Membrane topology of the human seipin protein

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Abstract The Berardinelli-Seip congenital lipodystrophy type 2 (BSCL2) gene encodes an integral membrane protein, called seipin, of unknown function localized to the endoplasmic reticulum of eukaryotic cells. Seipin is associated with the heterogeneous genetic disease BSCL2, and mutations in an N-glycosylation motif links the protein to two other disorders, autosomal-dominant distal hereditary motor neuropathy type V and Silver syndrome. Here, we report a topological study of seipin using an in vitro topology mapping assay. Our results suggest that the predominant form of seipin is 462 residues long and has an N\textsubscript{cyt}--C\textsubscript{cyt} orientation with a long luminal loop between the two transmembrane helices.

Keywords: Seipin; N-linked glycosylation; Oligosaccharyl transferase; Topology mapping; BSCL gene

1. Introduction

Seipin is associated with autosomal recessive Berardinelli-Seip congenital lipodystrophy type 2 (BSCL2) characterized by near absence of adipose tissue and severe insulin resistance [1]. Recently published results show that seipin is an integral membrane protein located in the endoplasmic reticulum (ER) and that two heterozygous mutations in the BSCL2 gene are associated with autosomal-dominant distal hereditary motor neuropathy type V and Silver syndrome [2,3]. The two known mutations affect a highly conserved glycosylation site, and result in aggregate formation leading to neurodegeneration. The occurrence of seipin mutations in two disorders affect primarily the peripheral nerves indicating an important role in the peripheral nervous system [4].

Presumably more than 50\% of all eukaryotic proteins are glycosylated [5] and defective protein glycosylation has been associated with a variety of human genetic diseases. Recently a pathogenic role through the gain of a glycosylation site by missense mutations has been demonstrated and several candidate genes were investigated [6].

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Abbreviations: ER, endoplasmic reticulum; TM, transmembrane; BSCL, Berardinelli-Seip congenital lipodystrophy; BHK, baby hamster kidney cells; SFV, Semliki Forest virus; TMHMM, transmembrane hidden Markov model

Northern blot analysis has shown that BSCL2 is transcribed into at least three different mRNAs with sizes ranging from 1.6, 1.8 to 2.2 kb [2,5]. Whereas the 1.8 kb mRNA, corresponding to AF052149, is exclusively expressed in brain and testis, the 1.6 and 2.2 kb (BC012140, BC009866) mRNAs are ubiquitously expressed.

The alignment of the human protein with other species suggests that seipin is at least 398 amino acids long [7] but might include an additional 64 residues at the N-terminus [8]. Due to an alternative 5'UTR exon, the 1.6 kb mRNA can only be translated into a 398 residues long protein. Because of two potential translation initiation sites, both the 1.8 and 2.2 kb mRNAs potentially code for proteins composed of both 398 and 462 amino acids. The Kozak consensus sequences for these two translation start sites are both predicted by www.hri.co.jp/atgpr/ as possible initiation sites.

The N- and C-terminal regions of seipin appear to be variable among species, but a highly conserved central region is 88\% identical among human, mouse, rat and chimpanzee. Seipin lacks any recognizable functional domains but has weak homology to the 550 kDa human and yeast midasin protein and to the surface protein PspC from Streptococcus pneumonia. Seipin has a conserved CAAX-motif sequence (CSSS) at the C-terminus, suggesting that it may be prenylated [9].

Despite its central role in severe disorders little information on the membrane topology of seipin is currently available. The N-V-S sequon at positions 152–154 (numbering refers to the 462 residues form of the protein) carry an N-linked glycan, and is therefore located on the luminal side of the ER membrane [2], but the number of transmembrane (TMs) helices and their orientations relative to the membrane have not been determined. Different topological prediction programs suggest that seipin has between one and three TM helices [9].

Here, we present an in vitro topological mapping study of human seipin, using engineered glycosylation sites at both ends of the protein and in the regions between the putative TM segments to determine the membrane orientation. We propose an N\textsubscript{cyt}--C\textsubscript{cyt} topology with two TM helices. We further provide both in vitro and in vivo data suggesting that the longer form of seipin comprising 462 residues is the predominant form of the protein.

2. Materials and methods

2.1. Plasmid constructions

Human seipin constructs were made as previously described [10]. Briefly, for cloning into and in vitro expression from the pGEM1 plasmid, the 5' end of the BSCL2 gene encoding seipin (462 amino acids...
long) was modified in two ways during PCR amplification: (i) by the introduction of a 5′ Xhol site, and (ii) by changing the context of the region immediately upstream of the initiator ATG codon to a Kozak consensus ribosome binding sequence, GCCACCATGG [10,11]: both changes were encoded within the 5′ PCR primer. The reverse primer encoded the 3′-end of BCSEL2, two stop codons, and a Snod site for cloning. The BCSEL2 gene was amplified using the Expand Long Template PCR System from Roche Diagnostics GmbH (Mannheim, FRG) and cloned into pGEM1 downstream of the SP6 promoter as an Xhol–Snod fragment. The amplified DNA products were purified using the QIAquick PCR Purification kit from QIAGEN (Hilden, FRG).

Glycosylation acceptor sites were designed as described previously [12], i.e. by replacing or inserting one or more appropriately positioned codons for the acceptor tripeptide Asn-Ser-Thr.

Site-specific mutagenesis was performed using the QuickChange™ Site-Directed Mutagenesis kit from Stratagene (La Jolla, USA). All mutants were confirmed by sequencing of plasmid DNA at BM labbet AB (Furulund, Sweden). Restriction enzymes were from Promega, Inc. (Madison, WI, USA).

2.2. In vitro expression

DNA template for in vitro transcription was prepared by transcription of the relevant pGEM1-derived plasmid with SP6 RNA polymerase for 1 h at 37°C. mRNA translation in nuclease-treated reticulocyte lysate supplemented with [35S]Met and dog pancreas rough microsomes was performed as described [13] at 30°C for 1 h. Samples were analyzed by SDS-PAGE and proteins were visualized in a Fuji FLA-3000 phosphorimager using the Image Reader V1.8J/Image Gauge V3.45 software.

2.3. In vivo expression

The seipin cDNA was cloned into the pcDNA™ 3.1/myc-His vector Invitrogen (CA, USA) in order to obtain a Myc tag in the C-terminus. Protein synthesis in baby hamster kidney (BHK) cells using the Semliki Forest virus (SFV) expression system was carried out as described previously [14,15]. Briefly, seipin constructs containing natural Kozak consensus ribosome binding sequence (AGGAAGATGG) under the SP6 promoter in the SFV vector was linearized for in vitro transcription. The resulting RNA was used to transfect BHK cells by electroporation. Six hours after electroporation, cells were starved of Met for 30 min, then labelled with [35S]-Met for 15 min. Cells were solubilized in lysate buffer containing 1% nonidet P-40 and protease inhibitors, immunoprecipitated using an anti-Myc antibody (Invitrogen) and analysed by SDS-PAGE. Quantifications were carried out on a phosphorimager as described above.

3. Results

As noted in Section 1, it is unclear whether seipin is translocated from the first or second Met in the open reading frame, corresponding to a total length of either 462 or 398 residues. To resolve this issue, we expressed seipin from a full-length cDNA clone that encodes both possible forms. When expressed in vitro in the presence of dog pancreas rough microsomes, the only product seen corresponds to the long form of the protein, Fig. 1A (lane 4). We also expressed a myc-tagged version of seipin in BHK cells using an SFV vector. In this case, one major and one minor product were observed, Fig. 1A (lane 1), corresponding to the long and short forms. Since the long form was the predominant one under both in vitro and in vivo conditions, we decided to focus our study on this form of seipin and therefore, all residue numbers below refer to the long form of the protein.

The membrane topology model for human seipin predicted by the transmembrane hidden Markov model method (TMHMM) [16] is shown in Fig. 1B. There are two strongly predicted TM helices (residues 95–117 and 294–316), with a third, weakly predicted candidate TM helix around residues 2282–2380. To experimentally determine the topology of seipin, we used a glycosylation mapping approach [17–19] to map the location of the N- and C-terminal ends of the protein and of the predicted loop relative to the ER membrane. When expressed in vitro in the absence of microsomes seipin migrated as a single product. In the presence of microsomes, it inserted into the microsomal membrane with the predicted loop in the lumen, as seen by the glycosylation of an endogenous acceptor site for N-linked glycosylation (Asn152-Val-Ser) present in this domain, Fig. 2A. No N-glycosylation was observed when the missense mutation N152S of seipin was expressed in the presence of microsomes. Therefore, the potential acceptor site Asn152-Phe-Thr that is situated in the predicted TM segment between residues 294 and 316 is, as expected, not glycosylated. N-linked glycosylation in the microsomal system requires a minimum distance of 10–15 residues between the glycosylation site and the nearest TM segment [20–22].

Since the wild-type protein has a modified endogenous N-linked glycosylation site, we used the N152S mutant for further topology mapping experiments. Single glycosylation acceptor sites (Asn-Ser-Thr) engineered into the N- and C-terminal tails at residues 3 and 423 were not modified, whereas an engineered site in the loop at position 254 was efficiently glycosylated, Fig. 2B. Fusion of the C-terminal part of seipin (residues 375–462) containing the engineered glycosylation acceptor site in position 423 to residue 425 in the luminal C-
terminal domain of the leader peptidase protein leads to efficient glycosylation of the engineered site (data not shown), demonstrating that it is not inherently refractive to modification. We conclude that the N- and C-termini are facing the cytosol, whereas the entire region between residues 117 and 294 is exposed to the ER lumen.

4. Discussion

This study reports the membrane topology and N-glycosylation of human seipin, and also provides data suggesting that the predominant form of seipin is 462 rather than, as originally proposed, 398 residues long.

We used engineered glycosylation sites to determine the topology of the seipin protein expressed in a mammalian in vitro transcription/translation system supplemented with dog pancreas microsomes. Glycosylation acceptor sites placed near the N- and C-termini were not modified, pointing to a cytosolic location of both the N- and C-terminus. An engineered glycosylation site in the central predicted luminal loop (L254) was efficiently modified, and the presence of an endogenous glycosylation site (N152) in the same loop also suggests a luminal location. Using all the experimental data as constraints on the TMHMM topology predictor [23], we obtained the TM model shown in Fig. 3. This model places the most highly conserved part of seipin, including the endogenous glycosylation site, in the lumen of the ER.

In summary, we present a model for the membrane topology of the 462-residues long form of seipin, with two TM helices, the N- and C-terminus in the cytosol, and the conserved central domain loop in the lumen of the ER. These results provide a necessary basis for future functional studies of the seipin protein.

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References


Fig. 2. Investigation of human seipin topology by in vitro translation. The position of the Asn-residue in the engineered NST glycosylation acceptor sites is indicated above each lane. (A) In vitro translation in the absence (−) or presence (+) of rough microsomes (RM) of the wild type protein and the N152S mutation. G, glycosylated product; UG, unglycosylated product. (B) In vitro translation in the absence (−) or presence (+) of rough microsomes (RM) of constructs N3 and C423 in the N152S and wild type backgrounds, and of construct N254 in the N152S background. G, glycosylated product; UG, unglycosylated product.

Fig. 3. Topology model for seipin. Experimentally analysed glycosylation sites are indicated in black letters. The dashed line indicates the weakly predicted TM segment (cf., Fig. 1B).


