Sterol regulatory element-binding proteins (SREBP-1 and SREBP-2) are proteins of ~1150 amino acids each that are attached to membranes of the endoplasmic reticulum (ER). In sterol-depleted cells, a protease releases an NH₂-terminal fragment of ~500 amino acids that contains a basic helix-loop-helix leucine zipper motif. This fragment enters the nucleus and stimulates transcription of genes encoding the low density lipoprotein receptor and enzymes of cholesterol biosynthesis. Prior evidence indicates that the SREBPs are attached to membranes by virtue of an 80-residue segment located ~80 amino acids to the COOH-terminal side of the leucine zipper. This segment contains two long hydrophobic sequences separated by a short hydrophilic sequence of ~30 amino acids. We have proposed a hairpin model in which the two hydrophobic sequences span the membrane, separated by the short hydrophilic sequence which projects into the lumen of the ER (the "lumenal loop"). The model predicts that the NH₂- and COOH-terminal segments face the cytosol. To test this model, we constructed a cDNA encoding human SREBP-2 with epitope tags at the NH₂ terminus and in the lumenal loop. The COOH-terminal region was visualized with a newly developed monoclonal antibody against this region. Sealed membrane vesicles were isolated from cells expressing the epitope-tagged version of SREBP-2. Trypsin treatment of these vesicles destroyed the NH₂- and COOH-terminal segments and reduced the lumenal epitope to a size consistent with protection of the lumenal sequence plus the two membrane-spanning segments. The lumenal epitope tag contained two potential sites for N-linked glycosylation. The size of the trypsin-protected fragment was reduced by treatment with N-glycanase and endoglycosidase H, indicating that this segment was located in the lumen of the ER where it was glycosylated. These data provide strong support for the hairpin model.

Sterol regulatory element-binding proteins (SREBPs) are membrane-bound transcription factors that regulate genes involved in cholesterol homeostasis (1-3). Each full-length SREBP contains between 1133 and 1147 amino acids. The NH₂-terminal portion, comprised of ~500 amino acids, includes an acidic domain that activates transcription and a basic helix-loop-helix leucine zipper (bHLH-Zip) domain that mediates homodimerization and DNA binding. This is followed by a hydrophobic segment of ~80 amino acids that attaches the protein to membranes of the endoplasmic reticulum (ER) and a COOH-terminal domain of ~550 amino acids whose function has not been assigned.

When cultured cells are depleted of cholesterol, the SREBPs are cleaved by a protease that releases the NH₂-terminal regions (4). These fragments of ~500 amino acids enter the nucleus and bind to a 10-base pair sterol regulatory element (SRE-1) in the promoter of the genes encoding 3-hydroxy-3-methylglutaryl-coenzyme A synthase and perhaps other cholesterol biosynthetic enzymes, thereby increasing cholesterol synthesis. They also bind to SRE-1 in the promoter of the gene encoding the low density lipoprotein receptor, thereby increasing cholesterol uptake from plasma lipoproteins. When steroids accumulate in the cell, proteolysis of the SREBPs is suppressed, and the residual nuclear fragments are rapidly degraded by a protease that is sensitive to inhibition by acetyl-leucinal-leucinal-norleucinal (ALLN) (4). As a result, transcription of the SRE-containing genes declines. The fate of the COOH-terminal segments of the SREBPs has not been studied because of the lack of antibodies that react with this fragment.

Cultured cells such as human HeLa cells and hamster fibroblasts produce two SREBPs, designated 1 and 2. The two human proteins are ~50% identical to each other, and they share all of the landmark features outlined above (1-3, 5). They bind to the same 10-base pair SRE-1, and they activate transcription of the same genes. The two SREBPs act independently in cultured cells, and there is no evidence that heterodimer formation is required (2). Proteolysis of both proteins is activated in parallel by sterol depletion and inhibited in parallel by overloading with steroids such as 25-hydroxycholesterol (4, 5).

Both SREBPs behave biochemically as integral membrane proteins. They are removed from membranes only by detergents and not by treatment with high salt or alkali (3, 4). The membrane attachment domain consists of two long hydrophobic segments of at least 20 residues each that are separated by a short hydrophilic sequence of ~30 residues. Deletion of this domain markedly reduces the proportion of SREBP-1 that is bound to membranes (3). Based on these observations, we have proposed a hairpin model for the orientation of SREBP in the membrane. The model postulates that the two hydrophobic segments span the membrane bilayer in opposite directions separated by the short hydrophilic sequence of ~30 amino acids, which projects into the lumen of the ER or the nuclear envelope. The NH₂- and COOH-terminal segments both face the cytosol (3, 4).

In the current experiments we use the classic method of protease protection (6) to test the hairpin model for the orientation of human SREBP-2 in the membrane. The ability to perform this test is based on two advances: 1) the development of a monoclonal antibody against the COOH-terminal domain...
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of human SREBP-2 and 2) the development of an epitope tag containing sites for N-linked glycosylation that can be inserted into the luminal loop. The results are consistent with the hairpin model.

EXPERIMENTAL PROCEDURES

Materials and Methods—Standard molecular biology techniques were used (7). DNA sequencing was performed with the dye chemistry termination method on an Applied Biosystems model 373A DNA sequencer. Newborn calf lipoprotein-deficient serum (d = 1.215 g/ml) was prepared by ultracentrifugation (8). Oligonucleotides were synthesized on a modified 380A DNA synthesizer (Applied Biosystems, Inc.). Oligonucleotide-directed mutagenesis was carried out with a Muta-Gene® phagemid in vitro mutagenesis version 2 kit (Bio-Rad) as described by Kunkel (9). We obtained trypsin (catalog number LS03740) from Worthington; soybean trypsin inhibitor (catalog number 650357) from Calbiochem; anti-BiP monoclonal antibody from StressGen Biotechnologies (Victoria, British Columbia, Canada); N-Glycanase from Genzyme; endoglycosidase H, neuraminidase, Klenow fragment, and restriction enzymes from New England Biolabs; HSV-Tag™ monoclonal antibody from Novagen; Takara DNA ligation kit from Panvera Corp; and Pfu DNA polymerase from Stratagene. All plasmid DNAs for transfection were prepared with Plasmid Mega or Maxi kits (Qiagen). Other reagents were obtained from sources as reported previously (4).

Construction of Plasmid pTK-HSV-BP2-7D4—This plasmid was constructed from pTK-HSV-BP2 (the parent plasmid)2 from pcDNA3 (Invitrogen), pT7K (Clontech), and human pSREBP2 (Ref. 2). Briefly, the parent plasmid contains a herpes simplex virus (HSV) thymidine kinase promoter in front of two tandem copies of the HSV epitope tag (QPELAPEDPED) (10) fused to the coding sequence of human SREBP-2 (amino acids 14–1141). To construct pTK-HSV-BP2-7D4, we inserted a cDNA segment encoding the 7D4 epitope into the loop region of the SREBP-2 sequence in the parent plasmid. First, we used oligonucleotide site-directed mutagenesis (9) to replace amino acids 505–513 in the loop region of human SREBP-2 in pTK-HSV-BP2 with the epitope YPYDVPDYA derived from amino acids 98–106 of influenza hemagglutinin (11). The hemagglutinin epitope contains a unique N-glycosylation site-directed mutagenesis (9) to replace amino acids 505–513 of the SREBP-2 sequence in the parent plasmid. First, we used oligonucleotide site-directed mutagenesis (9) to replace amino acids 505–513 in the loop region of human SREBP-2 in pTK-HSV-BP2 with the epitope YPYDVPDYA derived from amino acids 98–106 of influenza hemagglutinin (11). The hemagglutinin epitope contains a unique N-glycosylation site. A DNA segment encoding the 7D4 epitope (QPELAPEDPED) (10) was inserted into the loop region of human SREBP-2 at the unique AgeI site created by the Trp533Arg mutation (12), resulting in the construction of plasmid pTK-HSV-BP2-7D4. The sequence of the cloning junctions was confirmed by sequencing. To protect against polymerase chain reaction artifacts, two independent clones were used in each of the transfection and proteolysis experiments, and the results were always consistent.

Monoclonal Antibodies—Monoclonal antibody IgG-7D4 against amino acids 32–250 of hamster SREBP-2 has been described previously (12). This antibody recognizes hamster, but not human, SREBP-2. To produce a antibody against the NH2-terminal region of human SREBP-2, we produced in E. coli a fusion protein containing amino acids 48–403 of human SREBP-2 (Ref. 2) with six histidines following the initiator methionine. The protein was injected into Balb/c mice, and spleen cells were used (13) to produce a monoclonal antibody, designated IgG-3H8. A fusion protein containing six histidines after the initiator methionine was followed by amino acids 633–1141 of human SREBP-2, was expressed in E. coli and used to immunize mice to produce a monoclonal antibody, designated IgG-1C6. Prior to immunization, both fusion proteins were purified by Ni2+ affinity chromatography. All monoclonal antibodies were purified from ascites fluid by protein G chromatography. 2

Culture Transfection, and Cell Fractionation—Human HeLa cells were grown in spinner culture as described (4). On day 0, cells were set up in replicate spinner cultures at a density of 2.5 × 10^6 cells/ml in J oklik's minimum essential medium containing 100 units/ml penicillin, 100 μg/ml streptomycin sulfate, and 2.5% (v/v) newborn calf serum. On day 1, steroids dissolved in ethanol were added to each flask. The final concentration of ethanol in the medium was 0.2% (v/v). After incubation for 0.5–6 h, cells were harvested and fractionated into a nuclear extract and membrane fraction (10^7 × g pellet) as described previously for HeLa cells (4) with one modification: the extract from the crude nuclear pellet was centrifuged at 55,000 rpm for 30 min at 4°C in a Beckman TLSA100 rotor, and the supernatant from this spin was used. For proteolysis experiments, the membrane fraction was washed once with buffer A (50 mM Hepes-KOH, pH 7.4, 10 mM EDTA, 0.5 mM sodium EDTA, 0.5 mM sodium EGTA, and 100 mM NaCl) and then resuspended in buffer A.

Monolayers of human embryonic kidney 293 cells (3) were set up on day 0 (4 × 10^6 cells/60-mm dish) and cultured in 8–9% CO2 at 37°C in Dulbecco’s modified Eagle’s medium supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% (v/v) fetal calf serum. On day 2, the cells were transfected with 5 μg of pT7-HSV-BP2-7D4 using a modified bovine serum transfection kit (Stratagene) according to the manufacturer’s instructions, except that the monolayers were not washed prior to addition of 6% (v/v) modified bovine serum in Dulbecco’s modified Eagle’s medium. The transfection was carried out at 35°C for 3 h in 3% CO2, after which each dish of cells was washed once with 5 ml of phosphate-buffered saline and switched to 5 ml of Dulbecco’s modified Eagle’s medium supplemented with penicillin, streptomycin, 10% (v/v) calf lipoprotein-deficient serum, 1 μg/ml 25-hydroxycholesterol, 10 μg/ml cholesterol, 50 μg/ml compactin, and 50 μg/ml sodium mepalvonal. On day 3 (20 h later), 5 μl of solution containing 25 mg/ml ALLN was added to each dish, and the cells were harvested 3 h later. To prepare the nuclear fraction, the cells from 24–32 dishes were allowed to swell at 4°C for 10 min in buffer B (buffer A without NaCl and supplemented with 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 25 μg/ml ALLN, and 1 μg/ml dithiothreitol) and then homogenized with 12 strokes of a tight pestle in a Dounce homogenizer. The suspension was pelleted at 1000 × g for 10 min at 4°C, and the supernatant was centrifuged at 55,000 rpm for 30 min at 4°C in a TL10A100.2 rotor to obtain the membrane fraction (10^7 × g pellet). The pellets were washed once with buffer A and resuspended in buffer A for use in trypsin proteolysis experiments.

Trypsin Proteolysis and Glycosidase Sensitivity of Membrane-bound SREBP-2—For proteolysis sensitivity experiments, aliquots of the 10^5 (0.3 ml) of buffer A) were centrifuged at 10^7 × g at 4°C for 30 min. The resulting pellet was washed with 0.2 ml of buffer A and resuspended in 110 μl of the same buffer in the absence or presence of 1% (v/v) Triton X-100. The samples were incubated at room temperature in a model REAX 2000 mixer (Whatman Lab Sales) with mild shaking for 30 min. Varying amounts of trypsin were then added to each tube in 3 μl of Dulbecco’s phosphate-buffered saline (catalog number 14190–144, Life Technologies, Inc.). After incubation at room temperature for 30 min, 300 units of soybean trypsin inhibitor in 6 μl of phosphate-buffered saline were added to stop proteolysis, after which 40 μl of the suspension was subjected to SDS/PAGE, followed by immunoblot analysis.

For proteolysis of 293 cell membranes, aliquots of the 10^5 membrane fraction (0.1 mg of protein in 40 μl of buffer A) were incubated at room temperature in a model REAX 2000 mixer with mild shaking for 30 min in the absence or presence of 1% (v/v) Triton X-100. Trypsin and soybean trypsin inhibitor were added as described above, followed by SDS-PAGE and immunoblotting.

To determine the glycosidase sensitivity of the trypsin-resistant fragment, aliquots of the 10^5 g membrane fraction of transfected 293 cells (0.1 mg of protein in 40 μl of buffer A) were treated sequentially as follows: 1) incubation with 0.3 unit of trypsin (added in 3 μl) at room temperature for 30 min; 2) addition of 150 units of soybean trypsin inhibitor (added in 3 μl); 3) addition of Triton X-100 (final concentration, 1%); 4) boiling for 5 min in the presence (N-Glycanase) and absence (endo H) of the N-glycosidase F reaction, digestion with 5% (v/v) aminopeptidase N, (0.5% (v/v) and β-mercaptoethanol) and 5% (v/v) addition of the indicated glycosidase (added in 4 μl) and incubation at 37°C for 2 h as described in the legend to Fig. 8. Each sample (final volume, 59 μl) was mixed with 10 μl of 5 × SDS loading buffer (15) and used for SDS-PAGE and immunoblot analysis.

SDS/PAGE and Immunoblot Analysis—Gels were run with 5 × SDS loading buffer (15) prior to SDS-PAGE on 8% gels. After electrophoresis, the proteins were transferred to Hybond-C Extra nitrocellulose sheets (Amersham Corp.). After transfer with monoclonal antibodies at the concentrations indicated, the sheets were washed, and the antibodies were detected with horseradish peroxidase-conjugated anti-mouse IgG using the enhanced chemiluminescence (ECL) Western blotting detection system kit (Amersham) according to the instructions of the manufacturer with modification as described (4). Gels were calibrated with prestained molecular weight markers (New England Biolabs). Filters

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RESULTS

Fig. 1 shows hydrophobicity plots for human SREBP-1 and -2 according to the method of Kyte and Doolittle (16) with a window of 17 residues by the method of Kyte and Doddlittle (16) using the Genetics Computer Group sequence analysis software package, version 7.1 (21). Horizontal black bars denote bHLH-Zip domains. Numbers 1 and 2 denote the two putative transmembrane segments.

To compare the fates of the NH2- and COOH-terminal segments of SREBP-2, we took advantage of a newly developed monoclonal antibody, designated IgG-1C6, that was raised against a fragment of human SREBP-2 extending from residue 833 to the COOH terminus. In the experiment of Fig. 2, HeLa cells were partially deprived of sterols by growth in the presence of a low concentration of newborn calf serum (2.5%). We then overloaded the cells with sterols by adding a mixture of 25-hydroxycholesterol and cholesterol to the culture medium. At varying times, cells were harvested, and high salt nuclear extracts were subjected to SDS-PAGE and blotted with a monoclonal antibody directed against the NH2 terminus and with the COOH-terminal monoclonal IgG-1C6. At zero time, when the cells were in a sterol-depleted state, both antibodies stained the full-length precursor form of SREBP-2, which was found in the membrane fraction (designated P in Fig. 2, lane F). As expected, the NH2-terminal antibody also stained a fragment whose migration corresponded to a molecular mass of 68 kDa, which was found in the nuclear extracts (lane A). The NH2-terminal fragment was shown previously to migrate with anomalously slow mobility, owing to the acidic NH2-terminal activation domain (3). The COOH-terminal antibody stained a fragment of ~65 kDa that was bound to the membranes (lane F). Two h after sterol addition, the NH2-terminal fragment had almost disappeared from the nucleus (lane D). The COOH-terminal fragment was also rapidly degraded. By 2 h, it had been reduced by more than 80% (lane I), but a small amount continued to be detectable at 6 h (lane J). No COOH-terminal fragment was detected in immunoblots of the 10^5 × g supernatant fraction (data not shown).

The COOH-terminal fragment of SREBP-2 behaved as an integral membrane protein, just like the full-length precursor form (4). In the experiment of Fig. 3, membranes isolated from sterol-deprived cells were treated with various protein-solubilizing agents and subjected to centrifugation at 10^5 × g. The pellets and supernatants were subjected to SDS-PAGE and immunoblotted with IgG-1C6, which visualized both the precursor form and the COOH-terminal fragment of SREBP-2. The precursor and the COOH-terminal fragment were both solubilized partially with 1% SDS and 1% Triton X-100. Neither was solubilized with buffer alone, nor with 0.1 M Na2CO3 or 1 M hydroxylamine.

To determine the orientation of the COOH-terminal fragment with respect to the membrane, we homogenized HeLa cells gently with a Dounce homogenizer and isolated mem-
brane vesicles by centrifugation (Fig. 4). The vesicles were incubated with varying concentrations of trypsin in the absence or presence of Triton X-100 followed by SDS-PAGE and immunoblotting with IgG-1C6. Trypsin at a concentration of 1.7 units/ml obliterated the IgG-1C6 epitope, whether it was present on the precursor or on the COOH-terminal fragment (Fig. 4, upper panels). Disruption of the membranes with Triton X-100 increased trypsin sensitivity only slightly (Fig. 4, lower panels). This observation confirmed the inability of IgG-7D4 (Ref. 12). The antibody is specific for the hamster SREBP-2 (Ref. 5), and the monoclonal antibody that recognizes this segment consists of amino acids 33–250 of hamster SREBP-2 (Ref. 5); and the monoclonal antibody that recognizes this segment is part of the NH$_2$-terminal region of hamster SREBP-2 (Fig. 6) from the HA epitope and amino acids 33–250 of hamster SREBP-2. BHG PA denotes thebovine growth hormone polyadenylation sequence. Two putative N-linked glycosylation signals in the 74D epitope are underlined.

SDS-PAGE.

In a final attempt to circumvent this problem, we decided to insert a DNA sequence encoding a much longer segment of protein into the hydrophilic loop. We chose a 218-amino acid segment to which we already had a potent monoclonal antibody. This segment consisted of amino acids 33–250 of hamster SREBP-2 (Ref. 5), and the monoclonal antibody that recognizes this segment consists of two tandem copies of the 11-amino acid HSV epitope tag (QPELAPEDPED); this segment is inserted into the hydrophilic loop between the two transmembrane domains of SREBP-2. BHG PA denotes the bovine growth hormone polyadenylation sequence. Two putative N-linked glycosylation signals in the 74D epitope are underlined.

SDS-PAGE.

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To confirm the specificity of IgG-7D4, we transfected human 293 cells with pTK-HSV-BP2-7D4 or with a control plasmid (pTK-HSV-BP2) that contains the NH$_2$-terminal HSV tag but lacks the 74D epitope (Fig. 6). Membranes from the transfected cells were subjected to SDS-PAGE and immunoblotted with an antibody against the HSV tag or with IgG-7D4. Neither antibody visualized any protein in mock-transfected cells (lanes 1 and 4). In cells transfected with pTK-HSV-BP2, the protein was visualized with the anti-HSV antibody (lane 2), but not IgG-7D4 (lane 5). This result confirms the inability of IgG-7D4 to recognize the human protein. In cells transfected with pTK-
The current manuscript provides evidence to support the hairpin model for the orientation of SREBP-2 in the membrane. According to this model, the NH$_2$- and COOH-terminal regions of SREBP-2 are oriented toward the cytosol. They are separated by a membrane attachment domain that consists of two membrane-spanning segments separated by a short loop that projects into the lumen of the ER.

The evidence in support of this model comes from trypsin sensitivity experiments performed with membrane vesicles. The NH$_2$- and COOH-terminal regions were readily destroyed by trypsin, and the luminal loop epitope was reduced to a size consistent with protection of the glycosylated loop plus the two molecular mass was reduced to about 35 kDa by treatment with N-Glycanase$^\circ$ and endo H, but not neuraminidase. This pattern of glycosidase sensitivity indicates that the N-linked sugars remained in their high mannose unprocessed forms and that the protein had not been transported to the Golgi complex (20).

**DISCUSSION**

The apparent size of the trypsin-resistant fragment on SDS-PAGE, as visualized with IgG-7D4 (47 kDa) was greater than would be predicted if the protected fragment consisted of the 218 amino acid peptide containing the epitope plus the remaining luminal amino acids and the two transmembrane regions (∼35 kDa). We suspected that this might be attributable to glycosylation of the epitope tag at one or both of the N-linked sites shown in Fig. 5. To test this possibility, we digested intact membrane vesicles with trypsin as before, then solubilized the membranes and denatured the proteins by treatment with detergent and boiling. The trypsin-resistant fragment was then digested with one of three glycosidases, and its size was estimated by SDS-PAGE and immunoblotting with IgG-7D4. As shown in Fig. 8, in the absence of glycosidases the trypsin-resistant fragment migrated as a 47-kDa protein. The apparent

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**FIG. 6. Specificity of monoclonal antibody IgG-7D4 for hamster 7D4 epitope inserted into the loop region of human SREBP-2.** A, schematic diagram of protein encoded by pTK-HSV-BP2-7D4, showing site in loop between two transmembrane segments where hamster 7D4 epitope is inserted. B, immunoblot analysis of cells expressing protein encoded by pTK-HSV-BP2-7D4. 293 cells were transfected with 5 μg of one of the following plasmids: lanes 1 and 4, empty vector pTK-2; lanes 2 and 5, pTK-HSV-BP2 encoding HSV-tagged human SREBP-2; lanes 3 and 6, pTK-HSV-BP2-7D4 encoding HSV-tagged and 7D4-tagged human SREBP-2. All plasmids are consistent with the hairpin model for the orientation of SREBP-2 in the membrane.

**FIG. 7. Resistance of epitope-tagged loop region of SREBP-2 to trypsin proteolysis.** 293 cells were transfected with pTK-HSV-BP2-7D4, the 10$^4$ × g membrane fractions were prepared, and aliquots of these membranes (0.1 mg of protein) were resuspended in 40 μl of buffer A in the absence (lanes 1–4) or presence (lanes 5–8) of 1% Triton X-100 as described under "Experimental Procedures." After incubation at room temperature for 30 min, each sample received increasing amounts of trypsin. The final trypsin concentrations (units/ml) were as follows: lanes 1 and 5, 0; lanes 2 and 6, 2.5; lanes 3 and 7, 7.5; and lanes 4 and 8, 25. Triplicate samples for each reaction were incubated and processed for SDS-PAGE as described in the legend to Fig. 4. Immunoblot analysis was carried out with one of the following antibodies: upper panel, 0.5 μg/ml anti-NH$_2$-terminal antibody (HSV-Tag™ antibody); middle panel, 10 μg/ml anti-COOH-terminal antibody (IgG-1C6); and lower panel, 8 μg/ml of IgG-7D4. The filters were exposed to film for 10 s (upper and lower panels) or 3 min (middle panel). Arrows denote the position of migration of the endogenous and transfected precursor (P) forms of SREBP-2 and of the trypsin-resistant fragment.

The current manuscript provides evidence to support the hairpin model for the orientation of SREBP-2 in the membrane. According to this model, the NH$_2$- and COOH-terminal regions of SREBP-2 are oriented toward the cytosol. They are separated by a membrane attachment domain that consists of two membrane-spanning segments separated by a short loop that projects into the lumen of the ER.

The evidence in support of this model comes from trypsin sensitivity experiments performed with membrane vesicles. The NH$_2$- and COOH-terminal regions were readily destroyed by trypsin, and the luminal loop epitope was reduced to a size consistent with protection of the glycosylated loop plus the two
membrane-spanning segments. The addition of N-linked sugars to the loop was confirmed by the observation that the protected fragment was reduced in size by treatment with N-Glycanase and endo H.

In order to visualize the luminal loop in immunoblots, we had to resort to the unorthodox procedure of inserting a long 218-residue fragment of protein containing an epitope. This was necessary because short epitopes inserted into this region failed to react with their cognate monoclonal antibodies even after the protein was denatured by SDS-PAGE. It seems likely that this region of the protein must refold during transfer to nitrocellulose, perhaps through hydrophobic interactions between the two membrane-spanning segments, thereby occluding the luminal epitope. When a long protein segment was inserted, the epitope was no longer occluded.

Although some of these segments dip into the lipid bilayer, but we do not believe that any of them span the membrane.

Although all of the current experiments were performed with SREBP-2, we believe that the results apply to SREBP-1, since the hydrophobicity profiles of the two proteins are nearly identical (Fig. 1) and because proteolysis of the two proteins is regulated in parallel (4, 5, 12). Knowledge of the membrane orientation of these proteins is essential if we are to understand the site at which they are cleaved by the sterol-regulated protease and the mechanism of its regulation.

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