Membrane Topology of Human Insig-1, a Protein Regulator of Lipid Synthesis*

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Insig-1 is an intrinsic protein of the endoplasmic reticulum (ER) that regulates the proteolytic processing of membrane-bound sterol regulatory element-binding proteins (SREBPs), transcription factors that activate the synthesis of cholesterol and fatty acids in mammalian cells. When cellular levels of sterols rise, Insig-1 binds to the membranous sterol-sensing domain of SREBP cleavage-activating protein (SCAP), retaining the SCAP/SREBP complex in the ER and preventing it from moving to the Golgi for proteolytic processing. Under conditions of sterol excess, Insig-1 also binds to the ER enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase, facilitating its ubiquitination and proteosomal degradation. Here, we use protease protection, glycosylation site mapping, and cysteine derivatization to define the topology of the 277-amino acid human Insig-1. The data indicate that short segments at the N and C termini of Insig-1 face the cytosol. Most of the protein is buried within the membrane, forming six transmembrane segments separated by five short luminal and cytosolic loops that range from ~5 to 16 amino acids. The membranous nature of Insig-1 is consistent with its sterol-dependent binding to hydrophobic sterol-sensing domains in SCAP and HMG CoA reductase.

Insig-1 and Insig-2 were recently identified as membrane proteins that reside in the endoplasmic reticulum (ER) and play a central role in the regulation of cholesterol and fatty acid synthesis (1–4). When cellular cholesterol levels are elevated, Insigs bind to SCAP and 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG CoA reductase) through interactions with the sequence-related sterol-sensing domains in the membranous spanning regions of each protein. In the case of SCAP, Insig binding prevents movement of the SREBP/SCAP complex from the ER to the Golgi, thus blocking proteolytic cleavage and transcriptional activation of SREBP (1, 2). In the case of HMG CoA reductase, Insig binding promotes ubiquitination and degradation, thus slowing the rate-limiting enzymatic reaction in cholesterol synthesis (3, 4).

SREBPs are a family of membrane-bound transcription factors that control the synthesis of cholesterol and fatty acids in animal cells (5). The C terminus of SREBP binds to the C terminus of SCAP, the protein that transports SREBPs from the ER to the Golgi when cells are depleted of sterols. Within the Golgi, two resident proteases cleave SREBP through regulated intramembrane proteolysis (Rip), releasing the N terminus of SREBP from the membrane and allowing it to translocate to the nucleus and activate transcription of target genes (6).

In the presence of sterols, SCAP undergoes a conformational change, causing the SCAP/SREBP complex to be retained in the ER and thus abrogating SREBP-dependent transcription. Insig proteins have been found to facilitate both the ER retention of the SCAP/SREBP complex (1, 2) and the sterol-sensitive SCAP conformational change (7). These activities of Insig occur via an interaction with the sterol-sensing domain of SCAP, which comprises transmembrane segments 2–6. The sterol-dependent interaction between SCAP and Insig is abolished by point mutations in the membranous portion of SCAP (1, 2, 7). These same mutations render cholesterol synthesis resistant to feedback suppression by sterols (8).

Insig proteins also regulate the ER enzyme HMG CoA reductase, which produces mevalonate, the precursor of cholesterol and nonsterol isoprenoids (9). Overexpression of the sterol-sensing domain of SCAP prevents the sterol-accelerated, Insig-mediated degradation of HMG CoA reductase (4), suggesting that the two proteins bind to the same site on Insigs. The sterol-stimulated binding of HMG CoA reductase to Insig is required for sterol-dependent degradation of the enzyme, as evidenced by the failure of this regulatory process in cells that have been treated with RNAi that target Insig-1 plus Insig-2 (3).

Human Insig-1 is comprised of 277 amino acids (1), while human Insig-2 contains 225 amino acids (2). They demonstrate an amino acid identity of 59%, the differences confined mostly to the hydrophilic N- and C-terminal regions. Insig-2 lacks the N-terminal 50 amino acids of Insig-1. These structural differences are highly conserved across species. Insig-1 and Insig-2 are functionally similar in that both can cause the ER retention of the SCAP/SREBP complex (1, 2), and both activate sterol-sensitive HMG CoA reductase degradation (3, 4). Insig-1 expression is positively controlled by nuclear SREBPs, while Insig-2 expression is negatively regulated by insulin (10).

In order to fully understand the diverse and complex role that Insig proteins play in the regulation of lipid and cholesterol homeostasis, more complete information on the structure and functions of Insigs must be obtained. Sequence inspection suggests that Insig proteins are polytopic membrane-spanning.
proteins with a high proportion of hydrophobic residues. They are believed to interact with SCAP and HMG CoA reductase through binding to the polytopic membrane-spanning segments of these proteins. Transmembrane segments 2–6 of HMG CoA reductase show clear sequence identities to the sterol-sensing domain of SCAP (3, 11) Determining the membrane topology of Insig will provide a map to better explore the mechanism of sterol-induced binding of SCAP and HMG CoA reductase to Insig.

In the current studies we propose and test a model for the topology of Insig-1 through experiments involving protease protection, examination of N-linked glycosylation patterns, and cytostatin derivitization. The data are consistent with a model in which Insig-1 contains six membrane-spanning helices separated by very short hydrophilic loops and flanked by short hydrophilic N- and C-terminal extensions that protrude into the cytosol.

EXPERIMENTAL PROCEDURES

Materials—We obtained (2-trimethylammonium)ethyl methanethiosulfonate bromide (MTSET) from Toronto Research Chemicals, Inc.; Nα-(3-maleimidylpropionyl)biotin (biotin maleimide) from Molecular Probes, Inc.; Immobilized Neutravidin Biotin Binding Protein from Pierce; and anti-FLAG M2 antibody from Sigma. Monoclonal antibodies IgG-9D5 against hamster SCAP (12) and IgG-9E10 against c-Myc epitope (1) have been previously described. All other reagents were obtained from previously reported sources (13–16).

Plasmids—The following recombinant expression plasmids have been described: pTK-HSV-BP-2, encoding wild-type HSV-tagged human SREBP-2 under control of the thymidine kinase (TK) promoter (17); pCMV-SCAP, encoding wild-type hamster SCAP under control of the cytomegalovirus (CMV) promoter (12); pCMV-Insig-1-MycX6, encoding wild-type human Insig-1 followed by six tandem copies of a c-Myc epitope tag (EQKLISEEEDL) under control of the CMV promoter (2). The resulting plasmids were sequenced in their entirety prior to use in transfection experiments.

Transient Transfection of HEK-293 and SRD-13A Cells—HEK-293 cells were set up on day 0 in medium A at 7 × 10^5 cells per 100-mm dish. On day 2, the cells were transfected with 4 μl of FuGENE 6 reagent (Roche Applied Science) to 4 μl of DNA in DMEM (without antibiotics) in a final volume of 0.4 ml as described (15). Transfection was performed in DMEM (without antibiotics) incubated for 5 min at room temperature, and mixed with DNA. This mixture was then further incubated for 15 min at room temperature. Cells were refed with 8 ml of fresh medium A, treated with 0.4 μl of the FuGENE 6/DNA mixture, and incubated at 37 °C for 16–24 h. On day 3, the cells were harvested for cell fractionation.

SRD-13A cells were set up on day 0 in medium B at 7 × 10^5 cells per 60-mm dish. On day 1, the cells were transfected with 4 μl of DNA per dish by using FuGENE 6 reagent as described (15). After transfection, cells were incubated at 37 °C for 12 h. On day 3, the cells were washed once with phosphate-buffered saline (PBS), switched to medium C (a 1:1 mixture of Ham’s F-12 medium and DMEM containing 5% newborn calf serum),水中E1-1-11 (12) in DMEM (without antibiotics) incubated for 5 min at room temperature, and mixed with DNA. This mixture was further incubated for 15 min at room temperature. Cells were refed with 8 ml of fresh medium A, treated with 0.4 μl of the FuGENE 6/DNA mixture, and incubated at 37 °C for 16–24 h. On day 3, the cells were harvested for cell fractionation.

Cell Fractionation—SRD-13A cells were fractionated as described (18) with minor modifications: The cell pellets from duplicate dishes were resuspended in 0.4 ml of buffer A (10 mM Hepes-KOH (pH 7.4), 10 mM NaCl, 5 mM MgCl2, 5 mM sodium EDTA, 50 μM sodium molybdate, and 0.5 mM streptomycin sulfate), passed through a 22.5-gauge needle 20 times, and centrifuged at 1000 g for 5 min at 4 °C. The 1000 g spin was subjected to centrifugation at 10000 g for 5 min at 4 °C. The supernatant was designated the nuclear extract. The supernatant from the original 10000 g spin was used to prepare the membrane fraction by centrifugation at 105,000 g for 30 min at 4 °C. The resulting membrane pellets were resuspended in buffer B (10 mM Hepes-KOH (pH 7.6), 25% (v/v) glycerol, 0.42 mM NaCl, 1.5 mM MgCl2, 5 mM sodium EDTA, 5 mM sodium EGTA), rotated at 4 °C for 1 h, and centrifuged at 105,000 g for 30 min at 4 °C, April 1995. The supernatant from this centrifugation was designated the nuclear extract. The supernatant from the original 10000 × g spin was used to prepare the membrane fraction by centrifugation at 105,000 g for 30 min at 4 °C. The resulting membrane pellets were resuspended in buffer B (10 mM Tris-HCl (pH 7.4), 100 mM NaCl, 1% (w/v) SDS).
respectively. To prepare membranes for differential solubilization, protease treatment, glycosidase treatment, and cysteine derivitization, the supernatant from the original 1000 × g spin was subjected to centrifugation at 2 × 10^4 × g for 15 min at 4 °C. The resulting pellets were resuspended in buffer C (buffer A containing 100 mM NaCl) and designated as the membrane fraction. All the buffers used in the cell fractionation contained protease inhibitors (10 μg/ml leupeptin, 5 μg/ml pepstatin A, 2 μg/ml aprotinin, 25 μg/ml N-acetyl-leucinal-leucinal-norleucinal) except when the membrane fraction was prepared for protease treatment. Protein concentration of the nuclear extracts and membrane fractions was determined using the BCA Kit (Pierce) according to the manufacturer’s instructions.

**Protease Treatment**—Trypsin treatment of the membrane fraction was carried out as described (19). Briefly, aliquots of membranes (75–100 μg of protein) were treated with the indicated amount of trypsin in the absence or presence of 1% (v/v) Triton X-100 in a total volume of 58 μl of buffer C for 30 min at 30 °C. The reactions were then stopped by addition of 2 μl of soybean trypsin inhibitor (400 units), mixed with 5× SDS loading buffer (150 mM Tris-HCl (pH 6.8), 15% SDS, 25% glycerol, 0.02% (v/v) bromphenol blue, 12.5% (v/v) 2-mercaptoethanol), and boiled for 5 min. Proteinase K treatment of the membrane fraction was performed as follows: Aliquots of membranes (100 μg of protein) were treated with the indicated final concentration of proteinase K in the absence or presence of 1% Triton X-100 in a total volume of 58 μl of buffer C for 40 min at 30 °C. The reactions were stopped by addition of 2 μl of phenylmethylsulfonyl fluoride at a final concentration of 5 mM. The samples were then mixed with 5× SDS loading buffer and boiled for 5 min. All the samples were subjected to SDS-PAGE and immunoblot analysis.

**Glycosidase Treatment**—Glycosidase treatment of the membrane fraction was carried out as described (14). For treatment with endoglycosidase (endo H) and peptide N-glycosidase F (PNGase F), aliquots of the membranes (60 μg of protein) in 60 μl of buffer C received 10 μl of solution containing 3.5% SDS and 7% 2-mercaptoethanol. After boiling for 10 min, each sample received sequential additions as follows: endo H treatment; 9 μl of 0.5 mM sodium citrate (pH 5.5), 5 μl of water, followed by 1 μl of endo H (0.05 units); and PNGase F treatment; 7 μl of 0.5 mM sodium phosphate (pH 7.5), 7 μl of 10% (v/v) Nonidet P-40 in water, followed by 1 μl of PNGase F (7.7 × 10^3 units). All reactions were carried out overnight at 37 °C and stopped by addition of 20 μl of 5× SDS loading buffer. The mixtures were then boiled for 5 min and subjected to SDS-PAGE and immunoblot analysis.

**Cysteine Derivitization**—Aliquots of HEK-293 cell membrane fraction (100 μg of protein) were resuspended in 95 μl of buffer C with or without 5 μl (2-trimethylammonium)ethanol) methanethiosulfonate bromide (MTSET). (A 100 mM stock solution of MTSET was prepared fresh in water.) After incubation for 30 min at room temperature, the membranes were spun twice at 2 × 10^4 × g for 15 min at 4 °C, washed in 200 μl of buffer C, resuspended in 90 μl of buffer C containing 100 μM biotin maleimide, and incubated for 20 min at room temperature. (A 24 mM stock solution of biotin maleimide was prepared in dimethyl formamide and stored at −20 °C.) At the end of the incubation, the reaction was quenched with 10 mM (2-(trimethylammonium)ethyl) methanethiosulfonate (MTSET). (A 100 mM stock solution of MTSET was prepared fresh in water.) After incubation for 30 min at room temperature, the samples were incubated overnight under rotation at 4 °C and then centrifuged for 3 min at 2000 × g to obtain a supernatant fraction for immunoblot analysis (see below). After removal of the supernatant, the pellet fractions were resuspended in buffer B (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1.5% (v/v) Nonidet P-40, 0.1% (v/v) SDS) containing protease inhibitors as described above. Each sample then received a 35-μl aliquot of Immobilized Neutravidin Biotin Binding Protein (washed once in buffer B, and which the samples were incubated overnight under rotation at 4 °C) and then centrifuged for 3 min at 2000 × g to obtain a supernatant fraction for immunoblot analysis (see below). After removal of the supernatant, the pellet fractions were resuspended in 100 μl of SDS-containing buffer D, boiled for 5 min, and centrifuged for 30 s at 2000 × g to separate the eluted pellet fraction from the beads. Both the supernatant and the eluted pellet fractions were then subjected to SDS-PAGE and immunoblot analysis.

**Immunoblot Analysis**—Samples were subjected to SDS-PAGE, transferred to nitrocellulose filters, and subjected to immunoblot analysis with various antibodies. Gels were calibrated with prestained broad range protein markers (New England BioLabs), and antibodies were used at the following concentrations: anti-Myc IgG-9E10, 1 μg/ml; anti-HSV tag antibody, 0.67 μg/ml; anti-SCAP IgG-9D5s, 5 μg/ml; anti-KDEL antibody (StressGen Biotechnologies), 3 μg/ml; anti-transferrin receptor (Zymed Laboratories Inc.), 0.5 μg/ml; anti-FLAG M2 antibody, 1 μg/ml; and anti-mouse IgG (Jackson ImmunoResearch), 0.2 μl/ml. Bound antibodies were visualized by chemiluminescence using the Superpixel Substrate System (Pierce) according to manufacturer’s instructions. Filters were exposed to Kodak X-Omat Blue X-ray films at room temperature for various times as indicated in legends.

**RESULTS**

Fig. 1A shows a hydrophy plot of the amino acid sequence of human Insig-1. Based on this plot, Insig-1 is predicted to contain approximately six transmembrane segments, depicted by peak regions of hydrophobicity. Based on data from experiments described below, we suggest a membrane topology for human Insig-1 that is shown in Fig. 1B. The membrane-spanning segments correspond to hydrophobic sequences 1–6 in the hydrophy plot. The hydrophilic N-terminal and C-terminal ends are proposed to extend into the cytosol with the remaining portion, containing six membrane-spanning helices, embedded in the membrane.

As a first step in these studies, we documented the membrane association of Insig-1 by transfecting human embryonic kidney-293 (HEK-293) cells with pCMV-Insig-1-MycX6, which encodes human Insig-1 with six copies of a c-Myc epitope tag at its C terminus (Fig. 2). Previous studies demonstrated that this epitope-tagged version of Insig-1 has wild-type activity in its ability to bind SCAP in a sterol-dependent fashion and to confer sterol-regulated cleavage of SREBP-2 (1). Control-transfected cells (Fig. 2A, lanes 1–2) or cells transfected with pCMV-Insig-1-MycX6 (lanes 4–6) were harvested, lysed, and separated into three fractions: a 10^5 × g membrane pellet (lanes 1 and 4), 10^5 × g supernatant (lanes 2 and 5), and nuclear extract (lanes 3 and 6). Samples were then subjected to SDS-PAGE and immunoblot analysis with a monoclonal anti-Myc antibody. Insig-1 gives two bands on SDS-PAGE. The lower band, migrating at 37-kDa, is produced by alternative translation initiation at methionine 37 (1). The Insig-1 signal was localized exclusively to the 10^5 × g pellet (lane 4), which represents the membrane fraction of the cell. Both the nuclear extract fraction and the 10^5 × g supernatant were devoid of Insig-1 signal.

We next sought to determine the manner in which Insig-1 is associated with membranes (Fig. 2B). Membrane fractions of HEK-293 cells expressing epitope-tagged Insig-1 were isolated, exposed to various solubilizing agents, and subjected to centrifugation to separate the soluble (supernatant) and insoluble (pellet) fractions, which were then subjected to SDS-PAGE and immunoblot analysis.
immunoblotted for Insig-1. When exposed to aqueous buffer alone, Insig-1 was found in the pellet fraction (lane 3). Incubation with 1% SDS or 1% Triton X-100 released the majority of Insig-1 into the soluble fraction (lanes 2 and 12). The addition of 0.1 M sodium carbonate, 1 M hydroxylamine, or 1 M sodium chloride, reagents known to dissociate peripheral proteins from the membrane, failed to release Insig-1 into the supernatant (lanes 5–10). These results demonstrate that Insig-1 is an integral membrane protein.

To determine the membrane orientation of the hydrophilic N-terminal and C-terminal ends of Insig-1, we prepared an expression plasmid, pCMV-MycX3-Insig-1, that encodes human Insig-1 preceded by three copies of the c-Myc epitope (Fig. 3A). To compare the activities of Insig-1 tagged at the N or C terminus, we used SRD-13A cells, a line of SCAP-deficient CHO cells in which the amount of SCAP can be controlled by transfection (15). SRD-13A cells were transiently transfected with cDNAs encoding pCMV-SCAP, epitope-tagged SREBP-2, and Insig-1 tagged at either its N-terminal or C-terminal end (Fig. 3B). The cells were incubated for 5 h in the absence or presence of sterols, after which nuclear extract and membrane fractions were subjected to SDS/PAGE and immunoblot analysis. When SCAP and SREBP-2 were overexpressed, the nuclear form of SREBP-2 was not reduced in the presence of sterols (lanes 2 and 3). Earlier, we showed that this lack of reduction occurs because the overexpressed SCAP saturates the endogenous Insigs (1). When Insig-1 levels were elevated by transfection with pCMV-MycX3-Insig-1, sterols regained the ability to block SREBP-2 cleavage (lanes 4 and 5). Identical results were observed with pCMV-Insig-1-MycX6 (lanes 6 and 7). These data indicate that Insig-1 remains functional in trapping SCAP/SREBP when tagged at either its N terminus or C terminus.

To localize the N and C termini of Insig-1 with respect to the ER membrane, we transfected HEK-293 cells with pCMV-MycX3-Insig-1 or pCMV-Insig-1-MycX6, isolated intact ER membrane vesicles, and treated them with trypsin in the absence or presence of Triton X-100. The samples were then subjected to SDS/PAGE followed by immunoblotting with an antibody against the Myc tag. Membranes from control-transfected cells showed no reactivity with the anti-Myc antibody (Fig. 3, C, lanes 1–4 and D, lanes 1–4). In the absence of trypsin (Fig. 3C), the N-terminal epitope-tagged Insig-1 appeared as a single band at 34 kDa (lane 3), and small amounts of trypsin destroyed the epitope (lanes 6–8). The results were similar in the presence of Triton X-100, except slightly higher levels of trypsin were required to eliminate the Insig-1 signal (lanes 9–12). The C-terminal epitope-tagged Insig-1 appeared as two bands of 40- and 37-kDa in the absence of trypsin (Fig. 3D, lanes 5 and 9). As discussed above, the lower band is produced by alternative translational initiation at methionine-37 (1). The C-terminal Myc epitope was destroyed by trypsin either in the absence or presence of Triton X-100 (lanes 5–12). To control for the integrity of membrane vesicles, we visualized two ER luminal proteins, grp78 (BiP) and grp94, by blotting with an antibody against their common C-terminal KDEL sequences (Fig. 3, C and D, lower panels), which are known to be located in the ER lumen (20, 21). The KDEL epitope of grp94 is resistant to trypsin digestion in the absence of Triton X-100 (Fig. 3, C and D, lower panel, lanes 2, 6–8), but was destroyed in the presence of detergent (Fig. 3, C and D, lower panel, lanes 4, 10–12). These results confirm that the membrane vesicles were indeed sealed and impermeant to trypsin in the absence of detergent.

grp78 was not digested by trypsin, either in the absence or presence of detergent (Fig. 3, C and D, lower panel, lanes 1–12), indicating that this protein is intrinsically trypsin-resistant, even when solubilized with Triton X-100. This observation is consistent with previous studies (19, 22). Considered together, the results of Fig. 3 indicate that both the N- and C-terminal ends of Insig-1 are oriented toward the cytosol.

We next tested the orientation of the first predicted hydrophilic loop of Insig-1. For this purpose, we modified pCMV-Insig-1-MycX3 by inserting into the predicted hydrophilic loop two copies of the 8-amino acid FLAG epitope, separated by an N-linked glycosylation sequence (NGT). This plasmid is designated “FLAG A” (Fig. 4A). The Insig-1 protein produced by FLAG A was functional as determined by its ability to eliminate SREBP-2 from nuclear extracts in a sterol-dependent fashion when co-transfected into SRD-13A cells with excess SCAP and SREBP-2 (Fig. 4B, lanes 4–7).

To determine whether the first hydrophilic loop of Insig-1 is located in the ER lumen, we tested the ability of the NGT sequence in FLAG A to become N-glycosylated. HEK-293 cells were transfected with either wild-type or FLAG A versions of Insig-1, and membrane fractions were isolated and subjected to treatment with PNGase F, which cleaves between the core GlcNAc and the asparagine residue of N-linked glycoproteins (23). Although wild-type Insig-1 has two potential N-linked
glycosylation sites at residues 39 (NVN) and 110 (NTV), these sites are apparently not utilized, as indicated by the lack of change in the mobility of wild-type Insig-1 after PNGase F treatment (Fig. 4C top panel, lanes 5 and 6). In contrast, when the FLAG A version was expressed, both bands shifted after PNGase F treatment (Fig. 4C, bottom panel, lanes 5 and 6). In order to determine if the FLAG A Insig-1 reached the Golgi, we subjected the membranes to endo H digestion. N-linked carbohydrate chains become resistant to endo H digestion upon modification by mannosidase II in the Golgi (23). FLAG A Insig-1 increased in mobility upon endo H treatment (Fig. 4C, bottom panel, lanes 5–8). In contrast, when 0.2 μg of pCMV-Insig-1-MycX6 was transfected instead of pCMV-MycX3-Insig-1, Aliquots of the membranes (100 μg of protein) were treated with trypsin and analyzed as in C. Filters were exposed to film for 1–5 s.

The presence of Triton X-100, proteinase K destroyed the FLAG epitope (Fig. 4D, top panel, lanes 9–12). Again, to control for the integrity of the membrane vesicles, duplicate filters were blotted with the anti-KDEL antibody. Proteinase K treatment destroyed both grp78 and grp94 immunoreactivity only in the presence of detergent (Fig. 4D, bottom panel, lanes 5–8 and 9–12). Duplicate filters were blotted for the C-terminal Myc epitope. The immunoreactivity of the Myc tag was abolished upon proteinase K treatment both in the absence and presence of Triton X-100, consistent with its cytosolic orientation (Fig. 4D, middle panel, lanes 5–8 and 9–12). Together the data presented in Fig. 4, C and D indicate that the first predicted loop of human Insig-1 faces the lumen of the ER, consistent with the model shown in Fig. 1B.

Fig. 5 shows the results of proteinase K treatment of membranes isolated from HEK-293 cells transiently transfected with cDNAs expressing versions of pCMV-Insig-1-MycX3 containing an NGT sequence flanked by two copies of the FLAG epitope inserted into the N-terminal extension and two of the hydrophilic segments of the protein. In FLAG B the epitope is located between residues 61 and 62 (N terminus). In FLAG C it is between residues 151 and 152 (loop 2), and in FLAG D it is between residues 237 and 238 (loop 5) (Fig. 5A). Insertion of these FLAG epitopes did not disrupt the function of any of these proteins, as all conferred sterol-regulated SREBP-2 pro-
Fig. 4. Membrane orientation of the hydrophilic loop between the first and second transmembrane segments of Insig-1 as determined by glycosidase sensitivity and proteinase K treatment. A, schematic illustration of the fusion proteins expressed from cDNAs used in this figure. The wild-type (wt) plasmid is the same plasmid referred to in Fig. 3A as pCMV-Insig-1-MycinX6. The FLAG A version of pCMV-Insig-1-MycinX3 expresses a protein similar to that encoded by pCMV-Insig-1-MycinX3 except that two copies of a FLAG epitope tag, flanking a potential glycosylation site, were introduced between amino acids 112 and 113 of human Insig-1. B, on day 0, SRD-13A cells were set up as in Fig. 3B. On day 1, the cells were transfected with the following plasmids per dish: 2 μg of pTK-HSV-BP-2; 0.5 μg of pCMV-SCAP; and 0.15 μg of either the wild-type plasmid pCMV-Insig-1-MycinX6 or the FLAG A version of pCMV-Insig-1-MycinX3 as indicated. On day 2, cells were treated as described under "Experimental Procedures." The samples were then mixed with 5× SDS loading buffer, boiled for 5 min, and subjected to SDS/PAGE and immunoblot analysis with anti-HSV tag antibody (against SREBP-2), IgG-9D5 (against SCAP), and IgG-9E10 (against Insig-1). Filters were exposed to film for 1–30 s. N and P denote the cleaved nuclear and uncleaved precursor forms of SREBP-2, respectively. C, on day 0, HEK-293 cells were set up as in Fig. 2C. On day 2, the cells were transfected with 0.4 μg per dish of either the control vector (upper and lower panels, lanes 1–4), pCMV-Insig-1-MycinX3 (upper panel, lanes 5–8), or FLAG A plasmid (lower panel, lanes 5–8). On day 3, the cells were harvested for preparation of membranes. Aliquots of the membranes (60 μg of protein) were solubilized and subjected to treatment with the indicated glycosidase (F, PNGase F; H, endo H) as described under "Experimental Procedures." The samples were then mixed with 5× SDS loading buffer, boiled for 5 min, and subjected to SDS/PAGE and immunoblot analysis with IgG-9E10 (against Insig-1) and anti-transferrin receptor antibody. D, on day 0, HEK-293 cells were set up as in Fig. 2C. On day 2, the cells were transfected with 0.3 μg per dish of either the control vector (lanes 1–4) or FLAG A plasmid (lanes 5–12). On day 3, the cells were harvested for preparation of membranes as described. Aliquots of the membranes (100 μg of protein) were treated with the indicated concentration of proteinase K in a final volume of 58 μl of buffer B in the absence or presence of 1% Triton X-100 as indicated. After incubation for 40 min at 30 °C, the reactions were stopped by addition of phenylmethylsulfonyl fluoride at the final concentration of 5 mM. The samples were then mixed with 5× SDS loading buffer, boiled for 5 min, and subjected to SDS/PAGE and immunoblot analysis with the indicated antibody. Filters were exposed to film for 1–5 s.

In the absence of detergent, the FLAG epitopes in FLAG B and FLAG C were sensitive to proteinase K digestion, suggesting that these regions are oriented toward the cytosol (Fig. 5C, top and middle, lanes 5–8 and 9–12). In contrast, the FLAG D epitope was destroyed by proteinase K only in the presence of Triton X-100, suggesting its orientation in the ER lumen (Fig. 5C, bottom, lanes 5–8 and 9–12). As controls for these experiments, we blotted with antibodies against the Myc tag (Fig. 5C, middle panels) and the KDEL sequence (Fig. 5C, bottom panels), and these data revealed the expected patterns of protease sensitivity.

The FLAG epitope insertion method was useful for the localization of hydrophilic loops 1, 2, and 5, but it was not useful for the predicted short loops at positions 3 and 4 because insertions into these sites abrogated protein expression, presumably because of interference with protein folding. Accordingly, we turned to another technique, cysteine derivitization. Native Insig-1 contains seven cysteine residues. To facilitate the cysteine derivitization experiments, we first prepared an expression plasmid encoding a mutant version of Insig-1 in which all seven cysteines were mutated to serines. To this cysteine-less cDNA, we systematically introduced single cysteine residues at precise locations, as shown in Fig. 6A. We tested the functionality of each mutant Insig-1 for its ability to confer sterol-regulated SREBP-2 cleavage in SRD-13A cells. All of the cDNAs produced similar levels of Insig-1 protein, and all were functional in blocking SREBP-2 cleavage in a sterol-dependent fashion (Fig. 6B, lanes 5–30). For the cysteine derivitization experiments, we transfected these plasmids into HEK-293 cells. Sealed membrane vesicles were isolated and pretreated with a membrane impermeable cysteine derivitization agent MTSET (see "Experimental Procedures"). The vesicles were then treated with a membrane-permeable cysteine derivitization reagent, biotinylated maleimide. The membranes were

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then solubilized with Nonidet P-40 detergent, and biotinylated proteins were precipitated with neutravidin beads. The precipitated material was subjected to SDS-PAGE and immunoblot with anti-Myc.

We anticipated that the cysteine derivitization experiments would reveal three possible results, depending on the location of each cysteine: 1) cytosolic cysteines would be biotinylated in the absence of MTSET, and derivitization would be blocked by prior exposure to MTSET; 2) luminal cysteines would be biotinylated in the absence or presence of MTSET; and 3) intramembrane cysteines would not be biotinylated in either the absence or presence of MTSET because the biotinylation reaction can only occur in the water phase. As a test for the specificity of these reactions, we carried out labeling experiments in cells expressing Insig-1 with a single cysteine at position 74 in the N-terminal extension, which is known to be cytosolic. When cells expressed the cysteine-null mutant of Insig-1, there was no biotinylation and all of the Insig-1 was found in the supernatant fraction (Fig. 6C, lanes 1 and 2). The cysteine 74 mutant was biotinylated and precipitated in the absence of MTSET, and this reaction was blocked by MTSET (lanes 3 and 4), consistent with the cytosolic location of this cysteine. The same result was obtained with cysteines at positions 207 and 267, suggesting that hydrophilic loop 4 and the C terminus are oriented toward the cytosolic face of the ER membrane (Fig. 6C, lanes 9–10 and 13–14). In contrast, cysteines introduced at residues 111, 184, and 233 were biotinylated in the absence of MTSET incubation, and the reaction was not significantly blocked by MTSET incubation, and the reaction was not significantly blocked by MTSET (Fig. 6C, lanes 5–6, 7–8, 11–12) suggesting a luminal orientation for hydrophilic loops 1, 3, and 5. When cysteines were added at positions that were predicted to be located within transmembrane regions (residues 88, 93, 124, 203, 212, and 254), none of the proteins were biotinylated (Fig. 6C, lanes 15–26). This result supports the
localization of these residues to the membrane-spanning regions of Insig-1.

**DISCUSSION**

The current experiments define the membrane topology of Insig-1, a resident ER protein that is central to the control of lipid synthesis in animal cells. As summarized in Fig. 7, complete topographic mapping required the combined use of three methodologies; 1) protease protection, 2) N-glycosylation site mapping, and 3) cysteine derivitization. Fig. 7 shows the amino acid sequence of human Insig-1 in relation to the topographical map of the protein. Of the 277 total amino acids in the protein, approximately one-half are buried within the membrane. The vast majority of the water-exposed residues are located in the N- and C-terminal extensions. The hydrophilic loops that link the membrane-spanning helices are very short, ranging from 5 to 16 amino acids. The overall degree of amino acid conservation of Insig-2 with Insig-1 (59% identity) and the conserved hydrophobicity plots of the two Insigs (2) suggest a similar orientation. Indeed, trypsin digestion studies of a C-terminal epitope-tagged Insig-2 showed a cytosolic orientation (data not shown).
Although the current studies define the orientation of the hydrophilic loops, they do not indicate precisely the locations of the junctions between membrane-spanning helices and the various loops. We note that the second transmembrane helix is quite long (−27 residues) (Fig. 7), and some of the indicated residues may actually project into the water phase.

Insigs have been shown to bind to two intrinsic ER proteins, SCAP and HMG CoA reductase, in a sterol-dependent manner (1, 2, 4). Both Insig-interacting proteins have a similar organization: 1) an N-terminal polytopic membrane domain containing eight membrane-spanning segments, and 2) a long hydrophilic C-terminal extension that projects into the cytosol. In SCAP, this extension is composed of multiple WD-repeat domains that form propeller-like structures that bind SREBPs (12) and also coat proteins that cluster SCAP/SREBP complexes into CopII vesicles that bud from the ER (25). In HMG CoA reductase, the globular cytosolic domain contains all of the catalytic activity of the enzyme (26, 27). In both cases the polytopic membrane domain is the site of sterol regulation (8, 28), and in both cases the C-terminal extensions can be deleted without abolishing sterol-dependent binding to Insigs (3, 29). Inasmuch as most of Insig-1 is buried in the membrane, the current data support a model in which its sterol-dependent interactions with SCAP and HMG CoA reductase occur through binding to the transmembrane segments of these proteins. This conclusion is consistent with previous data showing that three point mutations in SCAP abolish sterol-dependent binding to Insig (1, 2). All three mutations (D443N, Y298C, and F315L) involve residues that are located either within or immediately adjacent to the predicted membrane-spanning helices.

Many questions about Insig function remain to be answered. These include the following: 1) How is Insig retained in the ER? 2) How does Insig binding lead to two very different responses (ER retention of SCAP/SREBP versus ubiquitin-mediated degradation of HMG CoA reductase)? 3) Why do cells produce two isoforms of Insig that are independently regulated, but seem to have the same functions? and 4) Does Insig play a direct role in sterol binding, or does it simply attach to SCAP and HMG CoA reductase after these proteins have bound sterols? These and other many questions should be solved more readily now that the membrane topology of Insig is established.

Acknowledgments—We thank our colleagues Chris Adams and Daisuke Yabe for helpful discussion; Angela Carroll, Jill Fairless, and Linda Donnelly for invaluable help with tissue culture; and Jeff Cormier for DNA sequencing.

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