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# Denervation Enhances the Expression of SHPS-1 in Rat Skeletal Muscle

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**SHPS-1 (Src homology 2 domain containing protein tyrosine phosphatase substrate 1) is a transmembrane glycoprotein containing three immunoglobulin-like motifs in its extracellular domain and immunoreceptor tyrosine–based inhibitory motifs (ITIM) that interact with SHP-2 (Src homology 2 domain containing protein tyrosine phosphatase-2) in its cytoplasmic region. SHPS-1 is highly expressed in brain, but at much lower levels in skeletal muscle. In this study, we found that the level of the SHPS-1 mRNA increases in rat skeletal muscle after denervation. Western blot analysis also confirmed the increase of SHPS-1 in denervated muscle. Moreover, it was found that the glycosylation of SHPS-1 is *N*-linked in a muscle-specific manner, and that this is altered upon innervation or denervation. Immunohistochemistry revealed SHPS-1 immunoreactivity at neuromuscular junctions (NMJs) under innervation, whereas immunoreactivity was observed extrasynaptically in muscle fibers after denervation. Our results indicate that the expression, glycosylation, and localization of SHPS-1 are strongly regulated by the nervous system, and that SHPS-1 may play an important role in denervated skeletal muscle.**

**Key words: denervation, glycosylation, neuromuscular junction, SHPS-1, skeletal muscle.**

Abbreviations: AchRα, acetylcholine receptor α-subunit; ARPP16/19, cAMP-regulated phosphoprotein 16/19; BIT, brain immunoglobulin-like molecule with tyrosine-based activation motifs; α-BTX, α-bungarotoxin; ConA, Concanavalin A; Den, denervation; EDL, extensor digitorum longus; EGF, epidermal growth factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IGF-I, insulin-like growth factor-I; Inn, innervation; MAP kinase, mitogen-activated protein kinase; MFR, macrophage fusion receptor; NCAM, neural cell adhesion molecule; NMJs, neuromuscular junctions; PBS, phosphate-buffered saline; PVDF, polyvinylidene difluoride; SH2, Src homology 2; SHP-1/2, Src homology 2 domain containing protein tyrosine phosphatase-1/2; SHPS-1, Src homology 2 domain containing protein tyrosine phosphatase substrate 1; SIRP, signal-regulatory protein.

The differentiation of skeletal muscle is regulated by the nervous system. Neural innervation sends myotubes into myofibers. Skeletal muscle size, phenotype, and composition are also regulated, in part, by neural factors. Eliminating neural stimuli to muscle *via* peripheral nerve axotomy (denervation) impairs the highly differentiated state of skeletal muscle, leading to muscle atrophy. In addition, denervation results in changes in the expressions of muscle-specific genes, notably myogenic regulatory factors (MRFs) (*1–5*), the type II myosin heavy chain (MHC) isoform (*6*, *7*), and the acetylcholine receptor α subunit (AchRα) (*1*, *8*). For example, AchR is composed of five subunits including the ε-subunit ($\alpha\alpha\beta\delta\varepsilon$), and is restricted to neuromuscular junctions (NMJs) under innervation. But following denervation, the expressions of all AchR subunit genes increase, and the fetal type receptor, including a γ-subunit ($\alpha\alpha\beta\delta\gamma$), localizes throughout the sarcolemma. This implies that skeletal muscle after denervation reverts to a fetal, undifferentiated state both structurally and functionally. The identification and characterization of genes that are activated in denervated muscles might provide clues to the molecular mechanisms of muscle atrophy and differentiation.

SHPS-1 (Src homology 2 domain–containing protein tyrosine phosphatase substrate 1) (*9*), also known as SIRPα (*10*), BIT (*11*), MFR (*12*), and p84 neural adhesion molecule (*13*), is a transmembrane glycoprotein member of the immunoglobulin superfamily. SHPS-1 is abundant in certain neuronal and hematopoietic cells (*13–15*). The tissue distribution of SHPS-1 shows that it is abundant in the brain and spleen, and much less abundant in skeletal muscle (*9*, *16*). SHPS-1 has three immunoglobulin-like domains with multiple *N*-linked glycosylation sites in the extracellular region, and four YXX(L/V/I) motifs, which are putative tyrosine phosphorylation sites and binding sites for the Src homology 2 (SH2) domains of the protein-tyrosine phosphatases SHP-2 and SHP-1 (*9*, *10*), in the cytoplasmic region. Since the binding of SHP-2 to the tyrosine-phosphorylated cytoplasmic domain of SHPS-1 increases the protein tyrosine phosphatase activity of SHP-2 *in vitro* (*11*, *17*), it is thought that SHPS-1 regulates intracellular signaling by recruiting and activating SHP-2 near the plasma membrane. For example, overexpression of SIRPα1, the human homolog of SHPS-1, inhibits the insulin- or EGF-induced activation of MAP kinases and cell growth (*10*). Furthermore,

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expression of SHPS-1 has been shown to be down-regulated in fibroblasts transformed by various oncogene products (*18*). Thus SHPS-1 may be involved in growth factor–induced mitogenesis. In addition, Maile and Clemmons demonstrated that SHPS-1 recruits SHP-2 at the plasma membrane, leading to the dephosphorylation of insulin-like growth factor-I (IGF-I) receptor by SHP-2 in porcine aortic smooth muscle cells (*19*). Timms *et al.* (1999) reported that SHPS-1 acts as a scaffold for the assembly of multiprotein complexs (*20*). These observations suggest a role for SHPS-1 as a signal transducer in various cell types.

The extracellular region of SHPS-1 mediates cell–cell adhesion through the immunoglobulin-like domains. It has been reported that SHPS-1 contributes to macrophage multinucleation (*21*), T-cell activation (*22*), and the tethering of apoptotic cells to phagocytes (*23*) through cell adhesion. Recently, it was shown that SHPS-1 may be involved in the formation of filopodia between neuroblastoma cells (*24*). Thus, SHPS-1 may play a role in the modulation of signal transduction through cell–cell communication. However, its function *in vivo*, especially in skeletal muscle, is not fully understood.

To find genes involved in muscle atrophy or differentiation, we investigated differentially expressed genes in rat extensor digitorum longus (EDL) and soleus muscles after denervation by DNA microarray analysis followed by Northern blot analysis. The results revealed that SHPS-1 is remarkably up-regulated by denervation. In addition, we found that the degree of glycosylation and the localization of SHPS-1 are altered in denervated muscles. SHPS-1 does not interact with SHP-2 in denervated muscles. Taken together, SHPS-1 in skeletal muscle is modulated depending on neural influences, and could play an important role in denervated muscles. This is the first report on the characterization of SHPS-1 in skeletal muscle.

## MATERIALS AND METHODS

*Animals and Surgical Procedures*—Adult male Wistar rats, 8 weeks of age and weighing approximately 250 g, were used in all experiments. Animals were anesthetized with nembutal (50 mg/kg), and the sciatic nerve on the right hindlimb was exposed. To maintain the denervated state for at least 2 weeks, a 1 cm segment of the sciatic nerve was surgically removed. At various time points, rats were deeply anesthetized and killed by decapitation. Extensor digitorum longus (EDL) and soleus muscles from both denervated (right) and innervated (left) legs were immediately removed, frozen in liquid nitrogen, and stored at –80°C

*RNA Extraction and Northern Blot Analysis*—Total RNA was extracted from frozen EDL and soleus with guanidium thiocyanate as described by Chomczynski and Sacchi (*25*). The total RNA in each sample (10–20 μg) was electrophoresed in a 1.0% agarose gel containing formaldehyde and then transferred to a nylon membrane (Biodyne B, KPL). The membranes were hybridized in hybridization solution (ULTRAhyb, Ambion) according to the manufacturer's instructions with $^{32}$P-labeled cDNA fragments encoding mouse SHPS-1 (NCBI Genbank #D87967, 1626–1993), mouse SHP-2 (NCBI Genbank #NM_011202, 1261–1849), human AchRα(NCBI Genbank #NM_000079, 375–887), and human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (NCBI Genbank #BC023632, 369–717). Autoradiographic signals were analyzed and quantified by a Bioimaging Analyzer System (BAS, Fujifilm).

*DNA Microarray Analysis*—DNA microarray analysis was performed with Atlas Glass Array Rat 1.0 (CLONTECH) containing 1,090 kinds of gene-specific 80 bp oligonucleotides.

*Western Blot Analysis and Deglycosylation*—Anti–SHPS-1 rabbit polyclonal antibodies were purchased from Upstate Biotechnology (Lake Placid, NY, USA). Anti–SHP-2 mouse monoclonal antibodies were purchased from Transduction Laboratories (Lexington, KY, USA).

Innervated and denervated muscles and brain from rats were homogenized on ice in 2 ml of homogenization buffer [50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 150 mM NaCl, 1% Nonidet P-40 containing 50 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate and 0.1% inhibitor mix (WAKO)] with a polytron homogenizer (HITACHI KOUKI). The homogenates were centrifuged at 1,000 × *g* for 2 min at 4°C, and the supernatants were collected. The supernatants were solubilized by rotation for 1 h at 4°C, and centrifuged at 10,000 × *g* for 15 min at 4°C. The resulting supernatants were subjected to immunoblot analysis. Protein concentration was determined using a DC protein assay kit (BIO-RAD). Approximately 20 μg of total homogenates were subjected to 7.5% SDS-PAGE and then transferred to PVDF membranes (finetrap NT-32, Nihon Eido) using a semi-dry electroblotting apparatus. The membranes were blocked for 1 h with 5% non-fat dry milk in phosphate-buffered saline (PBS) containing 0.05% Tween-20 at room temperature. The membranes were incubated with primary antibodies (anti–SHPS-1 at 1:1,000; anti–SHP-2 at 1:5,000) for 30 min at 37°C or overnight at 4°C. The primary antibodies were detected with anti–rabbit IgG horseradish peroxidase–conjugated antibodies (1:5,000) or anti–mouse IgG horseradish peroxidase–conjugated antibodies (1:5,000) for 30 min at 37°C, and then the membranes were incubated in freshly prepared chemiluminescence buffer [100 mM Tris-HCl (pH 8.5), 1.25 mM luminal, 0.2 mM *p*-coumaric acid, 0.009% $H_2O_2$] for 1 min at room temperature, and exposed to film (hyperfilm™ ECL, Amersham Biosciences).

To examine the glycosylation of SHPS-1, homogenates were boiled in the presence of 1% SDS and 1% 2-mercaptoethanol for 3 min and then subjected to deglycosylation with 2 U/ml of *N*-glycosidase F (Roche) in 50 mM Tris-HCl (pH 7.5) containing 50 mM EDTA, 1% 2-mercaptoethanol, and 1% TritonX-100 for 20 h at 37°C.

*Concanavalin A Sepharose Precipitation*—Innervated and denervated muscles were homogenized on ice in 2.5 ml of homogenization buffer [50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 5 mM EGTA, 50 mM NaCl, containing 50 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate and 0.1% inhibitor mix (WAKO)] with a polytron homogenizer (HITACHI KOUKI). The homogenates were centrifuged at 1,000 × *g* for 2 min at 4°C, and the supernatants were collected. The supernatants were solubilized by rotation for 1 h at 4°C, and centrifuged at 100,000 × *g* for 60 min at 4°C. The

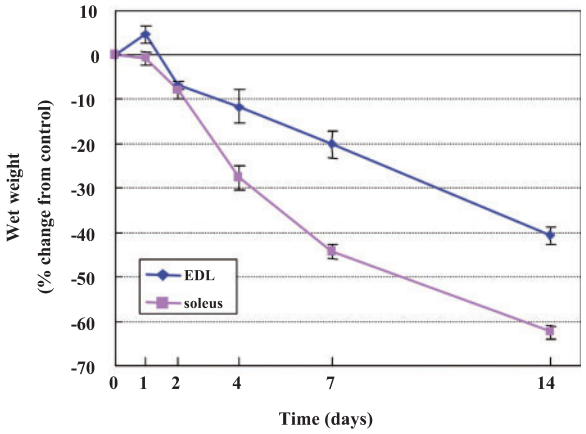
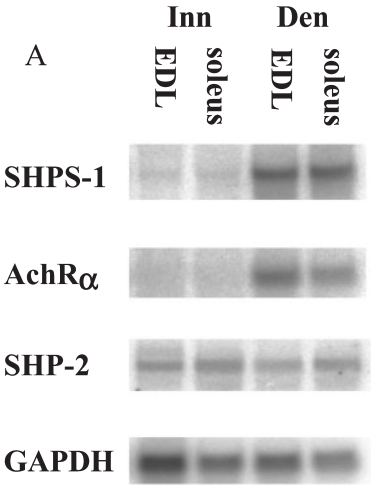



Fig. 1. **Time course of weight loss of rat EDL and soleus muscles after denervation.** Values are means ± SE; $n$ = 7.



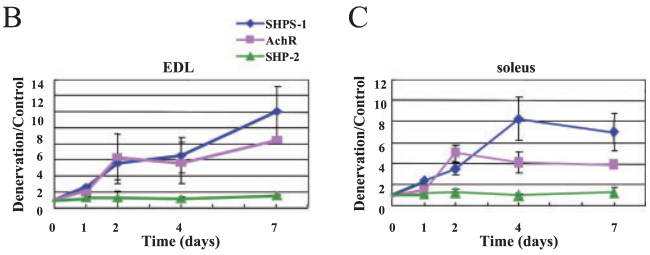




Fig. 2. **Northern blot analysis of SHPS-1, AchR*α*, and SHP-2.** (A) Bands are the signals of SHPS-1, AchRα and SHP-2 mRNA in innervated (Inn) and 7-day denervated (Den) EDL and soleus muscles. The ratios of mRNA expression of three genes to GAPDH in EDL (B) and soleus (C) muscles 1, 2, 4, and 7 days after denervation are shown. Values are means ± SE; $n$ = 3.

resulting supernatants were removed, and the pellets were suspended in 0.8 ml membrane solubilization buffer [20 mM Tris-HCl (pH 7.5), 1% Triton X-100, 150 mM NaCl, 1 mM $MgCl_2$ containing 50 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate and 0.1% inhibitor mix (WAKO)]. The suspensions were centrifuged at 100,000 × $g$ for 60 min at 4°C, and the resulting supernatants were referred to as the solubilized membrane fractions. The amount of total protein in the solubilized membrane fractions was standardized using a DC protein assay kit (BIO-RAD) before Concanavalin A (ConA) Sepharose precipitation, and the solubilized membrane fractions were incubated with 50 μl ConA Sepharose beads (Amersham Biosciences) overnight at 4°C. The beads were then washed three times with 0.5 ml membrane solubilization buffer, resuspended in SDS sample buffer [50 mM Tris-HCl (pH 6.8), 2% SDS, 6% 2-mercaptoethanol, 1% glycerol (v/v), 0.1% bromophenol blue], and boiled for 3 min at 100°C.

*Immunohistochemistry*—Tissues were excised, frozen in cold iso-pentane, and sectioned with a cryostat (6 μm). The sections were fixed in 4% paraformaldehyde in PBS for 15 min at 4°C. After pre-incubation with PBS containing 2% bovine serum albumin and 5% heat-inactivated normal goat serum, the sections were incubated with anti–SHPS-1 antibody at 1:250 or anti–SHP-2 antibody, C-18, at 1:250 (Santa Cruz Biotechnology) overnight at 4°C, and then incubated with anti-rabbit IgG antibody–conjugated Oregon green (Molecular Probes) at 1:500 and 2 μg/ml α-bungarotoxin–conjugated rhodamine (Molecular Probes) for 30 min at room temperature. Sections were observed under a fluorescence microscope (OLYMPUS IX70, OLYMPUS).

*Statistical Analysis*—All values are expressed as mean ± SE. Statistical analysis was performed by Student's $t$-test.

## RESULTS

*Weight Loss of Rat EDL and Soleus Muscles after Denervation*—The decreases in the wet weights of the EDL and soleus muscles with time after denervation are shown in Fig. 1. The wet weights of both muscles were unchanged 1 day after denervation and started to decrease constantly after 2 days. Soleus muscles decreased in wet weight at a faster rate than EDL muscles after 4 days. Finally, soleus muscles decreased to 37.4 ± 1.5% ($n$ = 7) of their initial weight and EDL muscles to 59.1 ± 1.9% ($n$ = 7) of their initial weight 2 weeks after denervation.

*Expression of SHPS-1 mRNA in Denervated Muscles*—To identify novel genes involved in the changes in muscles after denervation, we compared mRNA expression in EDL and soleus muscles 7 days after denervation with that in control muscles using DNA microarrays (data not shown). The expressions of several genes were shown to be increased in denervated muscles, and Northern blot analysis was performed for these genes. We found *SHPS-1* to be remarkably up-regulated in both EDL and soleus muscles 7 days after denervation (Fig. 2A). We also found that *AchR*α was dramatically up-regulated after denervation.

To further analyze the expression of these genes, we quantitated the expressions of SHPS-1, SHP-2 and AchRα using mRNA prepared from denervated muscles 1 to 7 days after denervation (Fig. 2, B and C). In EDL muscles, the expressions of SHPS-1 and AchRα increased constantly after denervation, reaching 12-fold (SHPS-1) and 8.5-fold (AchRα) elevations, respectively, after 7 days. The expression of SHP-2, which is known to interact with SHPS-1, did not change after denervation. In

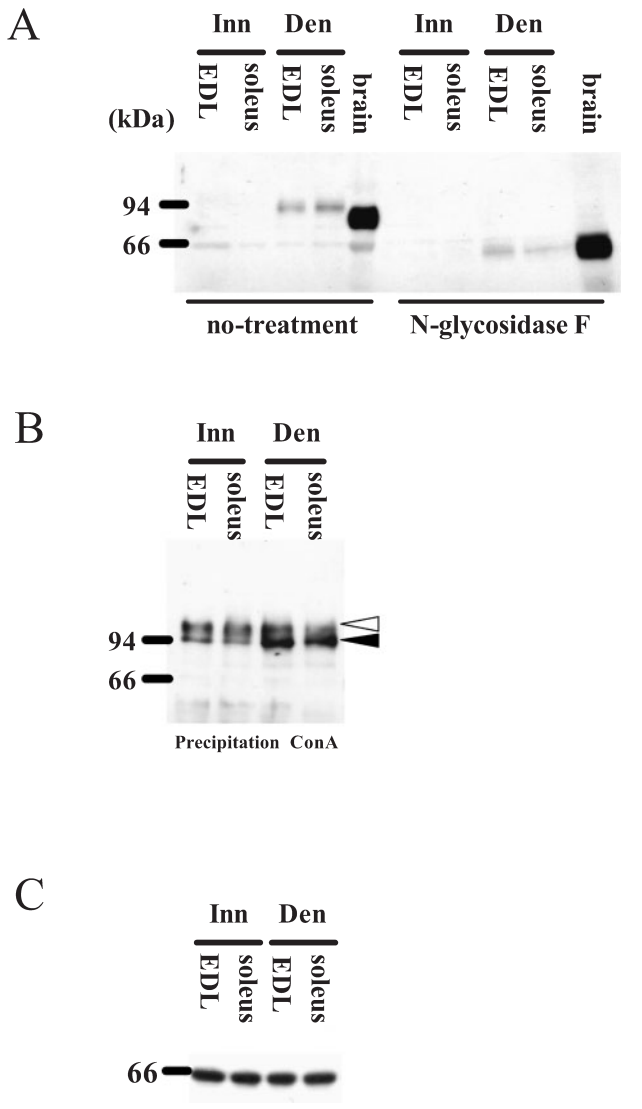



Fig. 3. **Western blot analysis of SHPS-1 and SHP-2 in rat skeletal muscles.** (A) Lysates were prepared from innervated (Inn) and 7-day denervated (Den) muscles, and immunoblotted with anti–SHPS-1 antibody. *N*-glycosidase F treatment was performed as described in "MATERIALS AND METHODS." (B) Solubilized membrane fractions of innervated (Inn) and denervated (Den) muscles were incubated with Con A-Sepharose beads. The proteins bound to Con A-Sepharose were separated by SDS-PAGE and immunoblotted with anti–SHPS-1 antibody. Two major bands were detected, "upper" (open arrowhead) and "lower" (filled arrowhead) SHPS-1. (C) Lysates from innervated (Inn) and 7-day denervated (Den) muscles were immunoblotted with anti–SHP-2 antibody.

soleus muscles, the expression of SHPS-1 increased constantly from 1 to 4 days after denervation, and remained elevated thereafter (a 7-fold increase after 7 days). The expression of AchRα increased rapidly to a 5-fold higher level after 2 days, and then remained high (a 4-fold elevation after 7days). The expression of SHP-2 was also unchanged in soleus muscles.

*Glycosylation of SHPS-1 in Innervated and Denervated Muscles*—To confirm that the level of the SHPS-1 protein increases in denervated muscles, Western blot analysis was performed with anti–SHPS-1 antibody. As shown in Fig. 3A (left), specific bands with molecular sizes of about 94 kDa were detected in denervated muscles, but not in innervated muscles. In rat brain, the antibody detected a band of about 90 kDa. Because it was thought that these differences in molecular size result from differential glycosylation, we examined shifts in the bands after deglycosylation with *N*-glycosidase F. Deglycosylation converted the molecular sizes of the bands in both denervated muscle and brain samples to about 65 kDa (Fig. 3A, right). Furthermore, we precipitated SHPS-1 with Concanavalin A (Con A) Sepharose. Con A precipitation revealed that a small amount of SHPS-1 protein exists in innervated muscles. Another SHPS-1 species with a molecular mass greater than 94 kDa ("upper" SHPS-1) was detected in both innervated and denervated muscles (Fig. 3B). It was thought that this is the more glycosylated form of SHPS-1. These results indicate that the SHPS-1 protein is expressed in both innervated and denervated muscles and modified in two distinct manners, and that the expression of a form of about 94 kDa ("lower" SHPS-1) increases after denervation, as in the case of the SHPS-1 mRNA. SHP-2 did not undergo any change in denervated muscles (Fig. 3C).

*Localization of SHPS-1 in Innervated and Denervated Muscles*—To examine whether SHPS-1 is expressed in muscle fibers, we observed EDL and soleus muscle sections immunostained with anti–SHPS-1 antibody. Immunoreactivity was observed as a few small dots in innervated muscles, but diffusely in the plasma membranes of muscle fibers after denervation (Fig. 4). While most fibers were immunoreactive in EDL muscles after denervation (Fig. 4B), only some fibers were immunoreactive in soleus muscles, showing patch-like staining (Fig. 4F). Moreover, anti–SHPS-1 immunoreactivity was also observed at neuromuscular junctions under innervation (Fig. 5A). This localization was confirmed by double-staining with anti–SHPS-1 antibody and rhodamine-conjugated α-bungarotoxin (α-BTX) (Fig. 5C). Since anti–SHPS-1 antibody and α-BTX stainings colocalized in denervated muscles, it was confirmed that anti–SHPS-1 immunoreactivity, like AchRs, localizes on plasma membranes in muscle fibers (Fig. 5, B and D).

We also stained muscle sections with anti–SHP-2 antibody. Immunoreactivity was observed in the cytoplasm in both innervated and denervated muscles (Fig. 6). SHP-2 was not localized at neuromuscular junctions. These observations imply that SHP-2 is not regulated by innervation.

## DISCUSSION

Previously, we found by DNA microarray analysis that another gene, ARPP16/19, is highly up-regulated in denervated rat muscle (*26*). However, this protein is a cytoplasmic adaptor and no physiological role was implicated. In this report, we provide the first demonstration that SHPS-1 is highly expressed in rat denervated skeletal muscles. We also show that the expression, glycosylation, and localization of SHPS-1 are altered after denervation. In contrast, SHP-2 does not change its expression or localization after denervation. These results suggest that SHP-2 is not involved in SHPS-1 function in denervated muscles. Taken together, SHPS-1 and SHP-2 may be regulated in different pathways in rat skeletal muscles.

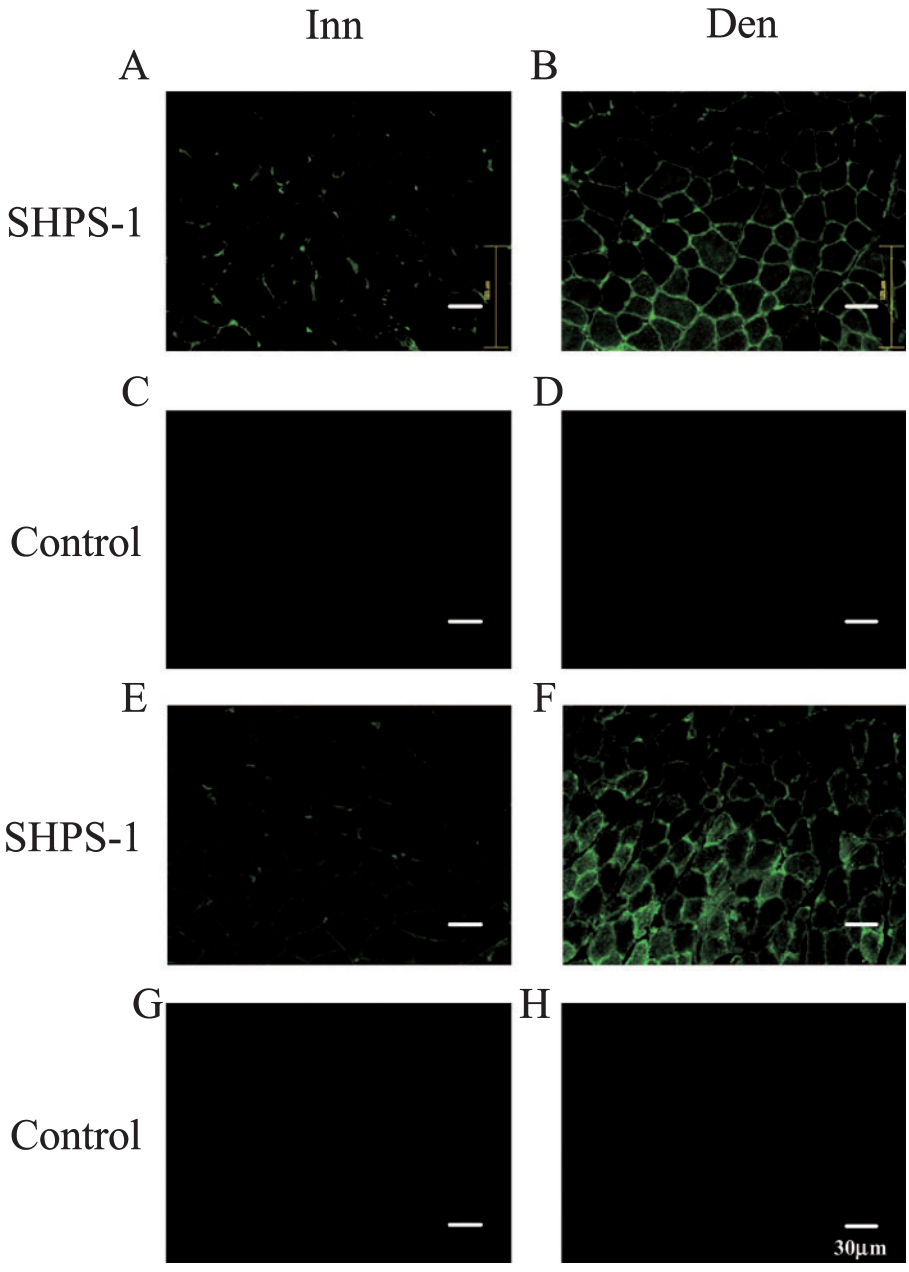



Fig. 4. **Localization of SHPS-1 in rat skeletal muscles.** Cross-sections of innervated (A, E) and 7-day denervated (B, F) muscles stained with anti–SHPS-1 antibody. A series of sections was stained without a primary antibody as a control (C, D, G, H). (A–D) EDL, (E–H) soleus ; Bar = 30 μm

We have demonstrated that the expression of SHPS-1, although very low in innervated muscles, increases in denervated muscle in a manner similar to that of AchRα. AchR is one of the most important cation channels for neuromuscular transmission. It is concentrated at NMJs, and is remarkably up-regulated after denervation (*27*). It is thought that this is a compensation mechanism for a loss of acetylcholine. Often, other proteins induced by denervation are up-regulated at far lower rates than the AchRα, but the SHPS-1 mRNA increases remarkably (Fig. 2). This implies that the increase in the expression of SHPS-1 may compensate for a loss of neural stimuli, and that SHPS-1 may play a role in innervation.

The levels of SHPS-1 and AChRαmRNA are relatively low under innervation and rise rapidly following denervation. In addition, we have observed that SHPS-1 immunoreactivity localizes at NMJs in innervated muscles and throughout the plasma membrane in denervated muscles. Although AchR and its interacting proteins, MuSK and rapsyn, are expressed at low levels and are restricted to NMJs under innervation, denervation induces increases in their expressions, and their localization becomes extrasynaptic (*28–30*). NCAM and BEN/SC1/DM-GRASP, members of the immunoglobulin superfamily, also show these alterations (*31–35*). NCAM is thought to play an important role in neurogenesis through cell–cell contacts (*36*, *37*). In recent studies, it was reported that SHPS-1 binds to CD47 *via* its extracellular domain, and this interaction is implicated in synapse formation or maintenance (*38*). Taken together, it is possible that SHPS-1 is involved in nerve–muscle cell interaction.

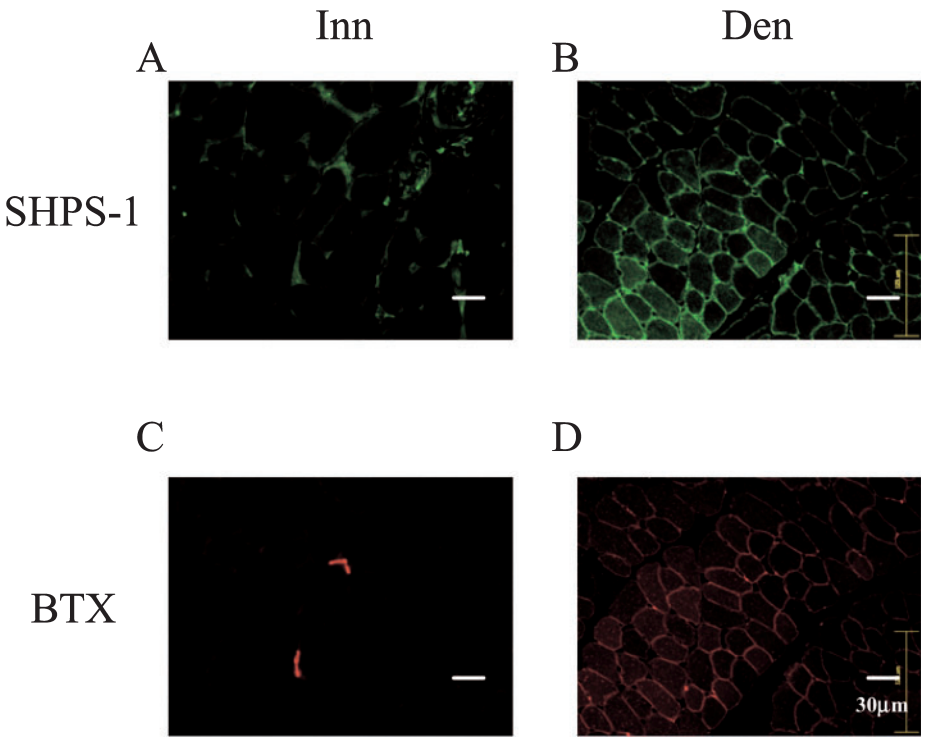



Fig. 5. **Co-localization of SHPS-1 and AchR in innervated and denervated muscles.** Cross-sections of innervated and 7-day denervated EDL muscle were double-stained with anti–SHPS-1 antibody (A, B) and rhodamine-conjugated α-bungarotoxin (BTX) (C, D). (A, C) innervated (B, D) denervated; Bar = 30 μm.

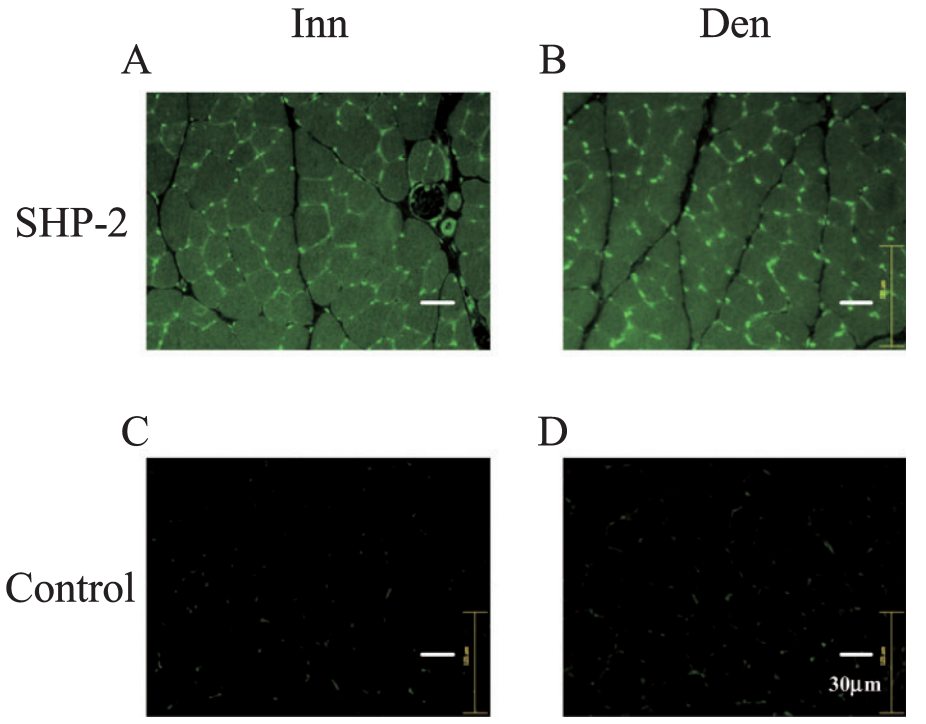



Fig. 6. **Immunohistochemistry of SHP-2 in EDL muscle.** Cross-sections of innervated and 7-day denervated EDL muscle were stained with anti–SHP-2 antibody (A, B). A series of sections was stained without a primary antibody as a control (C, D). (A, C)innervated (B, D)denervated ; Bar = 30 μm.

The rates of increase of SHPS-1 expression and immunoreactivity with the anti–SHPS-1 antibody differ slightly between EDL and soleus muscles. These differences may be due to the difference in fiber types. Muscle atrophy progresses at different rates in EDL and soleus muscles. Thus, differences in the nature of the muscle fibers might account for the small difference in SHPS-1 expression.

The glycosylation of SHPS-1 is regulated in tissue-specific manner, and the isoform in skeletal muscle is different from that in brain. Moreover, two distinct isoforms with different affinities for Con A exist in skeletal muscle, and the expression of only one form increases after denervation. Generally, glycosylation modulates the adhesion activity of glycoproteins, and it is reported that the aberrant *N*-glycosylation of SHPS-1 impairs its abil-

ity to bind CD47 (*39*). It is possible that SHPS-1 functions with different activities in innervated muscles and denervated muscles.

SHP-2 has been reported to interact with the tyrosine-phosphorylated cytoplasmic domain of SHPS-1 (*9*, *10*), and to regulate EGF, insulin, and the IGF-I signaling pathway (*10*, *19*). In our experiments, since the expression and localization of SHP-2 did not change after denervation, SHP-2 appears to be independent of the intracellular changes caused by denervation. We also demonstrated that SHP-2 does not concentrate at NMJs. Tanowitz *et al.* reported that SHP-2 is concentrated at NMJs in mouse diaphragm (*40*). But Mei *et al.* showed that SHP-2 localizes in the cytoplasm of muscle fibers in rat hindlimbs (*41*). We also examined SHP-2 in rat hindlimb muscles and our results are consistent with the latter results. Additionally, we performed co-immunoprecipitation experiments but could not detect any interaction between SHPS-1 and SHP-2 in either innervated or denervated muscles (data not shown). Our results suggest that SHPS-1 interacts with novel proteins other than SHP-2, and regulates intracellular signaling in response to changes caused by denervation in skeletal muscle.

Little is known about SHPS-1 except for its interactions with SHP-1 and SHP-2, and its participation in cell adhesion. Most previous studies utilized cultured cells; therefore, how SHPS-1 functions *in vivo* has remained unclear, even in brain where it is highly expressed. Our results contribute new information about the function of SHPS-1 *in vivo*, and suggest that SHPS-1 plays an important role in denervated and undifferentiated skeletal muscle.

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## REFERENCES


1. Adams, L., Carlson, B.M., Henderson, L., and Goldman, D. (1995) Adaptation of nicotinic acetylcholine receptor, myogenin, and MRF4 gene expression to long-term muscle denervation. *J. Cell Biol.* **131**, 1341–1349
2. Eftimie, R., Brenner, H.R., and Buonanno, A. (1991) Myogenin and MyoD join a family of skeletal muscle genes regulated by electrical activity. *Proc. Natl Acad. Sci. USA* **98**, 14440–14445
3. Weis, J. (1994) Jun, Fos, MyoD1, and myogenin proteins are increased in skeletal muscle fiber nuclei after denervation. *Acta Neuropathol. (Berl)* **87**, 63–70
4. Weis, J., Kaussen, M., Calvo, S., and Buonanno, A. (2000) Denervation induces a rapid nuclear accumulation of MRF4 in mature myofibers. *Dev. Dyn.* **218**, 438–451
5. Witzemann, V. and Sakmann, B. (1991) Differential regulation of MyoD and myogenin mRNA levels by nerve induced muscle activity. *FEBS Lett.* **282**, 259–264
6. Huey, K.A. and Bodine, S.C. (1998) Changes in myosin mRNA and protein expression in denervated rat soleus and tibialis anterior. *Eur. J. Biochem.* **256**, 45–50
7. Robin, N.M., David, J.P., and Shannon, E.D. (1996) Regulation of myosin heavy chain expression in adult rat hindlimb muscles during short-term paralysis : comparison of denervation and tetrodotoxin-induced neural inactivation. *FEBS Lett.* **391**, 39–44
8. Witzemann, V., Brenner, H.R., and Sakmann, B. (1991) Neural factors regulate AchR subunit mRNAs at rat neuromuscular synapses. *J. Cell Biol.* **114**, 125–141
9. Fujioka, Y., Matozaki, T., Noguchi, T., Iwamatsu, A., Yamao, T., Takahashi, N., Tsuda, M., Takada, T., Kasuga, M. (1996) A novel membrane glycoprotein, SHPS-1, that binds the SH2-domain-containing protein tyrosine phosphatase SHP-2 in response to mitogens and cell adhesion. *Mol. Cell. Biol.* **16**, 6887–6899
10. Kharitonenkov, A., Chen, Z., Sures, I., Wang, H., Schilling, J., and Ullrich, A. (1997) A family of proteins that inhibit signaling through tyrosine kinase receptors. *Nature* **386**, 181–186
11. Ohnishi, H., Kubota, M., Ohtake, A., Sato, K., and Sano, S. (1996) Activation of protein-tyrosine phosphatase SH-PTP2 by a tyrosine-based activation motif of a novel brain molecule. *J. Biol. Chem.* **271**, 25569–25574
12. Saginario, C., Sterling, H., Beckers, C., Kobayahi, R., Solimena, M., Ullu, E., and Vignery, A. (1998) MFR, a putative receptor mediating the fusion of macrophases. *Mol. Cell. Biol.* **18**, 6213–6223
13. Comu, S., Weng, W., Olinsky, S., Ishwad, P., Mi, Z., Hempel, J., Watkins, S., Lagenaur, C.F., and Narayanan, V. (1997) The murine P84 neural adhesion molecule is SHPS-1, a member of the phosphatase-binding protein family. *J. Neurosci.* **17**, 8702–8710
14. Veillette, A., Thibaudeau, E., and Latour, S. (1998) High expression of inhibitory receptor SHPS-1 and its association with protein-tyrosine phosphatase SHP-1 in macrophages. *J. Biol. Chem.* **273**, 22719–22728
15. Seiffert, M., Cant, C., Chen, Z., Rappold, I., Brugger, W., Kanz, L., Brown, E.J., Ullrich, A., and Bühring, H.J. (1999) Human signal-regulatory protein is expressed on normal, but not on subsets of leukemic myeloid cells and mediates cellular adhesion involving its counterreceptor CD47. *Blood* **94**, 3633–3643
16. Sano, S., Ohnishi, H., and Kubota, M. (1999) Gene structure of mouse BIT/SHPS-1. *Biochem. J.* **344**, 667–675
17. Takada, T., Matozaki, T., Takeda, H., Fukunaga, K., Noguchi, T., Fujioka, Y., Okazaki, I., Tsuda, M., Yamao, T., Ochi, F., and Kasuga, M. (1998) Roles of the complex formation of SHPS-1 with SHP-2 in insulin-stimulated mitogen-activated protein kinase activation. *J. Biol. Chem.* **273**, 9234–9242
18. Machida, K., Matsuda, S., Yamaki, K., Senga, T., Thant, A.A., Kurata, H., Miyazaki, K., Hayashi, K., Okuda, T., Kitamura, T., Hayakawa, T., and Hamaguchi M. (2000) v-Src suppresses SHPS-1 expression via the Ras-MAP kinase pathway to promote the oncogenic growth of cells. *Oncogene* **19**, 1710–1718
19. Maile, L.A. and Clemmons, D.R. (2002) Regulation of insulin-like growth factor I receptor dephosphorylation by SHPS-1 and the tyrosine phosphatase SHP-2. *J. Biol. Chem.* **277**, 8955–8960
20. Timms, J.F., Swanson, K.D., Marie-Cardine, A., Raab, M., Rudd, C.E., Schraven, B., and Neel, B.G. (1999) SHPS-1 is a scaffold for assembling distinct adhesion-regulated multi-protein complexes in macrophages. *Curr. Biol.* **9**, 927–930
21. Han, X., Sterling, H., Chen, Y., Saginario. C., Brown, E.J., Frazier, W.A., Lindberg, F.P., and Vignery, A. (2000) CD47, a ligand for the macrophage fusion receptor, participates in macrophage multinucleation. *J. Biol. Chem.* **275**, 37984–37992
22. Seiffert, M., Brossart, P., Cant, C., Cella, M., Colonna, M., Brugger, W., Kanz, L., Ullrich, A., and Bühring, H.-J. (2001) Signal-regulatory protein α (SIRPα) but not SIRPβ is involved in T-cell activation, binds to CD47 with high affinity, and is expressed on immature $CD34^+$ $CD38^-$ hematopoietic cells. *Blood* **97**, 2741–2749
23. Tada, K., Tanaka, M., Hanayama, R., Miwa, K., Shinohara, A., Iwamatsu, A., Nagata, S. (2003) Tethering of apoptotic cells to phagocytes through binding of CD47 to Src homology 2 domain-bearing protein tyrosine phosphatase substrate-1. *J. Immunol.* **171**, 5718–5726
24. Miyashita, M., Ohnishi, H., Okazawa, H., Tomonaga, H., Hayashi, A., Fujimoto, T.-T., Furuya, N., and Matozaki, T. (2004) Promotion of neurite and filopodium formation by CD47: roles of integrins, Rac, and Cdc42. *Mol. Biol. Cell* **15**, 3950–3963

25. Chomczynski, P. and Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**, 156–159
26. Yoshikawa, A., Mitsuhashi, H., Sasagawa, N., Tsukahara, T., Hayashi, Y., Nishino, I., Goto, Y., and Ishiura, S. (2003) Expression of ARPP-16/19 in rat denervated skeletal muscle. *J. Biochem.* **134**, 57–61
27. Tsay, H.-J. and Schmidt, J. (1989) Skeletal muscle denervation activates acetylcholine receptor genes. *J. Cell Biol.* **108**, 1523–1526
28. Baldwin, T.J., Theriot, J.A., Yoshihara, C.M., and Burden, S.J. (1998) Regulation of transcript encoding the 43K subsynaptic protein during development and after denervation. *Development* **104**, 557–564
29. Valenzuela, D.M., Stitt, T.N., DiStefano, P.S., Rojas, E., Mattsson, K., Compton, D.L., Nunez, L., Park, J.S., Stark, J.L., Gies, D.R., Thomas, S., LeBeau, M.M., Fernald, A.A., Copeland, N.G., Jenkins, N.A., Burden, S.J., Glass, D.J., and Yancopoulos, G.D. (1995) Receptor tyrosine kinase specific for the skeletal muscle lineage: expression in embryonic muscle, at the neuromuscular junction, and after injury. *Neuron* **15**, 573–584
30. Bowen, D.C., Park, J.S., Bodine, S., Stark, J.L., Valenzuela, D.M., Stitt, T.N., Yancopoulos, G.D., Lindsay, R.M., Glass, D.J., and DiStefano, P.S. (1998) Localization and regulation of MuSK at the neuromuscular junction. *Dev. Biol.* **199**, 309–319
31. Rieger, F., Grumet, M., and Edelman, G.M. (1985) N-CAM at the vertebrate neuromuscular junction. *J. Cell Biol.* **101**, 285–293
32. Covault, J., Merlie, J.P., Goridis, C., and Sanes, J.R. (1986) Molecular forms of N-CAM and its RNA in developing and denervated skeletal muscle. *J. Cell Biol.* **102**, 731–739
33. Covault, J. and Sanes J.R. (1985) Neural cell adhesion molecule (N-CAM) accumulates in denervated and paralyzed skeletal muscles. *Proc. Natl Acad. Sci. USA* **82**, 4544–4548
34. Daniloff, J.K., Levi, G., Grumet, M., Rieger, F., and Edelman, G.M. (1986) Altered expression of neural cell adhesion molecules induced by nerve injury and repair. *J. Cell Biol.* **103**, 929–945
35. Fournier-Thibault, C., Pourquié, O., Rouaud, T., and Le Douarin, N.M. (1999) BEN/SC1/DM-GRASP expression during neuromuscular development: a cell adhesion molecule regulated by innervation. *J. Neurosci.* **19**, 1382–1392
36. Seki, T. and Rutishauser, U. (1998) Removal of polysialic acid-neural cell adhesion molecule induces aberrant mossy fiber innervation and ectopic synaptogenesis in the hippocampus. *J. Neurosci.* **18**, 3757–3766
37. Rafuse, V.F., Polo-Parada, L., and Landmesser, L.T. (2000) Structural and functional alterations of neuromuscular junctions in NCAM-deficient mice. *J. Neurosci.* **20**, 6529–6539
38. Mi, Z.P., Jiang, P., Weng, W.L., Lindberg, F.P., Narayanan, V., and Lagenaur, C.F. (2000) Expression of a synapse-associated membrane protein, p84/SHPS-1, and its ligand, IAP/CD47, in mouse retina. *J. Comp. Neurol.* **416**, 335–344
39. Ogura, T., Noguchi, T., Murai-Takebe, R., Hosooka, T., Honma, N., and Kasuga, M. (2004) Resistance of B16 melanoma cells to CD47-induced negative regulation of motility as a result of aberrant *N*-glycosylation of SHPS-1. *J. Biol. Chem.* **279**, 13711–13720
40. Tanowitz, M., Si, J., Yu, D.-H., Feng, G.-S., and Mei, L. (1999) Regulation of neuregulin-mediated acetylcholine receptor synthesis by protein tyrosine phosphatase SHP-2. *J. Neurosci.* **19**, 9426–9435
41. Mei, L., Kachinsky, A.M., Seiden, J.E., Kuncl, R.W., Miller, J.B., and Huganir R.L. (1996) Differential expression of PTP1D, a protein tyrosine phosphatase with two SH2 domains, in slow and fast skeletal muscle fibers. *Exp. Cell Res.* **224**, 379–390