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# Sp-family of transcription factors regulates human SHIP2 gene expression

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**Abstract**

We have characterized the regulation of human SH-2 containing inositol 5′-phosphatase 2 (SHIP2) gene expression. First, the transcription initiation sites and the sequence of the 5′ upstream region of human SHIP2 gene were elucidated. Next, the minimal promoter of the human SHIP2 gene was identified by reporter gene assays in HL60 cells and differentiated human subcutaneous white adipocytes. An Sp1 element proximal to the transcription initiation site was indispensable for full promoter activity and bound specifically by Sp1 and Sp3 proteins. These findings suggest that human SHIP2 gene expression, like other housekeeping genes, is controlled by the Sp-family of transcription factors.


*Keywords:* SH-2 containing inositol 5'-phosphatase 2; Sp1; Sp3; Human adipocyte; HL60 cell

## 1. Introduction

Insulin binding to the extracellular α subunit of the insulin receptor activates the intrinsic tyrosine kinase activity of the intracellular β subunit. The activated insulin receptor phosphorylates the tyrosine residues of the insulin receptor substrate (IRS) family of proteins. IRS proteins propagate insulin signals to the p85 regulatory subunit of phosphatidylinositol (PI) 3-kinase, which activates the p110 catalytic subunit. PI 3-kinase functions as a lipid kinase which preferentially phosphorylates the D-3 position of PI, PI 4-phosphate [PI(4)P], and PI 4,5-bisphosphate [PI(4.5)P2] to produce PI(3)P, PI(3,4)P2, and PI 3,4,5-triphoshate [PI(3,4,5)P3], respectively. Insulin treatment increases amounts of cellular PI(3,4,5)P3 and PI(3,4)P2, which can serve as lipid second messengers to relay the signal to molecules downstream of PI 3-kinase such as Akt and atypical protein kinase C. These signals initiate GLUT4 translocation to the cytoplasmic membrane and activation of glycogen synthase, leading to glucose uptake and glycogen synthesis (Saltiel, 1996; Czech and Corvera, 1999; Virkamaki et al., 1999).

SH-2 containing inositol 5′-phosphatase 2 (SHIP2) is a family of inositol 5-phosphatases, which possess the 5'-phosphatase activity that hydrolyzes PI(3,4,5)P3 to PI(3,4)P2 (Pesesse et al., 1997; Ishihara et al., 1999; Schurmans et al., 1999). SHIP2 is a 140-kDa protein composed of an SH-2 domain at the N terminus, a central 5'-phosphatase catalytic domain, and a proline-rich region including a phosphotyrosine binding domain binding consensus at the C terminus (Wada et al., 2001). Although SHIP1 is a relatively hematopoietic cell-specific phosphoinositol 5′-phosphatase, SHIP2 is mainly expressed in the target tissues of insulin (Osborne et al., 1996).

SHIP2 was reported to negatively regulate metabolic signaling of insulin via 5'-phosphatase activity and to, therefore, be important for the regulation of insulin induced

*Abbreviations:* SHIP, SH-2 containing inositol 5'-phosphatase; IRS, insulin receptor substrate; PI, phosphatidylinositol; HPAd, human preadipocytes; NIDDM, non-insulin dependent diabetes mellitus.

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activation of molecules downstream of PI-3 kinase, leading to glucose uptake and glycogen synthesis (Wada et al., 2001; Sasaoka et al., 2001). Analysis with mice lacking the SHIP2 gene revealed that the loss of SHIP2 leads to an increased sensitivity to insulin, which was characterized by severe neonatal hypoglycemia, deregulated expression of genes involved in gluconeogenesis, and perinatal death (Clement et al., 2001). The enhanced expression of SHIP2 at the mRNA and protein levels was observed in the skeletal muscle and fat tissue of diabetic db/db mice. Treatment with the insulin-sensitizing agent rosiglitazone decreased the elevated expression of SHIP2 and normalized the sensitivity to insulin (Hori et al., 2002). These findings suggested a positive interaction between deregulated expression of SHIP2 and the diabetic pathology of insulin resistance. To elucidate how this kind of insulin resistance is acquired, it is necessary to clarify the regulatory mechanism of SHIP2 expression.

In this study, we examined the functional *cis*-acting elements in the human SHIP2 gene, and demonstrated that *trans*-activating factors Sp1 and Sp3 control human SHIP2 expression. Knowledge obtained from these studies may contribute to our understanding of the regulation of the human SHIP2 gene in vivo.

## 2. Materials and methods

### 2.1. Cloning of the human SHIP2 promoter

The screening was performed as previously described (Sambrook and Russell, 2001). Briefly, a human genomic library in the EMBL3 SP6/T7 vector was purchased from Clontech (Palo Alto, CA, USA). The library ($1\times10^6$ phages) was screened for positively hybridized plaques with a putative promoter fragment of the human SHIP2 gene (nucleotides from −284 to +238, numbered from the most upstream transcription start site).

### 2.2. Cell culture

The acute myelocytic leukemia cell line HL60 was grown in Dulbecco's modified Eagle's medium (Life Technologies, NY, USA) supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin in an atmosphere of 5% $CO_2$ at 37 °C. Human preadipocytes (HPAd) (origin: subcutaneous, white adipocytes) (Cell Applications, San Diego, CA, USA) were grown to confluence and induced to differentiate into adipocytes according to the manufacturer's instructions.

### 2.3. Identification of transcription start sites

The transcription start sites of the human SHIP2 gene in HPAd were identified by an oligonucleotide-capping method using an RLM-RACE kit (Life Technologies, NY, USA), following the manufacturer's instructions. Briefly, total RNA was extracted from HPAd. A primary PCR was conducted using the antisense gene-specific primer 1 (GSP1; 5′-GGATCGGGACTCAGCACTC-3′, complementary to nucleotides from +1506 to +1488 and a GeneRacer 5′ Primer (5′-CGACTGGAGCACGAGGACACTGA-3′). The PCR conditions were as follows: 35 cycles of 98 °C for 5 s, 60 °C for 30 s, 72 °C for 25 s. A secondary PCR was then conducted using GSP2 (5′-TGGGTGACCTCGGGCAAGTC-3′, complementary to the nucleotides from +415 to +396) and a GeneRacer 5' Nested Primer (5′-GGACACTGACATGGACTGAAGGAGTA-3′). The PCR conditions were as follows: 25 cycles of 98 °C for 5 s, 60 °C for 30 s, 72 °C for 25 s. The PCR products were cloned into the pCR4-TOPO vector (Life Technologies) and the nucleotide sequences were analyzed.

### 2.4. Luciferase activity assay

The reporter gene used in these studies was firefly luciferase in a pGL3-Basic vector (Promega). A genomic DNA fragment including part of the first exon and the 5′-flanking sequence was amplified by PCR with the upstream primers tagged with an *Mlu*I site and the downstream primer tagged with a *Bgl*II site. Oligonucleotides (restriction enzyme sequences are underlined) are: −1359, 5′-CGACGCGTGAGACAGATTCTACGGCT-3′; −595, 5′-CGACGCGTTTGCACACTCCTACTACA-3′; −419, 5′-CGACGCGTGTGGCTGATGAGAACGGTG-3′; −259, 5′-CGACGCGTTCAATGCATATTTGAAAT-3′; −111, 5′-CGACGCGTGGAGGCCTGCGCCTTTAA-3′; −63, 5′-CGACGCGTAGACGACTCCCCGGAGAC-3′; +19, 5′-CGACGCGTCCTTTGCGGCTGCCGCGTC-3′; +380, 5′-CGAGATCTGCGTCGGCTTCCCGCTCTG-3′. The amplified fragments were digested with *Mlu*I and *Bgl*II and ligated into the *Mlu*I/*Bgl*II sites of the pGL3-Basic vector. Then their sequences were confirmed.

PCR-mediated site-directed mutagenesis was performed to modify three putative Sp1 elements in the *Mlu*I/*Bgl*II tagged fragment from −111 to +380 (Sambrook and Russell, 2001). The PCR primers for mutagenesis were (only one strand was shown) 5′-CCAGGGATCCGAGGATCCGGGCAG-3′ for the Sp1a mutant, 5′-TTTGGGAGGGATCCGGCCAGACCC-3′ for the Sp1b mutant, and 5′-GGAGCCGCTGGATCCGGGGCGAGTG-3′ for the Sp1c mutant. Mutagenesis primers were designed as reported (Yamabe et al., 1998) and confirmed by DNA sequencing after insertion into the plasmid pGL3-Basic vector *Mlu*I/*Bgl*II sites.

HL60 cells were transfected with 0.5 μg of pRL-TK plasmid (Promega) as an internal standard along with 10 μg of pGL3-Basic or pGL3-Basic-derived plasmids by electroporation at 250 V, 960 μF. The differentiated human adipocytes were transfected using cationic liposome Tfx-20 reagent (Promega), according to the manufacturer's instructions. At 24–28 h after transfection, luciferase reporter assays were performed using the luciferase assay