycholesterol was purchased from Steraloids Inc. (Wilton, NH). Streptolysin O (SLO) was obtained from Wellcome Diagnostics (Dartford, England). 1,4-diazobicyclo-[2.2.2]-octane was from Aldrich Chemical Co. (Milwaukee, IL). [^{35}S]-methionine (sp. act. > 1,000 Ci/mmol) and oligonucleotide-directed in vitro mutagenesis kit were from Amersham Corp. (Arlington Heights, IL). pTZ18R phagemid was from Pharmacia LKB Biotechnology Inc. (Piscataway, NJ). Protein A Bacterial Adsorbent, Tran^{35}S-label metabolic labeling reagent (sp. act. > 1,000 Ci/mmol) and mouse anti-β-tubulin mAb (TUB2.1) were from ICN Biomedicals (Irvine, CA). Mouse anti-β-galactosidase mAb was purchased from Promega (Madison, WI) and rabbit anti β-galactosidase polyclonal antibodies were from 5 prime -> 3 prime, Inc. (Boulder, CO.). Oligonucleotides, synthesized on a DNA synthesizer (model 380B; Applied Biosystems, Foster City, CA) in the Department of Biological Sciences, Stanford University (Stanford, CA) were purified on OPC columns. Compactin was a gift from Akira Endo (Tokyo-Noko University, Tokyo, Japan). Rabbit polyclonal antibodies against the catalytic domain of HMG-CoA reductase were kindly provided by Hans-Stephan Jenke, (Institute für Zellchemie, München, Federal Republic of Germany). Anti HMG-CoA reductase mAb (A9) was a generous gift from Y. K. Ho (University of Texas Health Science Center, Dallas, TX).

Cells

CHO cells expressing the HMGal fusion protein (CHO-HMGal) (Skalnik et al., 1988; Chun et al., 1990) or the Span7-HMGal mutant (Chun and Simoni, 1992) were maintained in MEM supplemented with 5% FCS and 250 μg/ml Geneticin. Elevated levels of HMG-CoA reductase and HMGal were induced by switching the cells to MEM supplemented with 5% lipid-poor serum (Rothblat et al., 1976) and 1 μM compactin and 100 μM mevalonate (Induction Medium; Chun et al., 1990; Roitelman et al., 1991). C100, an SV-40-transformed BHK cell line adapted to grow in the presence of 225 μM compactin (Hardeman et al., 1983), was maintained in MEM supplemented with 5% lipid-poor serum and 64 μM compactin. COS cells were maintained in DME supplemented with 5% FCS.

Generation of Anti-Peptide Antibodies

The dry peptides, synthesized with an extra NH_2-terminal lysine, were directly dissolved at 1 mg/ml in PPD, which is supplied by the manufacturer as a 1 mg/ml solution in PBS. The relative molecular weight of the protein component in PPD is about 10,000 (Lachmann et al., 1986), therefore the molar ratio between a 20-residue long peptide and the carrier protein is ~5. Glutaraldehyde was added to a final concentration of 0.1% (vol/vol), and the mixture was stirred overnight at room temperature. Aliquots of the mixture were acetone precipitated, PPD conjugates were collected by centrifugation, dried under reduced pressure, redissolved in PBS at 1 mg/ml, and kept frozen at −20°C until use.

New Zealand White rabbits were first immunized by intramuscular injection of 250 μg of PPD-coupled peptides emulsified in complete Freund's adjuvant. Rabbits were subsequently injected with 500 μg of coupled peptides, emulsified in incomplete Freund's adjuvant, at 4-wk intervals. Two rabbits were immunized with each peptide, blood was collected from the ear vein, and sera were prepared and pooled. The serum was tested by ELISA for anti-peptide antibodies one week after each injection. IgG fractions were purified on Agarose-Protein A columns (Pierce Chemical Co.) according to the manufacturer's protocol.

Metabolic Labeling and Immunoprecipitation

Cells were metabolically labeled with [^{35}S]methionine or Tran^{35}S-label, and HMG-CoA reductase and HMGal were immunoprecipitated, as previously described (Chun et al., 1990).

Indirect Immunofluorescence

Cells were grown on 12-mm glass coverslips in 24-well cell culture dishes.

Elevated cellular levels of HMG-CoA reductase and HMGal were induced, as described above. Cells were washed twice in PBS and fixed in 2% (wt/vol) paraformaldehyde in PBS for 15 min at room temperature. Excess paraformaldehyde was neutralized with 100 mM NH_4Cl for 10 min, and the cells were further washed three times with PBS. Selective permeabilization of the plasma membrane was achieved by one of the following protocols: (*a*) 15 min on ice with 10 μg/ml digitonin in 0.3 M sucrose/0.1 M KCl/2.5 mM $MgCl_2$/1 mM EDTA/10 mM Pipes, pH 6.8; (*b*) 15 min on ice with 10 U/ml of SLO in PBS/2 mM DTT. The SLO solution was then removed and cells were incubated in fresh PBS at 37°C for an additional 10 min; (*c*) semi-intact cells prepared by the nitrocellulose overlay method (Beckers et al., 1987; see below). Complete permeabilization of cellular membranes of fixed cells was by treatment with 0.25% Triton X-100 in PBS for 5 min on ice. After permeabilization by either protocol, the cells were washed three times with PBS and incubated with 5% BSA in PBS for 1 h at room temperature. The blocking solution was removed and fresh BSA solutions containing primary antibodies, at the appropriate dilution, were added and allowed to incubate at room temperature for 1 h. The cells were washed three times in 0.5% BSA/PBS, and fluorescent secondary antibodies were added in 5% BSA/PBS for 2 h. After three washes in 0.5% BSA/PBS and an additional three times in PBS, the coverslips were rinsed in H_2O and mounted on glass slides in a drop of mounting solution (25 mg/ml of 1,4-diazobicyclo[2.2.2]-octane in 90% glycerol/0.1 M Tris-Cl, pH 9; Johnson et al., 1982). Cells were viewed and photographed in a Zeiss epifluorescence microscope (Carl Zeiss, Oberkochen, Germany).

Semi-intact Cells

The medium of CHO-HMGal cells, grown as a monolayer in 30 mm dishes, was aspirated and circles of nitrocellulose, pre-wetted in PBS, and blotted between sheets of Whatman paper for 5 s under a pressure of about 1 kg, were overlaid on top of the monolayer for 60 s. The nitrocellulose was then gently lifted from the monolayer and the cells were processed for immunofluorescence analysis, as described above. For microscopy, a drop of mounting glycerol solution was added to the dish and a glass coverslip was placed on top of the monolayer. Cells were viewed while on the dish.

Construction of HMGal/Dins(Δ)

HMGal/Dins(Δ) is a mutant of HMGal in which the sequence His^{268} through Glu^{302} (overlapping the H epitope and the native glycosylation site of HMG-CoA reductase) has been moved from its native position into the D loop segment connecting the putative 3rd and 4th membrane spans (Liscum et al., 1985) of HMG-CoA reductase (i.e., in between residues Thr^{119} and Glu^{123}; see Figs. 1 and 8). This mutant was constructed as follows: the EcoRI/PstI DNA fragment of pSRα-HMGal (HMGal cloned in pcDLSRα296 (Takebe et al., 1988); Chun and Simoni, 1992), which encodes the entire membrane domain of HMG-CoA reductase (residues 1–449), was subcloned into the multifunctional phagemid pTZ18R to yield pTZ-HM. Through site-directed mutagenesis, a unique NheI site (underlined) was created after Thr^{119}, using the mutagenic oligonucleotide 5′-AAAGAACTGACAGGGCTAGCTGAAGCTTTGCCC-3′. The native H epitope (the sequence Thr^{284} through Glu^{302}) was destroyed by site-directed deletion using the 36mer 5′-TCCCCTCAGAACAGCACA/CCAAGTGTTTCTCTCTGG-3′ (where / indicates the site of deletion), bridging Thr^{283} and Pro^{303}. The mutated membrane domain was swapped, as an EcoRI-PstI fragment, with the wild-type membrane domain back into pSRαHMGal, creating pSRαHMGal(NheΔH). A DNA which includes the sequence encoding His^{268} through Glu^{302} was generated by annealing four oligonucleotides comprising two self-complementary sets (underlined nucleotides denote NheI overhangs): 5′-CTAGGGTCTAGACATGCTCACAGTCGCTGGATAGCTGATCCTTCCCCTCAG-3′ and 3′-CCAGATCTGTACGAGTGTCAGCGACCTATCGACTAGGAAGGGGAGTCTTGTCGTGT-5′ and 5′-AACAGCACAACAGAACATTGTAAAGTTTCCTTGGGACTAGATGAAGATGTGTCCAAGAGAATTGAA-3′ and 3′-TGTCTTGTAACATTTCAAAGGAACCCTGATCTACTTCTACACAGGTTCTCTTAACTTGATC-5′. This 113-bp DNA was inserted into the NheI-cut pSRαHMGal(NheΔ). The modified sequence in this region reads: . . . Thr^{119}(Gly)(Ser)(Arg)-His^{268} . . . . Glu^{302}-(Leu)Glu^{123}. . . . , where the residues indicated in parentheses were engineered into the oligonucleotides to create a diagnostic XbaI restriction site and to maintain the reading frame. The desired mutations were confirmed by restriction analyses and DNA sequencing of the relevant regions.

Transient expression of HMGal and HMGal/Dins(Δ) in COS Cells

For metabolic labeling, sub-confluent COS cells, grown in 150 mm dishes in DME containing 5% FCS, were transfected with 100 μg of wild-type pSRαHMGal or pSRαHMGal/Dins(Δ) by the calcium phosphate precipitation method (Graham and van der Eb, 1973). In separate experiments we have established that maximal expression of β-galactosidase activity of the chimeric proteins is reached 3 d after transfection. Two days after transfection, the cells were refed with Induction Medium. On the third day, the cells were switched to methionine-free MEM containing 5% LPS and compactin and mevalonate and starved for 1 h. Cells were then pulsed with 250 μCi of Tran^{35}S-label (125 μCi/ml) for 2 h at 37°C. Cells were washed three times in PBS and lysed and processed for immunoprecipitation, as previously described (Chun et al., 1990).

For immunofluorescence studies, COS cells were plated on glass coverslips in 60-mm dishes and transfected with plasmid DNA's, as described above. On the second day posttransfection, the coverslips were removed and inserted to individual wells of 24-well dishes and the cells were refed with 1 ml of Induction Medium. On the third day, cells were washed and processed for immunofluorescence inspection.

Immunogold Cytochemistry

Rats, adapted to 12 h dark/12 h light cycle, were fed ad libitum powdered chow containing 5% (wt/wt) cholestyramine resin. After 5 d, the diet was switched to chow containing 5% (wt/wt) cholestyramine and 0.2% (wt/wt) compactin for an additional 2 d. This diet regimen has been shown to increase hepatic levels of HMG-CoA reductase (Tanaka et al., 1982; Clark et al., 1984). Near the end of the dark cycle, the animals were anesthetized with ether and their livers perfused in situ with saline then with fixative solution (0.1% glutaraldehyde/0.2% picric acid/0.5 mM $CaCl_2$ in 0.1 M $NaPO_4$, pH 7.4). Portions of the liver, minced into 1 mm^3 cubes, were postfixed for an additional 4 h. The cubes were washed, stained en bloc with uranyl acetate, dehydrated, and embedded in LRGold, as described by Berryman and Rodewald (1990). Immunocytochemical localization of the G and H epitopes was done following procedures outlined by Berryman and Rodewald to enhance cell membrane contrast (Berryman and Rodewald, 1990). Briefly, ultra-thin sections of embedded tissue were incubated overnight with the primary antibodies diluted in 5% normal goat serum in TBS. After washes, the sections were incubated with goat anti-rabbit IgG conjugated to 5 nm colloidal gold, postfixed with 2% glutaraldehyde and 2% osmium tetroxide, and then stained with lead citrate.

Results

Antibodies against Peptides in the Membrane Domain of HMG-CoA Reductase

The current topological model for the membrane domain of hamster HMG-CoA reductase, schematically drawn in Fig. 1 *A*, is based on hydropathy plots of the amino acid sequence of the NH_2-terminal 350 residues (Liscum et al., 1985). An alternative model, which has recently emerged from our laboratory (Olender and Simoni, 1992), is depicted in Fig. 1 *B*. The major difference between the two models is the existence of an additional membrane span between the putative 6th and 7th spans of the original model, increasing the total number of spans to 8, and thereby reversing the orientation of spans 1–6 (Olender and Simoni, 1992).

To test the 8-span model by an independent approach, we prepared antibodies against synthetic peptides corresponding to several regions in the membrane domain of HMG-CoA reductase. The peptides were coupled to PPD which elicits a strong immune response, yet does not give rise to antibodies against itself (Lachmann et al., 1986). Out of four different peptides injected, only two anti-peptide antisera recognized HMG-CoA reductase in cells (see below), although all antibodies recognized their respective antigenic peptides in ELISA (data not shown). The peptides that produced anti-reductase antibodies correspond to residues Arg^{224} through Leu^{242} in loop G (H_2N-RESREGRPIWQLSHFARVL-COOH, peptide G; Fig. 1 *B*) and to residues Thr^{284} through Glu^{302} in loop H (H_2N-TEHSKVSLGLDE-

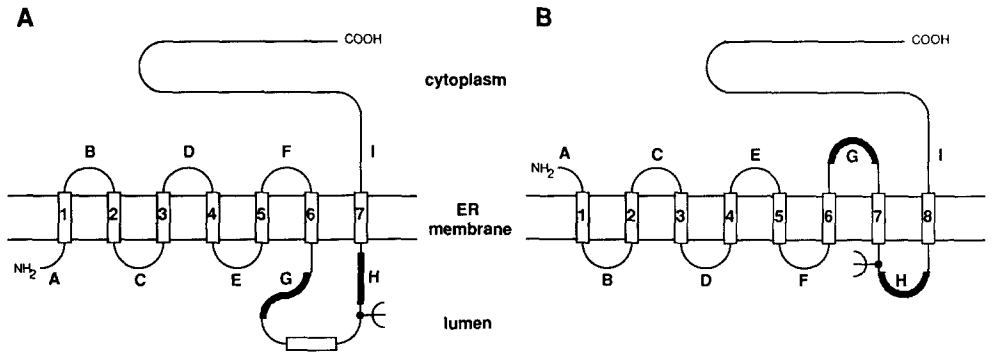


Figure 1. Schematic models for the topology of the membrane domain of HMG-CoA reductase. The seven transmembrane spans model proposed by Liscum et al. (1985). (*B*) The eight transmembrane spans proposed in this report. The transmembrane spans are represented by open boxes transversing the ER membrane and are numbered 1-7 (*A*) or 1-8 (*B*). The hydrophilic loop segments between adjacent spans are designated *A–H*. The *fork* symbol indicates the glycosylation site at Asn^{281}. Peptides G and H are represented by the heavy line in loops G and H, respectively.

DVSKRIE-COOH, peptide H; Fig. 1 *B*) in the sequence of both Chinese and Syrian hamster HMG-CoA reductase (Chin et al., 1984; Skalnik and Simoni, 1985). The antibodies produced are designated anti-G and anti-H, respectively.

Both anti-G (Fig. 2, lane *1*) or anti-H (Fig. 2, lane *3*) immunoprecipitate two proteins of M_r 97,000 and 150,000 from ^{35}S-labeled CHO-HMGal lysates. The mobilities of these bands in SDS gels are consistent with the sizes of HMG-CoA reductase (97 kD; Hardeman et al., 1983; Chin et al., 1984; Skalnik and Simoni, 1985) and HMGal (150 kD; Skalnik et al., 1988). These proteins were specifically precipitated as they were competed by an excess of the corresponding free peptides (Fig. 2, lanes *2* and *4*). Furthermore, the same proteins were precipitated with a mixture of antibodies directed against β-galactosidase and the cytoplasmic domain of HMG-CoA reductase (Fig. 2, lane *5*). The latter antibody mixture, unlike the anti-peptide antibodies, also precipitated a ~63-kD protein (Fig. 2), most likely the cytoplasm-facing proteolytic fragment of HMG-CoA reductase (Hardeman et al., 1983; Liscum et al., 1985).

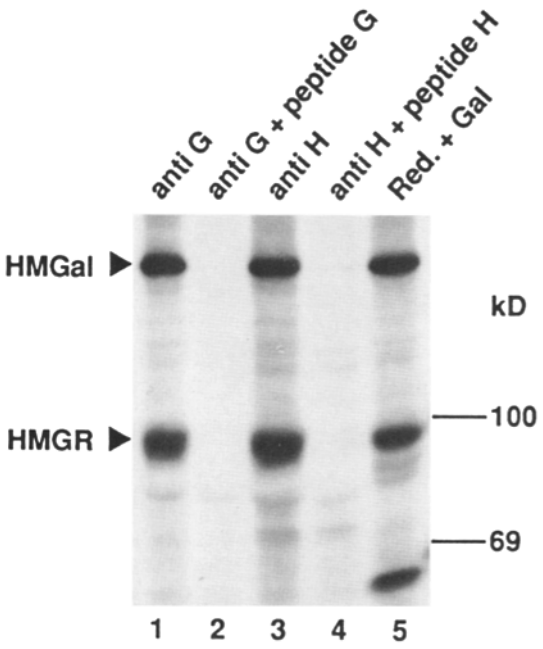


Figure 2. Specificity of anti-G and anti-H antibodies. CHO-HMGal cells were grown in Induction Medium for 24 h before the experiment. Cells were starved for 1 h in methionine-free MEM and then labeled for 4 h with 150 μCi of [^{35}S]methionine (0.5 mCi/ml; 0.3 ml). Cells were washed and lysed, as described (Chun et al., 1990). After centrifugation, the cleared post-nuclear lysate was divided to five equal aliquots and 5 μl of anti-G (lanes *1* and *2*), anti-H (lanes *3* and *4*) IgGs or a 1:1 mixture of anti-reductase and anti-β-galactosidase (lane *5*) antibodies were added. For peptide competition, 50 μg of free peptide G (lane *2*) or peptide H (lane *4*) were added to the cell lysate. Immunoprecipitation was carried out as described (Chun et al., 1990), and precipitates were resolved by 8% SDS-PAGE. Relative molecular weight standards are indicated on the right hand side. *HMGR*, HMG-CoA reductase and *HMGal*, the fusion protein between the membrane domain of HMG-CoA reductase and β-galactosidase, are indicated by arrowheads.

To demonstrate unequivocally that the anti-peptide antibodies react with HMG-CoA reductase and HMGal, we carried out an immunodepletion experiment in which ^{35}S-labeled lysates of CHO-HMGal cells were first incubated with one antibody followed by removal of the immune complex. The supernatant solution was then taken through a second round of immunoprecipitation with a different antibody (Fig. 3). When pre-immune sera were used in the first round of precipitation (Fig. 3 *A*, lanes *1* and *9*), anti-G and anti-H precipitated the 97- and 150-kD proteins in the second precipitation step (Fig. 3 *A*, lanes *2* and *10*, respectively). When the lysate was first treated with anti-G (Fig. 3 *A*, lanes *3*, *5*, and *7*), neither bands were precipitated in the second step with either anti-H (Fig. 3 *A*, lane *4*), anti-HMG-CoA reductase (Fig. 3 *A*, lane *6*), or anti-β-galactosidase antibodies (Fig. 3 *A*, lane *8*). Conversely, immunoprecipitation with the anti-H antibody (Fig. 3 *A*, lanes *11*, *13*, and *15*) depleted the lysate of the 97- and 150-kD proteins, such that they could not be further immunoprecipitated by either anti-G (Fig. 3 *A*, lane *12*), anti-HMG-CoA reductase (Fig. 2 *A*, lane *14*), or anti-β-galactosidase (Fig. 3 *A*, lane *16*). In Fig. 3 *B* it is demonstrated that initial immunoprecipitations with anti-HMG-CoA reductase (Fig. 3 *B*, lanes *3* and *9*) or with anti-β-galactosidase (Fig. 3 *B*, lanes *5* and *11*) antibodies do not allow the subsequent precipitation of the 97- and 150-kD proteins by either anti-G (Fig. 3 *B*, lanes *4* and *6*) or anti-H (Fig. 3 *B*, lanes *10* and *12*) antibodies, respectively. It should be noted that anti-HMG-CoA reductase antibody also immunoprecipitates HMGal (Fig. 3 *B*, lanes *3* and *9*). This polyclonal antibody was raised against the purified 52-kD cytoplasmic fragment of rat liver HMG-CoA reductase (Jenke et al., 1981) which has been recently shown to be generated by proteolysis at Ser^{423} of the hamster sequence (Clarke and Hardie, 1990). In the construction of the

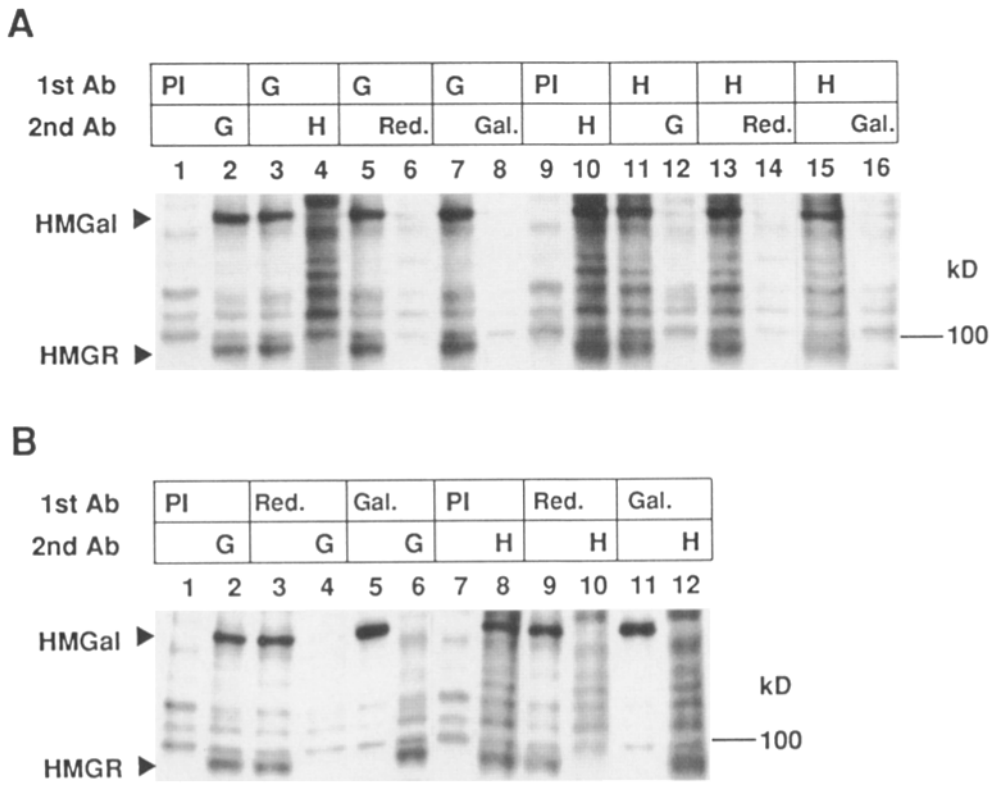


Figure 3. Anti-G and anti-H antibodies identify the same proteins in CHO-MHGal cells as do anti-HMG-CoA reductase or anti-β-galactosidase antibodies. CHO-HMGal cells were grown, induced and labeled with [^{35}S]methionine as described in the legend of Fig. 2. The cleared cell lysate was divided to 14 aliquots and 5 μl of IgG fraction were added, as follows: (*Ist Ab*) Preimmune (*PI*) serum of rabbits injected with peptide G, lanes *1* and *2* in *A* and *B*); anti-peptide G (*G*), lanes *3–8* in *A*; preimmune (*PI*) serum of rabbits injected with peptide H, *A*, lanes *9* and *10* and *B*, lanes *7* and *8*; anti-peptide H (*H*), lanes *11–16* in *A*; Anti-HMG-CoA reductase antiserum (*Red*), *B*, lanes *3*, *4*, *9*, and *10*; anti-β-galactosidase antibody (*Gal*), *B*, lanes *5*, *6*, *11*, and *12*. After incubation overnight at 4°C, Protein A bacterial adsorbent was added and further incubated for 2 h at 4°C. The tubes were spun at 16,000 *g* for 10 min, the supernatants were carefully transferred into new tubes and were either analyzed by SDS-PAGE or supplemented with different antibodies, as follows: (*2nd Ab*): Anti-G, *A*, lanes *2* and *12* and *B*, lanes *2*, *4*, and *6*; anti-H, *A*, lanes *4* and *10* and *B*, lanes *8*, *10*, and *12*; anti-HMG-CoA reductase, *A*, lanes *6* and *14*; anti-β-galactosidase, *A*, lanes *8* and *16*. The second immunoprecipitation step was carried out overnight at 4°C followed by addition of Protein A bacterial adsorbent, as described above. All immunoprecipitates were processed and resolved simultaneously by 8% SDS-PAGE. Relative molecular weight standard is indicated on the right hand side. HMG-CoA reductase (*HMGR*) and HMGal (*HMGal*) are indicated by the arrowheads.

HMGal chimera, β-galactosidase was cloned at position 449 in the hamster reductase sequence (Skalnik et al., 1988), therefore this anti-HMG-CoA reductase polyclonal antibody most likely reacts also with the region between Ser^{423} and Gln^{449} in HMGal.

These results demonstrate that the 97- and 150-kD proteins correspond to HMG-CoA reductase and HMGal, respectively, and that anti-G and anti-H antibodies specifically recognize the membrane domain of these proteins.

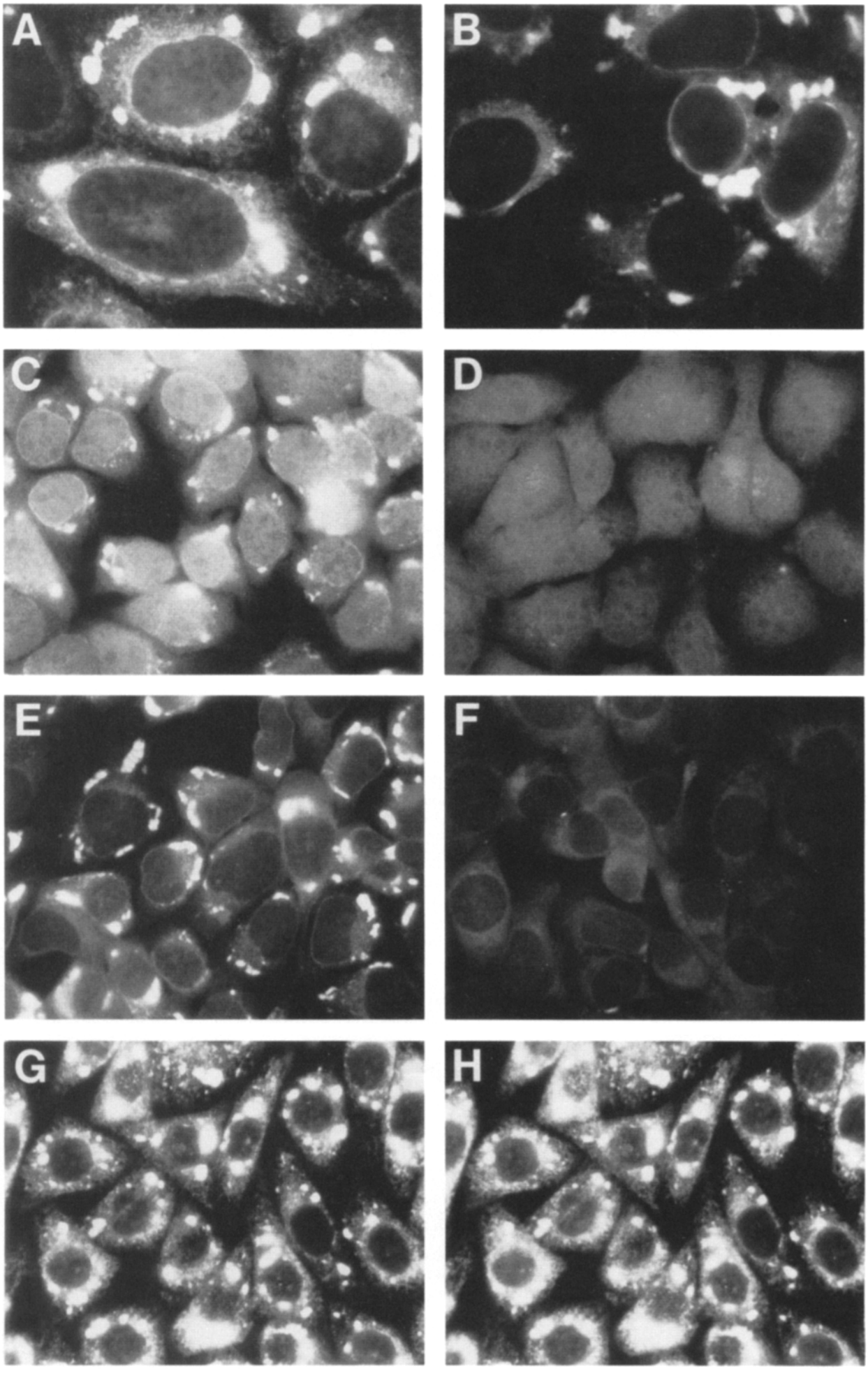
Anti-G and Anti-H Stain ER

By indirect immunofluorescence (Fig. 4, *A–F*), anti-G (*C*), and anti-H (*E*) stain fixed and Triton X-100-permeabilized C100 cells in a manner indistinguishable from the staining obtained with either polyclonal (*A*) or mAbs (*B*) directed against the cytoplasmic domain of HMG-CoA reductase. C100 cells are a variant of an SV-40–transformed BHK cell line which was adapted to grow in the presence of 225 μM compactin. As a result, these cells exhibit some 100-fold increased HMG-CoA reductase activity (Hardeman et al., 1983). Anti-G and anti-H, as well as the anti-HMG-CoA reductase antibodies, stain the nuclear envelope and a perinuclear reticulum of C100 cells. Associated with this reticulum and surrounding the nucleus are numerous intensely labeled inclusions resembling the appearance of crystalloid ER in UT-1 cells (Chin et al., 1982; Anderson et al., 1983; Pathak et al., 1986). The binding of anti-G or anti-H is specific since it could be blocked by an excess of the corresponding antigenic peptides (Fig. 4, *D* and *F*, respectively).

Additionally, the images in Fig. 4 *G* show that in CHO-HMGal cells, staining with anti-β–galactosidase antibodies also reveals a lacy structure throughout the cytoplasm, characteristic of ER, with occasional staining of the nuclear envelope. Associated with the ER are also several regions of intense fluorescence that surround or appear on opposite sides of the nucleus (Fig. 4 *G*), similar to the ones observed in C100 cells. The A9 monoclonal anti–reductase antibody identifies the same structures in CHO-HMGal cells (Fig. 4 *H*), indicating that HMGal and HMG-CoA reductase are colocalized in the same intracellular membranes. It should be noted that CHO-HMGal cells have been taken through several rounds of fluorescence-activated cell sorting to isolate population of cells expressing high HMGal activity (Chun et al., 1990). Thus, the elevated levels of HMGal in these cells may have caused proliferation of the ER similar to that observed in cells that express high levels of HMG-CoA reductase (Chin et al., 1982).

G Epitope Faces the Cytoplasm in Cultured Cells and in Rat Liver

To determine the sidedness of the binding of anti-G and anti-H antibodies to the membrane domain of HMGal/HMG-CoA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reductase, we used three different protocols for permeabilizing the plasma membrane without compromising the integrity of the ER (see Materials and Methods).

Digitonin Permeabilization. Fixed CHO-HMGal cells were permeabilized with low concentrations of digitonin. Digitonin, which complexes with β-hydroxysterols, should perforate preferentially (but not exclusively) the plasma membrane because of its high content (~90%) of cellular free cholesterol (Lange, 1991). The permeabilized cells were stained simultaneously with mouse monoclonal anti-β-galactosidase antibody and with rabbit anti-G or anti-H antipeptide antibodies, followed by species-specific fluorescent secondary antibodies. Inasmuch as anti-H antibody reacts with a lumenal epitope adjacent to the single N-glycosylation site of HMG-CoA reductase (Liscum et al., 1983*b*; Brown and Simoni, 1984) or HMGal (Skalnik et al., 1988; Olender and Simoni, 1992), this antibody should not gain access to its epitope when the ER membrane remains intact. The mouse antibody, which reacts with the cytoplasmically disposed β-galactosidase domain of HMGal, serves as a control for accessibility of antibodies to the cell interior. Mild treatment with digitonin (5–10 μg/ml) allows staining of HMGal by the anti-β-galactosidase antibody, with no significant binding of anti-H antibody to the membrane domain of the enzyme (Fig. 5, *C* and *D*). *A* and *B* of Fig. 5 show that, in cells which are first permeabilized with digitonin and then treated with Triton X-100, anti-H stains the same intracellular structures as does anti-β-galactosidase antibody, demonstrating that the inability of anti-H to stain the cells in *D* is because of inaccessibility of the antibody to its cognate epitope in the lumen of the ER (see below). Anti-G antibody, on the other hand, recognizes HMGal in digitonin-permeabilized cells (Fig. 5 *F*) as effectively as it does in Triton X-100–treated cells (Fig. 5 *H*), and stains the ER as intensely as does anti-β-galactosidase antibody (Fig. 5, *E* and *G*). These results demonstrate that, under conditions where lumenal epitopes are inaccessible to antibodies, the G epitope is readily exposed to antibodies in the cytoplasm. Similar differential staining with anti-H and anti-G in digitonin-permeabilized CHO-HMGal cells was obtained when we used the TUB2.1 monoclonal anti-β-tubulin antibody to assess accessibility to cytoplasmic epitopes (data not shown).

To rule out the remote possibility that the antigenicity of the G or H epitopes in the membrane domain of HMG-CoA reductase/HMGal has been altered by digitonin, we used alternative protocols for selective permeabilization of the plasma membrane.

Permeabilization by Streptolysin O. Streptolysin O (SLO), a pore-forming toxin produced by hemolytic streptococcus, was successfully used for selective permeabilization of the plasma membrane (Ahnert-Hilger et al., 1989). We applied reduced SLO to fixed CHO-HMGal cells at 0°C. After removing excess toxin, the cells were warmed to 37°C, and processed for immunofluorescent analysis, as described under Materials and Methods. That pores large enough to allow passage of antibodies to the cytoplasmic compartment were generated is indicated by the specific staining by the anti-β-galactosidase antibody (Fig. 6, *A* and *C*). Under these conditions, however, ER lumenal epitopes remain cryptic, as indicated by lack of staining by anti-H antibodies (Fig. 6 *B*). Nevertheless, the G epitope is fully exposed to anti-G antibodies, as demonstrated by the fluorescent labeling of the same structures that house HMGal (Fig. 6, compare *D* with *C*).

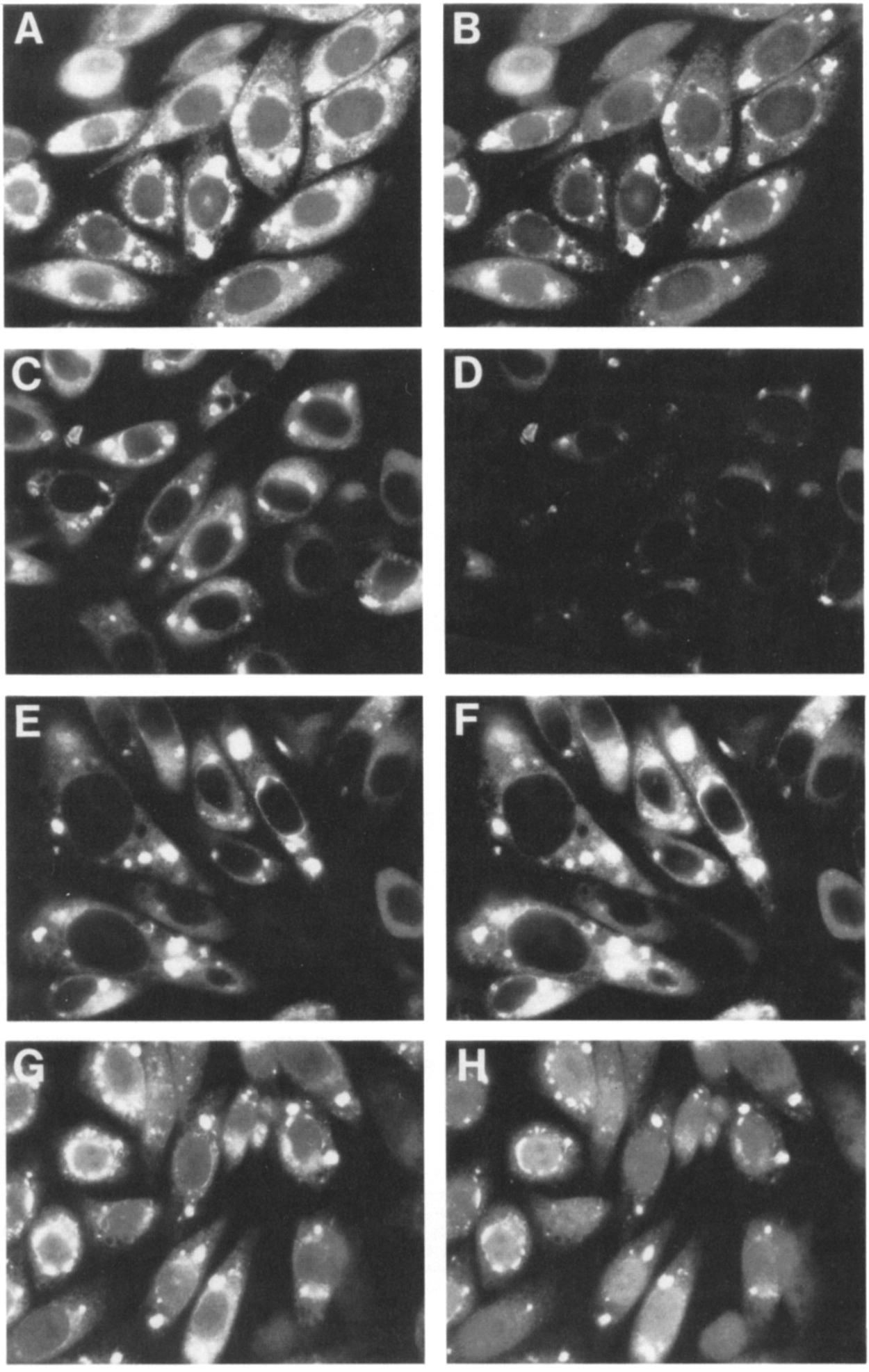
Semi-intact Cells. CHO-HMGal cells, grown on plastic dishes, were rendered semi-intact by the nitrocellulose overlay method, essentially as described by Beckers et al. (1987). By this method large perforations are created in the cell surface when portions of the plasma membrane are peeled away by an overlaid nitrocellulose filter. Such large pores allow direct access of antibodies to the cytoplasmic domain of the cell (Beckers et al., 1987). In Fig. 6 we show that antibodies did indeed gain access to the cytoplasm of the semi-intact CHO-HMGal cells, as indicated by the staining of HMGal by anti-β-galactosidase antibody (Fig. 6, *E* and *G*). That the integrity of the ER membrane was maintained is indicated by the lack of specific staining by anti-H antibody (Fig. 6 *F*). Again, the G epitope was cytoplasmically accessible to anti-G antibody (Fig. 6 *H*).

Rat Liver. Next, we examined the membrane sidedness of the G and H epitopes in situ by immunogold labeling of rat liver sections with the anti-peptide antibodies (Fig. 7). Both anti-G and anti-H antibodies label almost exclusively smooth tubules and vesicles of variable sizes and shapes located throughout the cell. Labeling was not present over mitochondria, peroxisomes, nuclei, or Golgi cisternae, but was found over some regions of rough ER (not shown). When using anti-G antibody, labeling is restricted to the cytoplasmic surfaces of these vesicles (Fig. 7 *A*). Contrary, the epitope recognized by anti-H antibody is found almost exclusively on the lumenal surfaces (Fig. 7 *B*). Thus, the results obtained from rat liver are consistent with those observed in cultured cells, demonstrating that epitope G faces the cytoplasmic surface of the ER.

The Loop Region between the 3rd and 4th Membrane Spans Is Lumenal

The results presented above demonstrate that the G epitope is exposed to the cytoplasm whereas the H epitope is lumenal, indicating that the G and H epitopes are separated by a membrane spanning segment (see Fig. 1 *B*). This is in contrast to the current topological model for the membrane spanning domain of HMG-CoA reductase in which both epitopes reside in the lumen of the ER (Liscum et al., 1985; see Fig. 1 *A*). Moreover, these data strongly suggest that the

Figure 4. Anti-G and anti-H antibodies specifically identify endoplasmic reticulum in C100 and CHO-HMGal cells. C100 (*A–F*) or CHO-HMGal (*G* and *H*) cells, grown on glass coverslips, were fixed and permeabilized with 0.25% Triton X-100, as described under Materials and Methods. C100 cells were stained with polyclonal rabbit anti-rat HMG-CoA reductase antibodies (*A*) or with the A9 mouse anti-hamster HMG-CoA reductase mAb (*B*). Cells were stained with anti-G antibody with (*D*) or without (*C*) 50 μg of competing peptide G, or with anti-H antibody with (*F*) or without (*E*) 50 μg of competing peptide H. CHO-HMGal cells were stained simultaneously with rabbit anti-β-galactosidase antibodies (*G*, FITC-labeled secondary antibody) or with the A9 antibody (*H*, rhodamine-labeled secondary antibody).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orientations of the membrane spans NH_2-terminal to epitope G are reversed relative to the plane of the ER membrane. To test this directly, we deleted the H epitope from its native position between the glycosylation site and the last membrane span, and moved it into the loop region bridging spans three and four (see scheme in Fig. 8, *bottom*). According to the seven spans model, this epitope should be exposed to the cytoplasm. COS cells were transfected with pcDLSRα plasmid carrying this mutated HMGal/Dins(Δ) chimeric gene. In parallel, we transfected COS cells with the wild-type pSRαHMGal. Fig. 8 shows that HMGal/Dins(Δ) is expressed in amounts comparable to those of wild type HMGal (compare lanes *1* and *4*). Moreover, both anti-G (lanes *2* and *5*), and anti-H (lanes *3* and *6*) antibodies precipitate the mutant and the wild type HMGal. These results suggest that the mutation in HMGal/Dins(Δ) does not grossly affect its recognition by antibodies or stability. (The higher molecular size of HMGal/Dins(Δ) is expected due to the fact that a net of 20 residues were added to the protein (see Materials and Methods).

Next, the plasma membrane of the cells expressing HMGal/Dins(Δ) was selectively permeabilized with SLO, or cells were fully permeabilized with Triton X-100. The cells were immunofluorescently stained with a mixture of anti-β-galactosidase and anti-H antibodies (Fig. 9). Clearly, while the H epitope was fully accessible to anti-H antibodies in Triton X-100–permeabilized cells (Fig. 9 *F*), this epitope was not recognized in SLO-permeabilized HMGal/Dins(Δ) cells (Fig. 9 *D*), demonstrating that the H epitope in its altered position is still sequestered in the lumen of the ER, just as it is in its native position (Fig. 9 *B*). Taken together with our recent finding that a similar mutant, HMGal/Dins(-), is glycosylated in vivo (Olender and Simoni, 1992), these results demonstrate that the loop region between the putative 3rd and 4th membrane spans is lumenally disposed.

The Sequence between the G and the H Epitopes Is Involved in the Sterol-accelerated Degradation of HMGal

The results presented above demonstrate that the G and the H epitopes are separated by a transmembrane-spanning region. Since the membrane domain of HMG-CoA reductase plays a critical role in the turnover of HMG-CoA reductase and of HMGal, we compared the regulated degradation of a mutant in which this putative transmembrane segment (Gln^{254} through Trp^{273}) has been replaced with the first transmembrane span of bacteriorhodopsin (Span7-HMGal; Chun and Simoni, 1992). Fig. 10 shows that the β-galactosidase activity of wild type HMGal rapidly drops to ~25–30% of initial activity within 24 h after addition of excess sterols to CHO-HMGal cells. We have previously shown that this drop in activity is a result of accelerated degradation of HMGal (Skalnik et al., 1988; Chun et al., 1990; Inoue et al., 1991; Roitelman et al., 1991). The Span7-HMGal mutant, however, is totally unresponsive to added sterols (Fig. 10), indicating that the sequence between Gln^{254} and Trp^{273} is critical for the sterol-accelerated degradation of HMG-CoA reductase.

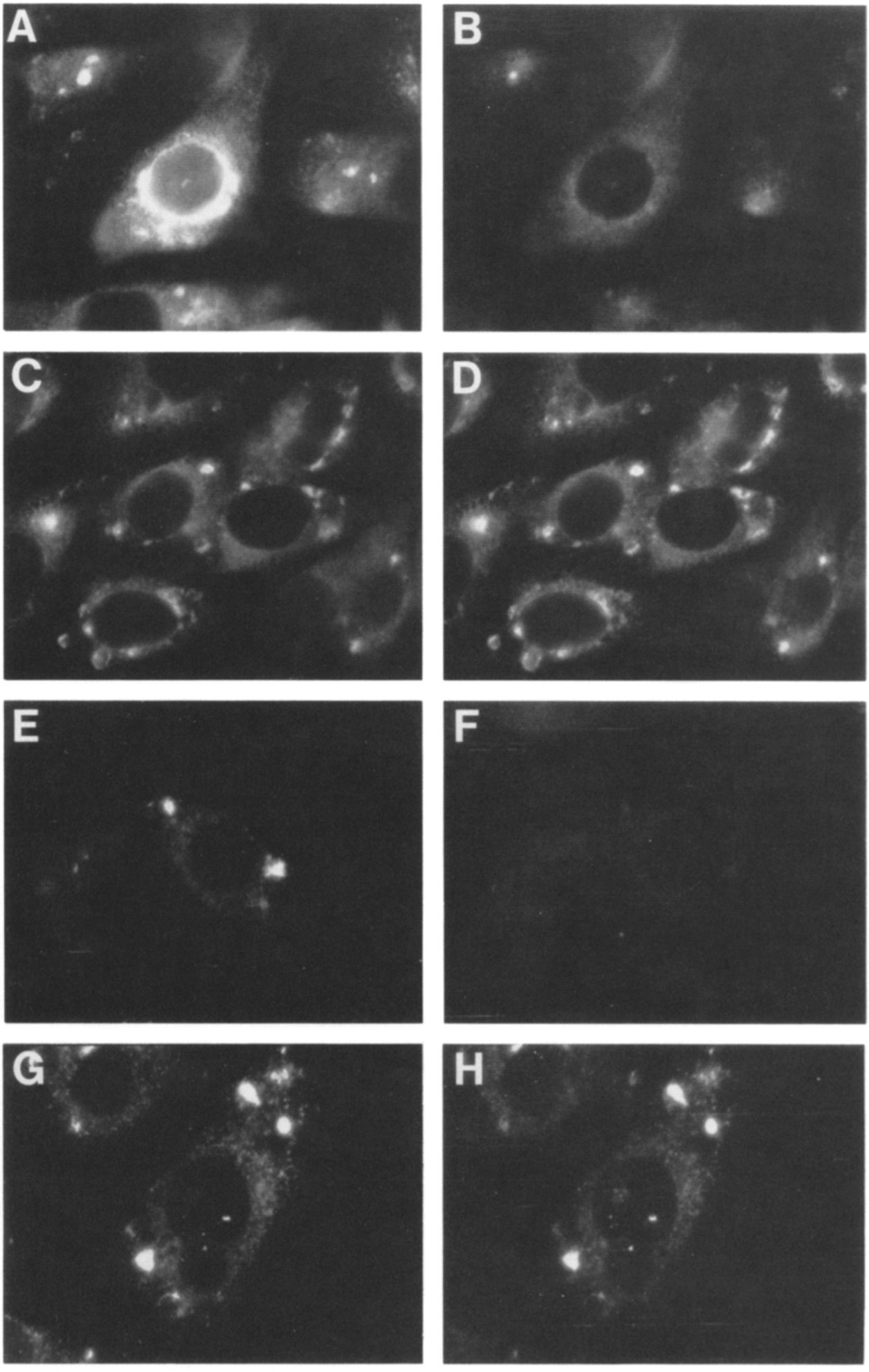
Discussion

In this report, we undertook an immunological approach to study the topology of the membrane domain of the ER glycoprotein HMG-CoA reductase, the rate-limiting enzyme in the cholesterogenic pathway. Such an approach has proven successful in studies of the topology of several membrane proteins (reviewed by Jennings, 1989).

We describe the generation and characterization of monospecific rabbit antibodies against synthetic peptides derived from the membrane-bound domain of HMG-CoA reductase. Using these antibodies, we demonstrate that the sequences Arg^{224}–Leu^{242} and Thr^{284}–Glu^{302} are on opposite sides of the ER membrane both in cultured CHO cells and in rat liver. We find that the region Arg^{224}–Leu^{242} (peptide G) faces the cytoplasm whereas peptide H (Thr^{284}–Glu^{302}) resides, as expected from its proximity to the native N-glycosylation site, in the lumen of the ER. These results indicate that a sequence between Leu^{242} and Thr^{284} in the primary structure of hamster HMG-CoA reductase constitutes a previously unrecognized membrane spanning segment of the enzyme. This region, which appears as a minor spike in hydropathy plots of HMG-CoA reductase amino acid sequences from many species (Liscum et al., 1985; Skalnik and Simoni, 1985; Rajkovic et al., 1989), was interpreted to be a part of an extended β-structure in the long lumenal loop that connects the putative 6th and 7th membrane spans in the model proposed by Liscum et al. (1985).

We have recently shown that, in in vitro translation/translocation assays, this sequence (hereafter referred to as span 7, see Figs. 1 *B* and 11) exhibits some signal recognition particle–independent membrane association and has stop transfer activity. Also, the first transmembrane span of HMG-CoA reductase (residues 1–39), which acts as the signal sequence for the protein, assumes an N-*cis* orientation both in vitro and in vivo (Olender and Simoni, 1992). Moreover, mutants of HMGal in which the native glycosylation site was eliminated and a new glycosylation site has been introduced separately into loops B, D, or F (see Fig. 1 *B*) were all glycosylated in vivo, whereas sites at loops C, E, or G were not. Taken together with the results presented here, these findings constitute evidence that the NH_2 domain of HMG-CoA reductase spans the ER membrane eight times, as shown schematically in Fig. 11. Consequently, the membrane spanning regions NH_2-terminal to span 7 are reversed in their orientation, relative to the plane of the ER membrane, as compared to the 7 span model (Liscum et al., 1985). We tested this conclusion directly using the mutant HMGal/Dins(Δ) in which the loop segment connecting membrane spans 3 and 4 was tagged with epitope H. The results

Figure 5. Anti-G antibody recognizes cytoplasmic epitopes in digitonin-permeabilized CHO-HMGal cells. CHO-HMGal cells were fixed and permeabilized with 10 μg/ml of digitonin, as described under Materials and Methods. In *A*, *B*, *G*, and *H*, cells were further treated with 0.25% Triton X-100. Cells were doubly stained with a mixture of mouse anti-β-galactosidase (*A*, *C*, *E*, and *G*, rhodamine-labeled secondary antibodies) with either anti-H antibodies (*B* and *D*) or anti-G antibodies (*F* and *H*, FITC-labeled secondary antibodies). Each pair of panels was photographed with the same exposure time and prints were made with the same time and contrast values.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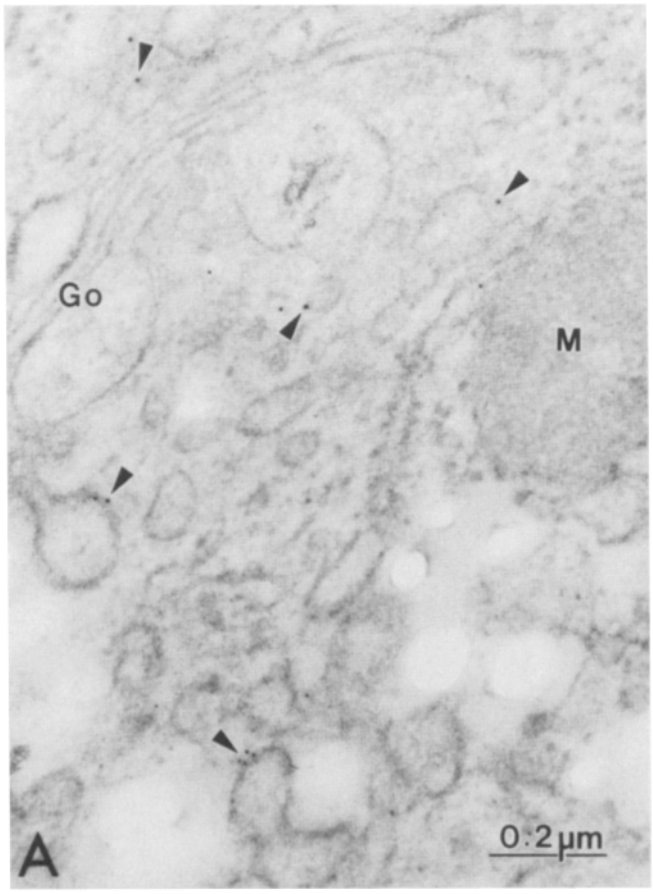

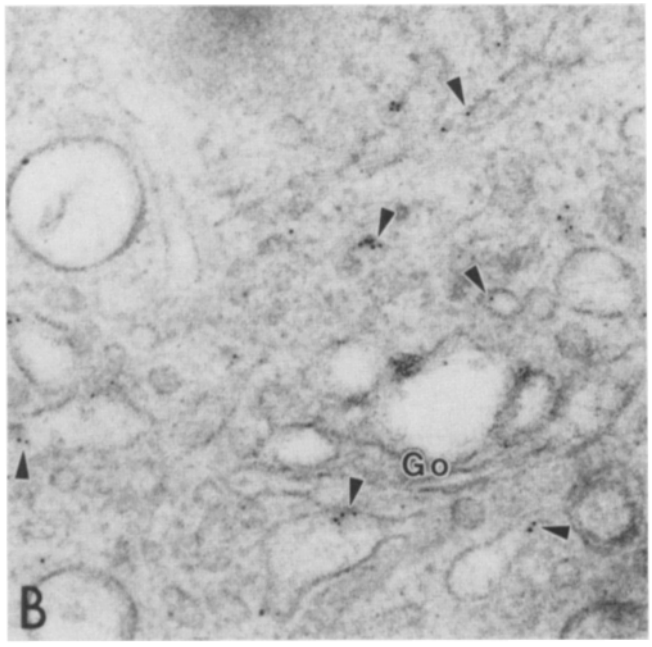

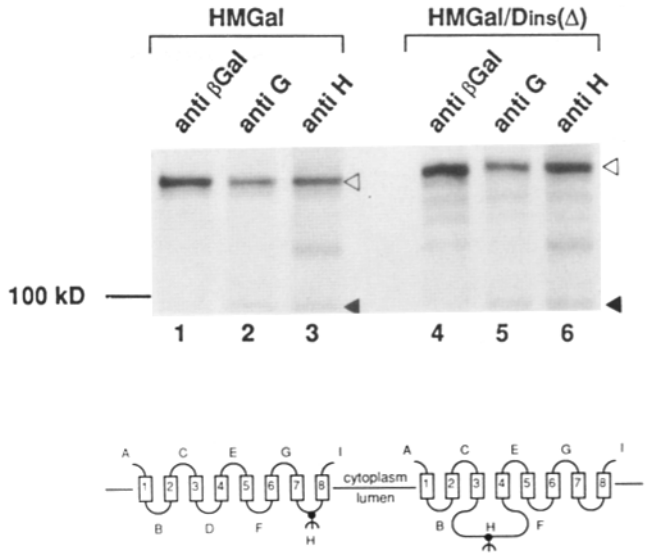

Figure 8. Expression of HMGal and HMGal/Dins(Δ) in COS cells. On Day 1, subconfluent COS cells were transfected with pSRαHMGal or with pSRαHMGal/Dins(Δ) plasmids, as described in Materials and Methods. On Day 2, the medium was removed and replaced with Induction Medium. On Day 3, cells were starved for methionine for 1 h and then labeled with 250 μCi of Trans^{35}S-label (0.5 mCi/ml) for 2 h. Cells were lysed and processed for immunoprecipitation using either anti-β-galactosidase antibody (lanes *1* and *4*), anti-G (lanes *2* and *5*), or anti-H (lanes *3* and *6*) antibodies. Immunoprecipitates were resolved by 5–15% SDS-PAGE. Closed and open arrowheads indicate HMG-CoA reductase and HMGal [or HMGal/Dins(Δ)], respectively. The migration of the ^{14}C-labeled phosphorylase *b* molecular weight standard is indicated on the left. A schematic structure of HMGal and HMGal/Dins(Δ) is drawn at the bottom.

clearly show that in its new position epitope H is still cryptic in the ER lumen, in agreement with the 8 membrane spans model.

The current analysis does not allow us to define the precise boundaries of span 7. It is possible that this span is very short, as has been suggested for several membrane spans of the α subunit of the *E. coli* F_1F_0-ATPase (Lewis et al.,

Figure 7. Immunogold cytochemical localization of the G and H epitopes in rat liver sections. LRGold-embedded ultra-thin section of livers from rats fed a cholestyramine/compactin diet were incubated with anti-G (*A*) or anti-H (*B*) antibodies followed by goat anti-rabbit IgG conjugated to 5-nm colloidal gold, as described under Materials and Methods. (*A*) Anti-G antibody routinely label the cytoplasmic surfaces of smooth tubules and vesicles (*arrowheads*). (*B*) Labeling with anti-H antibody is localized to the lumenal surfaces of smooth tubules and vesicles (*arrowheads*). *M*, mitochondrion; *Go*, Golgi complex. Bar, 0.2 μm.

Figure 6. Anti-G antibody recognizes cytoplasmic epitopes in streptolysin O- or mechanically permeabilized CHO-HMGal cells. CHO-HMGal cells were permeabilized with SLO (*A–D*) or by the nitrocellulose overlay method (*E–H*), as described under Materials and Methods. Cells were doubly stained with a mixture of mouse anti-β-galactosidase (*A*, *C*, *E*, and *G*) with either anti-H (*B* and *F*) or anti-G antibody (*D* and *H*), as described in the legend to Fig. 5. Each pair of panels was photographed with the same exposure time and prints were made with the same time and contrast values.

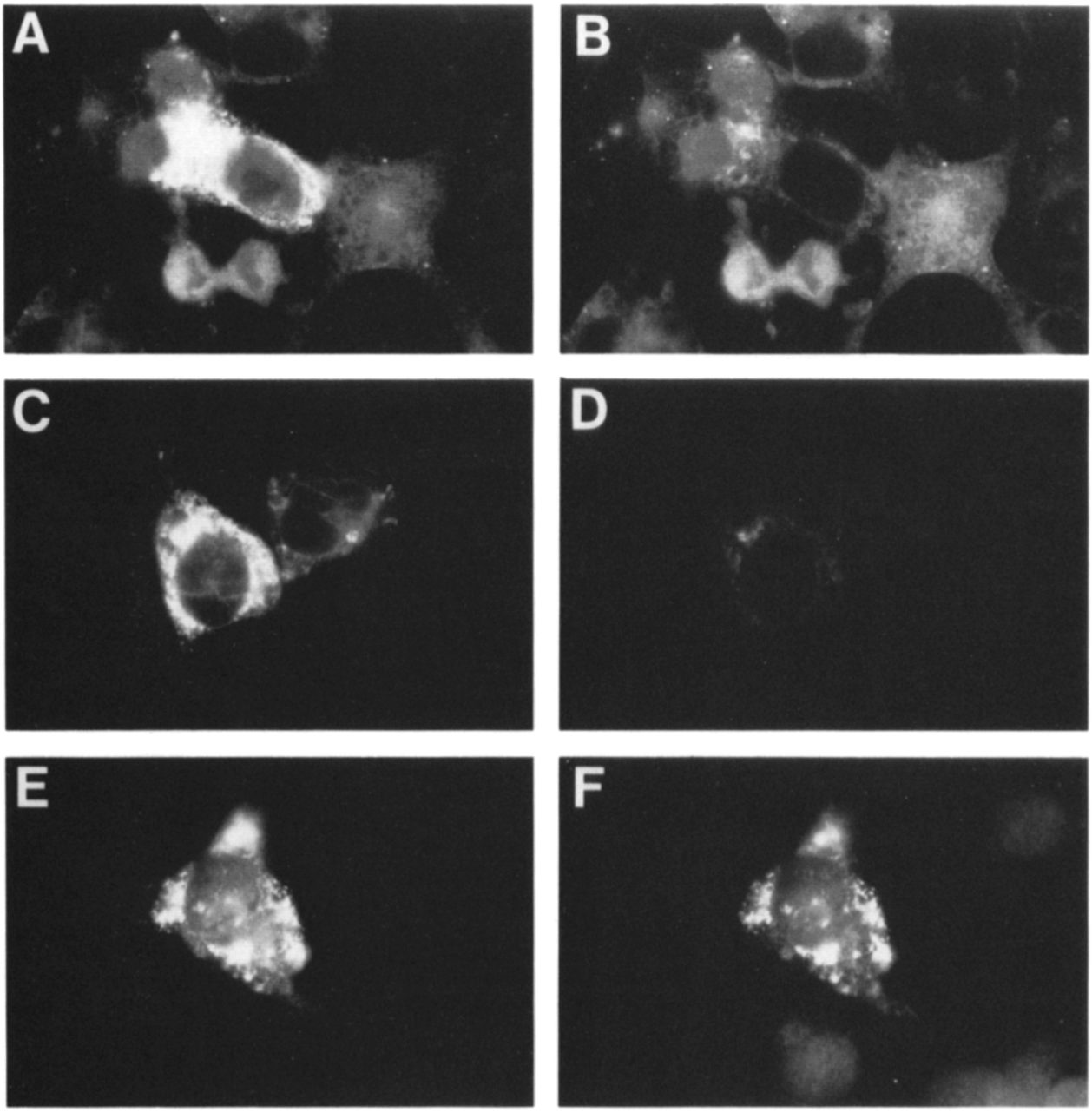

Figure 9. Sidedness of the H epitope in HMGal and HMGal/Dins(Δ) expressed in COS cells. COS cells, grown on glass coverslips were transfected with the wild-type pSRαHMGal (*A* and *B*) or the mutant pSRαHMGal/Dins(Δ) (*C–F*) plasmids, as described under Materials and Methods. On the 3rd day, cells were permeabilized with SLO (*A–D*) or with Triton X-100 *E* and *F*). The cells were doubly stained with anti-β-galactosidase antibody (*A*, *C*, and *E*) and anti-H antibodies (*B*, *D*, and *F*), as described in the legend to Fig. 5. Each pair of panels was photographed with the same exposure time and prints were made with the same time and contrast values.

1990). Indeed, a stretch of 10 hydrophobic residues in this region (Met^{258}–Val^{267}) may agree with this possibility, if this core assumes an extended non-α-helical conformation. Such a membrane span would be flanked on both sides by a cluster of charged residues, consistent with the general structure of spanning regions of polytopic membrane proteins (Jennings, 1989). Alternatively, it is reasonable that the sequence between Pro^{249} or Val^{252} and Ala^{275} constitutes this 7th membrane span since it is preceded by the highly charged stretch . .EEEENK- and followed by a -DPSP. . , 5 residues NH_2-terminal to the native glycosylation site at Asn^{281} (Fig. 11). This region of 24-27 residues is of sufficient length to span the ER membrane in an α-helical configuration and contains the above mentioned hydrophobic core of 10 contiguous amino acids. In this model, however, there are five basic residues buried in the lipid bilayer (Fig. 11) which would make this segment the most charged span in the membrane domain of the protein. Yet, potentially charged residues also occur in other membrane segments of HMG-CoA reductase, regardless of the model proposed.

It has been demonstrated that the membrane domain of the reductase is responsible for the regulated ER degradation of

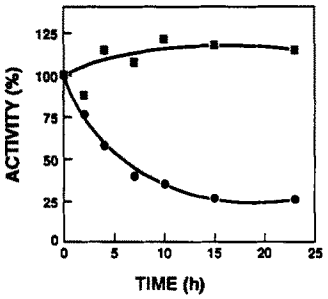

Figure 10. Replacement of transmembrane Span 7 with the first membrane span of bacteriorhodopsin abolishes the sterol-accelerated degradation of HMGal. The growth medium of CHO cells expressing wild type HMGal (●) or Span 7-HNGal (■) (Chun and Simoni, 1992) was switched to Induction Medium 24 h before addition of sterols. A mixture of 25-hydroxycholesterol and cholesterol was given in ethanol (final concentration are 1.5 μg/ml and 15 μg/ml, respectively) in a staggered fashion, so that all time points could be assayed simultaneously. The β-galactosidase activity of the chimeric proteins was assayed as previously described (Skalnik et al., 1988).

the enzyme and of HMGal (Gil et al., 1985; Skalnik et al., 1988; Chun et al., 1990). The results presented in this report demonstrate the role of span 7 in this level of enzyme regulation. Replacement of wild type span 7 with the first membrane span of bacteriorhodopsin creates an enzyme with a short half-life, ~2 h (Chun and Simoni, 1992; Roitelman and Simoni, unpublished results), and completely abolishes the sterol-accelerated ER degradation of HMGal. Interestingly, a similar phenotype is created by deleting spans 4 and 5 in the membrane domains of HMG-CoA reductase and HMGal (Jingami et al., 1987; Skalnik et al., 1988; Chun and Simoni, 1992). The replacement mutation, which introduces the sequence GWIWLALGTALMGLGTLYFLVKG in place of residues Gln^{254} through Trp^{273}, does not disturb the intracellular targeting or transmembrane orientation of span 7-HMGal since the mutant enzyme is active and is localized correctly in the ER (Chun and Simoni, 1992). However, this replacement eliminates most of the charged residues in this segment. Therefore, it is attractive to speculate that the charged residues in span 7 play a role in the sterol-regulated degradation of HMG-CoA reductase as they do in the ER degradation of the α subunit of the T cell antigen receptor (Bonifacino et al., 1991; Bonifacino and Lippincott-Schwartz, 1991). Possibly these residues are engaged in intramolecular charge interaction between adjacent membrane spans that might stabilize the enzyme or in intermolecular interactions involved in monomer-dimer interconversion between subunits of the reductase (Edwards et al., 1985; Ness et al., 1985; Roitelman and Shechter, 1986), similar to the association between subunits of the T cell antigen receptor and CD3 (Cosson et al., 1991). Alternatively, membrane span 7 may interact directly with sterols or indirectly with an auxiliary protein(s) that tag HMG-CoA reductase for degradation in response to sterols.

Liscum et al. have noted that in HMG-CoA reductase gene, as in the bovine rhodopsin gene (Nathans and Hogness, 1983), the introns are located near the junction of a membrane span and an external loop, such that each membrane span is separated from the adjacent span by a single intron (Liscum et al., 1985). The position of intron 8 of the reductase gene (Reynolds et al., 1984) in span 7 conforms with this general relation between gene organization and protein structure (Fig. 11).

HMG-CoA reductase and HMGal, large proteins of M_r ≥100,000, are rapidly degraded in the ER when sterols or mevalonate are added to the cells. The degradation pathway undertaken by these proteins, and the fate of the polypeptide chains during proteolysis is unknown. No degradation intermediates have been detected in pulse–chase experiments when immunoprecipitation was performed with antibodies directed against the cytoplasmic domains of reductase or of HMGal. Hence, detection and identification of such proteolytic fragments is essential for elucidating the process of

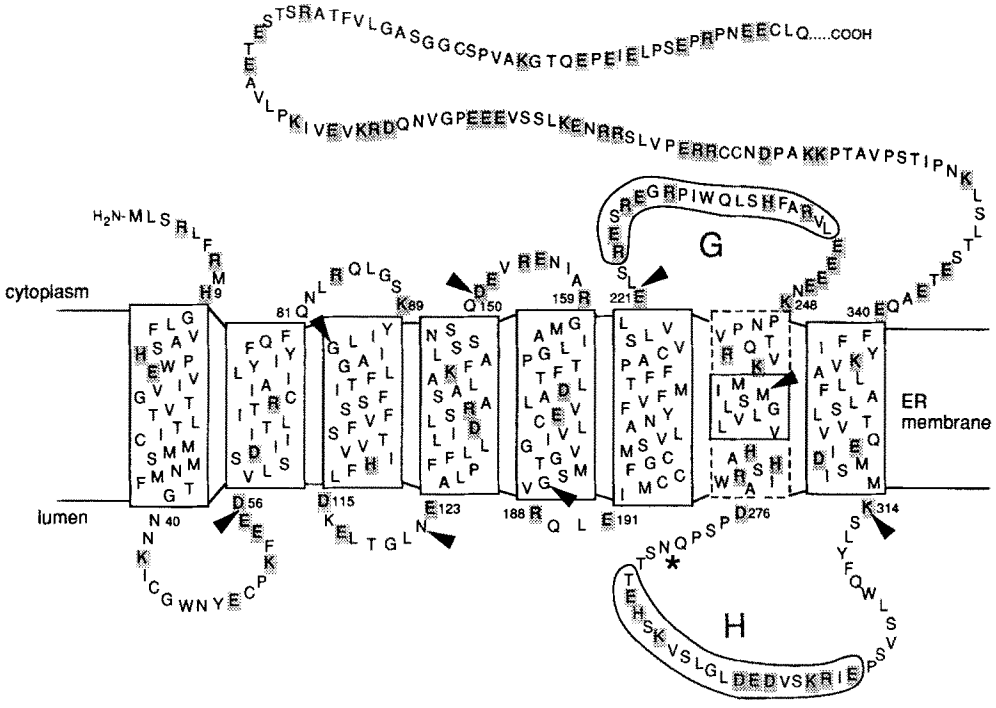

Figure 11. Proposed model for the orientation of the transmembrane spans of HMG-CoA reductase. Amino acids residues are shown in the single-letter code. Charged residues are *shaded*. Residues that potentially mark the boundaries of membrane spans are numbered. Sequences comprising peptides G and H are circled. The N-glycosylation site is indicated by an *asterisk*. Possible boundaries of Span 7 are *dashed* and the core of 10 hydrophobic amino acids in this span is *boxed*. Intron-exon boundaries are indicated by *arrowheads*.

regulated ER degradation. We anticipate that the antibodies against the membrane domain described in this report will prove useful also towards this goal.

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