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Genomic structure and chromosomal mapping of the human Site-1 protease (*S1P*) gene

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Abstract Site-1 protease (S1P) is a subtilisin-related enzyme that cleaves sterol regulatory element-binding proteins (SREBPs) in the lumen of endoplasmic reticulum, thereby initiating the release of transcriptionally active NH₂-terminal fragments of SREBPs from membranes. In the experiments reported here, we localized the human *S1P* gene to chromosome 16q24 by fluorescent in situ hybridization and radiation-hybrid mapping, and determined its genomic structure. This gene is more than 60kb long and contains 23 exons and 22 introns. Its transcription-initiation site within exon 1 is separate from the initiation codon in exon 2. Analysis of the exon/intron structure revealed that the *S1P* gene consists of a mosaic of functional units: exon 1 encodes the 5' non-translated region; exon 2 encodes the NH₂-terminal signal sequence; and exons 2 and 3 encode the pro-peptide sequence that is released when *S1P* is self-activated by intramolecular cleavage. Exons 5–10 encode the subtilisin-homology domain that is critical for catalytic activity, and exon 23 encodes the transmembrane region. Analysis of the putative promoter region revealed a highly G/C-rich region containing a binding site for ADD1/SREBP-1, as well as Sp1 and AP2 sites. Therefore, expression of the *S1P* gene may be under the control of SREBP-1, a key regulator of the expression of genes essential for intracellular lipid metabolism. Our data establish a basis for investigations to detect molecular variants in this gene that may alter levels of plasma lipoproteins and/or otherwise disrupt intracellular lipid metabolism.

Key words Site-1 protease (S1P) · Sterol regulatory element binding proteins (SREBPs)

Introduction

Cholesterol metabolism in mammalian cells is controlled by the sterol-regulated proteolysis of membrane-bound transcription factors called sterol regulatory element-binding proteins (SREBPs) (Sakai et al. 1996; Brown and Goldstein 1997; Sakai et al. 1998a,b). In this regulatory pathway, the first cleavage in SREBPs is made by site-1 protease (S1P), a membrane-bound subtilisin-related serine protease that cleaves SREBPs in a hydrophilic loop that projects into the lumen of the endoplasmic reticulum and other organelles. Cleavage by S1P separates the SREBP into two fragments, each of which has a single membrane-spanning sequence. This separation allows a second protease, designated site-2 protease (S2P), to cleave the NH₂-terminal fragment at a position within its membrane-spanning sequence. The release of the NH₂-terminal segment allows an SREBP to enter the nucleus, where it binds to enhancers and activates the transcription of genes encoding the low-density lipoprotein (LDL) receptor, as well as multiple enzymes of cholesterol and fatty acid biosynthesis (Yokoyama 1993; Guan et al. 1995; Osborne 1995; Ericsson et al. 1996; Kim and Spiegelman 1996; Vallett et al. 1996; Brown and Goldstein 1997). When sterols accumulate in cells, the cleavage of SREBPs by S1P is abolished; SREBPs remain attached to membranes, and the transcription of their target genes declines.

The site-1 processing reaction is the target for the feedback-regulation of lipid biosynthesis and uptake in animal cells. This regulation is mediated by the sterol-sensing domain of SREBP cleavage-activating protein (SCAP) (Hua et al. 1996). This feedback regulation is disrupted in SRD-12B cells that harbor mutations in the *S1P* gene (Sakai et al. 1998a).

Given the central role of S1P in the regulation of lipid metabolism, molecular variants in the human *S1P* gene

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would likely result in altered concentrations of plasma lipoproteins and/or in the derangement of lipid metabolism within tissues. As a first step in determining whether molecular variants in the *SIP* gene contribute to human disease, we determined the structure and chromosomal localization of the human *SIP* gene.

Materials and methods

Isolation of the human *SIP* gene. We used a bacteriophage P1-derived artificial chromosome (PAC) library containing human genomic DNA pooled in a three-dimensional structure (Genome Systems, St. Louis, MO, USA), to isolate human *SIP* genomic clones. The three-dimensional PAC library was screened by a polymerase chain reaction (PCR) method described previously (Tsukamoto et al. 1998), using two oppositely oriented oligonucleotides (forward: 5'-AAACCATCTTCATCGGTACTCCA-3' and reverse: 5'-CTCGTTTAAAGGCTGTGGCTTG-3') that correspond, respectively, to nucleotides 3202-3224 and 3295-3316 of the human *SIP* cDNA (Genbank accession number, NM_003791). *Escherichia coli* containing positive clones were cultured in the presence of ampicillin, and PAC DNA was isolated as described elsewhere (Tsukamoto et al. 1998).

Sequencing of exon/intron junctions and the 5'-flanking region. Nucleotide sequences of exons and their bound-

aries were determined by sequencing the PAC clone directly, using primers designed from the *SIP* cDNA sequence. Sequencing was performed by the BigDye Terminator cycle sequencing method with a 377 ABI Prism automated DNA sequencer (Perkin Elmer, Norwalk, CT, USA). The size of each intron was determined by direct sequencing of the PAC genomic clone, or was estimated by PCR amplification of each intron, using LA Taq (Takara, Tokyo, Japan) and two oppositely oriented primers located in the exons immediately flanking each intron.

Fluorescence in situ hybridization (FISH). To assign the human *SIP* gene to its chromosomal location, a PAC clone carrying the human gene was used as the probe. Fluorescence in situ hybridization was carried out as described by Mukae et al. (1998). The PAC DNA was labeled with biotin-16-deoxyuridine triphosphate (dUTP) by nick-translation and hybridized to denatured metaphase chromosomes at a final concentration of 25 ng/ml in 50% formamide, 10% dextran sulfate, 2× standard saline citrate (SSC), 0.2 mg/ml Cot-1 DNA (GIBCO/BRL, Frederick, MD, USA), 2 mg/ml salmon sperm DNA, and 2 mg/ml *E. coli* tRNA. Hybridized signals were detected with fluorescein isothiocyanate-avidin (Boehringer Mannheim, Indianapolis, IN, USA). Cells were counterstained with 4',6-diamidino-2-phenylindole, and the slides were examined through a Nikon epifluorescence microscope equipped with a charge-coupled device (CCD) camera (Photometrics, Tucson, AZ, USA). Images were captured with Quips (Vysis, Tokyo, Japan) software and processed with Adobe Photoshop 3.0 software.

Table 1. Exon/intron organization of the human *SIP* gene

Exon		Sequence at exon/intron junction ^a		
number	Exon size (bp)	5' Splice donor	3' Splice acceptor	Intron size ^b (kb)
1	~172	GGCGGCGCAGgtacgtcagc•••	•••tggttcacagATTGGCTCAC	13.0
2	487	GTGGAATATGgtaatgatgt•••	•••ttgggttcagAATATATTGT	2.3
3	258	TATGCTGAATgtgagtattg•••	•••ttgcctgtagCTGACCCAC	3.0
4	204	GGATATACAGgtgtgtgcta•••	•••tccgaataagGTGCTAATGT	1.7
5	111	CTGGACGATGgtgggtctca•••	•••tggactgcagGGTTGGGCCA	0.4
6	110	CAATAATCAGgtaggtgtti•••	•••atttaactagGTATCTTACA	1.3
7	117	TGTTGACAAGgtttgtggag•••	•••cctttttagGTGTGGGAAT	2.8
8	68	CTCTTTATGGtaagtacgc•••	•••ctcaatagCACTCTGAAT	0.7
9	103	GACTACCTGGgtacgtctt•••	•••ttattcatagGAGCTACCAG	2.0
10	152	TGTTAGTGAGgtgagtgtg•••	•••tttctccagCACAGTCCAG	4.8
11	162	CACAGGCAAGgtcagtgca•••	•••tttatttcagTTTGAGCCCC	10.0
12	145	TGTAGATAAGgtgaaacttc•••	•••tttctgcagCCTGACTGGC	2.8
13	189	AGAGACAGAGgtaggatai•••	•••tttttttagTCAAAAAATG	1.1
14	173	CTTTAGACTGgtaagcacct•••	•••tggttttcagGAATGGTGAT	1.4
15	116	AGTCAGTATGtaagtggca•••	•••tggttcacagGCACTTTGCT	0.6
16	157	AAAACACAAGgtactgttga•••	•••actttccagGCAGTGGTGG	1.2
17	125	AACCATGACAgtaaggctct•••	•••tcttctagTGTATTATGC	0.7
18	78	AAGGACCAAGgtaaaggatg•••	•••gtgttttcagGATTGGAGGT	2.2
19	141	CGACAGAAGgtaggaaaaa•••	•••gacttcgagACTGCTTTTG	2.5
20	132	AGGATGGAAGgtgagtggat•••	•••ctaaccacagGAAACCATCT	1.2
21	127	CGGCGCCCAGgttagtgtcc•••	•••tttctgaagTAACCTTTGG	2.8
22	131	ATTCTGGAGgtgagttctc•••	•••cctcttacagGGATCATGCC	1.2
23	~880			

^aThe sequences of exon/intron boundaries were determined as described in Materials and methods. Lowercase letters refer to intronic sequences, and uppercase letters to exonic sequences

^bIntron sizes were determined by direct sequencing of the P1-derived artificial chromosome (PAC) genomic clone, or were estimated by polymerase chain reaction (PCR) amplification, using LA Taq (TaKaRa, Tokyo, Japan)

A **B**

Fig. 1. A Genomic structure of the human *SIP* gene. **B** Relationship between exon organization and functional domains of *SIP*. A translation initiation codon (*ATG*) is present in exon 2, and a translation stop

codon (*TGA*) in exon 23. Upward arrows indicate *SIP* processing sites. *SS*, Signal sequence; *TM*, transmembrane region

Fig. 2. Chromosomal localization of the human *SIP* gene to 16q by fluorescence in situ hybridization on metaphase chromosomes

Radiation-hybrid mapping. The G3 RH mapping panel from the Stanford Human Genome Center (Boehnke et al. 1991), was used to confirm and define further the localization of the human *SIP* gene. Typing was carried out using opposing primers located in the introns flanking exon 17 (forward: 5'-TGTTCCCTGTGTGTCTGTTACCAC-3'; reverse: 5'-GCTCTCACACCTAAGACCACGC-3'). The results of maximum-likelihood analysis were obtained by submitting the raw scores to <http://www-shgc.stanford.edu>.

Results and discussion

Cloning of the human *SIP* gene

More than 110kb of the genomic region containing the entire human *SIP* gene was present in an isolated PAC clone. The gene is more than 60kb long, about 15 times the size of its corresponding cDNA. It consists of 23 exons interrupted by 22 introns (Table 1 and Fig. 1A), exon 8 being the smallest, at 68bp. Other exons ranged from 78 to around 880bp. Sequences at the exon-intron boundaries for

AGTTGTGGGATTACAT

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-1200 CATGCGCTACCACTCAGCTAAGTTTTTATATTTTGGTAGAGCCGGCGTTTCACCCAT
-1140 GTTGACCAGGCTGGTCTGAACTTCTGACCTCAAGTGATCCGGCTGCCTCGGCCTCCCCA
      SREBP-1
-1080 AGTGCTGGGATTACAGGCGCGAGCCACCCCGCCTGGCTAGTACCCCTATTTTGTAAATGA
      Sp1
-1020 AGAAGCTGAGGTACAAAGAGGTTAAACAACCTGCTCAAAATTACGAAGTAGAAGCCTGAG
-960 GTGGGGTATGGTAATTTGTGATTCTACATTTATCCACCATATTAAGTCAACTGTGTTAAA
-900 TCATGTTGAAGGAAGATGATGCGCTGTGATTGTAATATTGGTGCAGTTCAGCATTACCGT
-840 GGTGCAGTGTCAAGTTCCAAAAGACACAAAACGTCTGATCGGCCTGTAGCGCATCACCG
-780 CCCACCAGCAGTACCTGAAGACGCTTTCAGTCCCCTGCTAGTGCCGTACGGAGTCCGGGC
      AP2
-720 TGGTCCATTTACATTGAAAACAACTGATCCTAGCATCTAAGTCACCCAGACATCTTTTA
-660 GGC GGCAAGCTTTTTCAGACAGTTTAAACATTTATTCTTGTTTTTTCAAAAAGAGCAAAC
      HNF3B
-600 ACGTGGCGTTACAAAGGTCCTCCGTTTCGCACGCACCGGTGTCCCGGCAGCGCGCTGCGC
      AP2
-540 GTCGGGAGGCCAGTCTGTCTCCGTTGGGGCCGGGGGTGAGAGGTCGGGCAGGCGGGA
-480 TTTGGGGTTTCGCGTGTTCGGACCGGACACTTCGCCATCTTAACGAGCTACCGCCCGGG
-420 TTAAGGGGCACCCAGGGCAGGTGCCGGGCCGGGGGTGGGGTGTCCGAGCCGCCGGTGCCT
      Sp1
-360 GGACGTGGACCCCTGCAGGAAGGGTCCGCGCGGGAAGCGAGCTCTCCACGAGTCCATGAC
-300 AGCAGGCCCGCGGGGGCAGGGAGCCTGCCGCCAGGCAGTCTTTCTAGATCCTGGCCCAGCC
      AP2
-240 TGGCCGCTTTTCGCGCCCGTGCTCCTTTTCGGGGATCCTAGAGCGACGCGCAGAGCGGACC
-180 AAGGGTTCCCCAGCGCGTTCTCGCCGCTGGCAGCTCGCTGGCCACGCCCTCCGCTTC
      Sp1
-120 CACCCTGCCACCGGCCCGCGCGCAAATGTCGCGAGACCTTCGTCCATCCTCCCGGC
      Sp1
-60 GTGGTCCCGCTGCCCACGGCGTCCAGAGCGCGCACGCAAGCCCCGGGAGCGCGGGACAGA
      AP2
+1 CAGGGCACGCTGGGTCGGCGGAGCTGAGGCTCCAGCTGTGGGCCTCGCTGGCCCGGTCG
+61 CCCAGTCTCGCGAGAGTTGGGAGTAAACAGCCCCGAAA***

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Fig. 3. Nucleotide sequence of the 5' flanking region of *SIP*. Boldface letters indicate 5' sequences of exon 1. Nucleotide 1 is assigned to the putative transcription-initiation site. Putative binding motifs for Sp1,

AP2, sterol regulatory element-binding protein (*SREBP*)-1, and HNF3B are indicated with *underlines*

all 22 introns are compatible with the consensus sequence for splicing junctions, including AG-GT (Mount 1982).

Comparison of the genomic sequence with the cDNA revealed that exon 1 and the 5' half of exon 2 encode the 5' untranslated region (UTR); the protein-coding region extends from the 3' portion of exon 2 through exon 22, and exon 23 encodes both the carboxy-terminal coding region and the 3'UTR (Fig. 1B). Exon 23 contains a polyadenylation site preceded by a polyadenylation signal.

Chromosomal mapping of the *SIP* gene

A PAC genomic clone served as the probe to localize the *SIP* gene by fluorescence in situ hybridization on

metaphase chromosomes. Clear fluorescent signals were visualized on chromosomal band 16q24 (Fig. 2). The *SIP* gene was mapped more precisely by means of the G3 RH mapping panel of 83 hybrid cell lines from the Stanford Human Genome Center (Boehnke et al. 1991), through linkage to marker SHGC-30993 with a logarithm of differences (lod) score of more than 14.83). These results support data reported by Nagase et al. (1995).

Exon organization and protein domains

The introns of *SIP* interrupt the protein-coding sequence in such a way that many domains are encoded by distinct exons, as Fig. 1B indicates. Exon 1 encodes the 5' non-

translated region; part of exon 2 encodes the 22 NH₂-terminal amino acids that constitute a signal sequence; the remainder of exon 2 and all of exon 3 encode the propeptide sequence that is synthesized as an inactive membrane-bound precursor and is self-activated by intramolecular cleavage to produce active enzyme (Cheng et al. 1999; Espenshade et al. 1999; Sakai et al. 1998a). Exons 5–10 encode the subtilisin-homology domain, whose aspartate 218, histidine 249, and serine 414 residues are required for the catalytic activity of *SIP* (Sakai et al. 1998a).

A region with no resemblance to other proteins is encoded by exons 11–22, and exon 23 encodes the transmembrane region and the 3'UTR.

Sequence of the 5'-flanking region of *SIP*

The nucleotide sequence of the putative promoter of the *SIP* gene is given in Fig. 3. This sequence was examined with MatInspector V2.2 (Quandt et al. 1995) to identify any binding sites for transcription factors. This region is highly G/C-rich and contains Sp1 sites and AP2 sites, but no CAAT or TATA box characteristic of promoters of housekeeping genes (Dyanan 1986). However, we identified an ADD1/SREBP-1 site (Yokoyama et al. 1993; Kim et al. 1995) in the 5'-flanking region of the *SIP* gene. The SREBPs are a family of three closely related membrane-bound transcription factors that activate genes involved in the synthesis and uptake from plasma of cholesterol and unsaturated fatty acids, and regulate the transcription of genes encoding several enzymes in the cholesterol biosynthetic pathway (e.g., 3-hydroxy-3-methylglutaryl-coenzyme A [HMG CoA] reductase, HMG CoA synthase, farnesyl diphosphate synthase, and squalene synthase; Guan et al. 1995; Osborne 1995; Ericsson et al. 1996; Vallett et al. 1996; Brown and Goldstein 1997). SREBPs also regulate the LDL receptor, which supplies cholesterol through receptor-mediated endocytosis (Yokoyama 1993; Kim and Spiegelman 1996; Brown and Goldstein 1997). Transcriptional regulation by SREBP-1 therefore has a central role in lipid metabolism. For its part, S1P-related proteolysis releases active fragments of SREBPs from membranes of the endoplasmic reticulum, and allows them to enter the nucleus (Hua et al. 1996). Although the transcriptional regulation of the *SIP* gene via SREBP-1 may be crucial for the regulation of lipid metabolism, its functional significance remains to be determined. The Sp1 sites present in its 5'-flanking region may act synergistically with SREBPs to activate expression of the LDL receptor gene (Sanchez et al. 1995; Yieh et al. 1995).

Our data establish a basis for investigations to discover molecular variants of the *SIP* gene that may alter plasma lipoprotein levels and/or derange intracellular lipid metabolism.

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