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Secreted frizzled related protein 1 (Sfrp1) and Wnt signaling in innervated and denervated skeletal muscle

Anna Svensson · Marlene Norrby · Rolf Libelius · Sven Tågerud

Abstract Wnts are secreted proteins with functions in differentiation, development and cell proliferation. Wnt signaling has also been implicated in neuromuscular junction formation and may function in synaptic plasticity in the adult as well. Secreted frizzled-related proteins (Sfrps) such as Sfrp1 can function as inhibitors of Wnt signaling. In the present study a potential role of Wnt signaling in denervation was examined by comparing the expression levels of Sfrp1 and key proteins in the canonical Wnt pathway, Dishevelled, glycogen synthase kinase 3β and β-catenin, in innervated and denervated rodent skeletal muscle. Sfrp1 mRNA and immunoreactivity were found to be up-regulated in mouse hemidiaphragm muscle following denervation. Immunoreactivity, detected by Western blots, and mRNA, detected by Northern blots, were both expressed in extrasynaptic as well as perisynaptic parts of the denervated muscle. Immunoreactivity on tissue sections was, however, found to be concentrated postsynaptically at neuromuscular junctions. Using β-catenin levels as a readout for canonical Wnt signaling no evidence for decreased canonical Wnt signaling was obtained in denervated muscle. A role for Sfrp1 in denervated muscle, other than interfering with canonical Wnt signaling, is discussed.

Keywords Sfrp1 · Wnt signaling · Skeletal muscle · Denervation · Neuromuscular junction

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Introduction

Wnts are secreted proteins with functions in differentiation, development and cell proliferation (see Wodarz and Nusse 1998). Wnt signaling may also function in synaptic plasticity in the adult (Chen et al. 2006; see also Tang 2007). Wnts interact with and activate the seven transmembrane Frizzled (Fzd) receptors (see Wodarz and Nusse 1998). This interaction activates the cytoplasmic scaffold protein Dishevelled (Dvl), which becomes phosphorylated (see Ciani and Salinas 2005; Lee et al. 1999; González-Sancho et al. 2004). Several different Wnt and Fzd proteins exist and signaling is widespread among many species (see Ciani and Salinas 2005). Downstream of Dvl, Wnt signaling diverges into several pathways. In the most studied, the so called canonical pathway, Dvl promotes disruption of a complex comprising glycogen synthase kinase 3β (GSK-3β), axin, the adenomatous polyposis coli (APC) protein and β-catenin (see Wodarz and Nusse 1998; Ciani and Salinas 2005). Wnt activation causes GSK-3β inhibition, leading to stabilization and accumulation of unphosphorylated β-catenin in the cytosol. β-catenin then translocates into the nucleus and activates the transcription factor T-cell factor (Tcf)/lymphoid enhancer factor (Lef), leading to transcription of Wnt responsive genes. In the absence of Wnts, GSK-3β phosphorylates β-catenin,

leading to its ubiquitin-mediated degradation (see Wodarz and Nusse 1998; Ciani and Salinas 2005).

Secreted frizzled-related proteins (Sfrps) are secreted proteins containing a region homologous to the cysteine-rich ligand-binding domain found in Fzd receptors (Finch et al. 1997; Rattner et al. 1997). Sfrp1 can function as an inhibitor of Wnt signaling (Finch et al. 1997; Bafico et al. 1999) but Sfrp1 may also serve functions in axonal guidance unrelated to Wnt inhibition (Rodriguez et al. 2005). Sfrp1 mRNA expression has been detected in various tissues such as brain, kidney, and heart, and the transcript is present both in the adult and during embryogenesis (Finch et al. 1997; Rattner et al. 1997). Only weak expression has been reported in skeletal muscle (Finch et al. 1997; Melkonyan et al. 1997).

Formation of the neuromuscular junction requires the presence of the components muscle-specific kinase (MuSK), agrin and rapsyn. The proteoglycan agrin promotes activation of the post-synaptic protein MuSK, leading to clustering of acetylcholine receptors (AChRs) (see Witzemann 2006). Rapsyn forms complexes with AChRs and stabilizes the AChR clusters. AChR clusters are absent in MuSK or rapsyn mutants (see Witzemann 2006). Wnt signaling has also been implicated in neuromuscular junction formation (Luo et al. 2002; Packard et al. 2002). For example MuSK expression can be regulated by Wnt signaling (Kim et al. 2003) and Dvl, APC, GSK-3β and β-catenin have all been implicated in AChR clustering. Dvl1 interacts with MuSK (Luo et al. 2002) and APC with the AChR β-subunit (Wang et al. 2003) and inhibitors of GSK-3β inhibit agrin-induced AChR clustering (Sharma and Wallace 2003). β-catenin interacts with rapsyn and inhibiting β-catenin attenuates agrin-induced AChR clustering (Zhang et al. 2007). Dvl1, APC and β-catenin have all been reported to be concentrated at neuromuscular junctions (Luo et al. 2003; Wang et al. 2003; Zhang et al. 2007).

A previous microarray study indicated reduced Wnt signaling in mouse skeletal muscle following denervation (Magnusson et al. 2005). For example, the Frizzled receptor Fzd9 appeared to be down-regulated after denervation (Magnusson et al. 2005). Additional analyses of the microarray data indicated that Sfrp1 was up-regulated in denervated mouse hemidiaphragm muscle (unpublished). In the present study a potential role of Wnt signaling in denervation was examined by comparing the expression levels of Sfrp1 and key proteins in the canonical Wnt pathway, Dvl1, GSK-3β and β-catenin, in innervated and denervated rodent skeletal muscle. Sfrp1 mRNA and immunoreactivity were found to be up-regulated in skeletal muscle following denervation. Expression of Dvl1, GSK-3β and β-catenin, however, did not indicate reduced canonical Wnt signaling in denervated skeletal muscle.

Materials and methods

Animals

All experiments were performed on adult male NMRI mice or SD rats (immunohistochemistry, B & K Universal, Sollentuna, Sweden or Charles River, Uppsala, Sweden). Before surgery the animals were anaesthetized by an intraperitoneal injection of a mixture of ketamine and xylazine or by inhalation of isoflurane. Denervation of the left hind-limb or the left hemidiaphragm was performed by sectioning and removing a few mm of the sciatic nerve or phrenic nerve as described previously (Magnusson et al. 2001). Six days (RNA extraction) or 6, 13 or 21 days (protein extraction) after denervation mice were killed by cervical dislocation whereas rats where killed by carbon dioxide inhalation with subsequent decapitation. The experimental manipulations have been approved by the Ethical Committee for Animal Experiments at Lund and Linköping Universities, Sweden.

RNA extraction

For RNA extraction diaphragms from separate innervated or denervated mice were dissected and rinsed in phosphate buffered saline (PBS) with calcium (2 mM) added or containing calcium and magnesium (0.9 and 0.5 mM, respectively). A region considered to contain the majority of endplates (perisynaptic region, about the middle third of the muscle) and regions devoid of endplates (extrasynaptic regions) were cut out from innervated or denervated left hemidiaphragms under a dissecting microscope. Muscle regions from 7 denervated (hypertrophic) or 16–20 innervated left hemidiaphragms were pooled.

Gastrocnemius, soleus, anterior tibial and extensor digitorum longus muscles from the denervated hind-limb were dissected, pooled and then processed together for RNA extraction. The same muscles from the contralateral leg were pooled separately and used as innervated controls. RNA was extracted as described in Magnusson et al. (2001).

Cloning of complementary DNA fragments

Complementary DNA (cDNA) fragments to be used for synthesis of labeled complementary RNA probes were amplified and cloned as described previously (Magnusson et al. 2001). Sfrp1, Dvl1 and β-actin cDNA were obtained with the GeneAmp RNA PCR kit (Perkin Elmer, Roche Molecular Systems Inc., Brachbury, NJ) where 1 μg of total RNA extracted from mouse hemidiaphragm (denervated perisynaptic region for Sfrp1, innervated perisynaptic region for Dvl1) or hind-limb muscles (for β-actin) was used for reverse transcription together with random

hexamers as primers. Reverse transcription and the following amplification by PCR were performed according to the manufacturer's instructions. Sfrp1 primers (forward: 5′-CGA CAA CGA GTT GAA GTC AGA G and reverse: 5′-TTT CTT GTC CCA CTT GTG AAT G), were designed to amplify a 304 bp cDNA fragment corresponding to nucleotides 823–1126 of the mouse Sfrp1 mRNA sequence (GenBank Accession number U88566, Rattner et al. 1997). A Dvl1 cDNA fragment was amplified with primers (forward: 5′-GCT ACT ATG TCT TTG GCG ACC T and reverse: 5′-TGG TCT GAT TCA CTG CCA CTA C) designed to amplify a 337 bp cDNA fragment corresponding to nucleotides 1801–2137 of the mouse Dvl1 mRNA sequence (GenBank Accession number NM_010091, Fan et al. 2004). β-actin primers (forward: 5′-CAC CCC CAC TCC TAA GAG GA and reverse: 5′-GGT AAG GTG TGC ACT TTT ATT GGT), were designed to amplify a 296 bp cDNA fragment corresponding to nucleotides 1587–1882 of the mouse β-actin mRNA sequence (GenBank Accession number NM_007393). The amplified cDNA fragments were cloned into the pGEM-T vector and JM 109 cells (Promega, Madison, WI) were transformed with the ligated plasmids. Qiagen Plasmid Midi Kit (Qiagen Inc, Chatsworth, CA) was used for plasmid purification from bacterial cultures. The plasmid inserts were sequenced using an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA) and the BigDye Terminator Cycle Sequencing Ready Reaction DNA Sequencing kit (PE Applied Biosystems, Warrington, UK) according to the manufacturer's instructions. The cloned cDNA sequences were identical to the sequences expected from the reported mouse mRNA sequences.

Transcription of ^{32}P-labeled RNA probes and Northern blots

The purified plasmids with the cloned cDNA fragments were linearized by cutting with NotI and antisense RNA probes were transcribed with T7 RNA polymerase. Transcription, RNA separation and hybridization were performed as described previously (Magnusson et al. 2001).

Protein extraction

Mouse hemidiaphragm and anterior tibial muscles were used for protein extraction. Perisynaptic and extrasynaptic regions were cut out from innervated or denervated hemidiaphragm muscle and processed separately. Muscle regions from three left hemidiaphragms were pooled. The tissues were homogenized, centrifuged and the protein concentration was determined as described in Magnusson et al. (2003). For determination of protein phosphorylation status hemidiaphragm tissues were homogenized in 1 ml of a buffer containing 100 mM Tris–HCl, pH 7.6, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.1% sodium deoxycholate, 2 mM Na_3VO_4 and 100 mM NaF with one Protease Inhibitor Cocktail Mini Tablet (Roche Diagnostics GmbH, Mannheim, Germany) per 10 ml of extraction buffer.

Western blots

Western blots were prepared essentially as described in Magnusson et al. (2003). Five to 28 μg protein were reduced, denatured and electrophoretically separated on a 12% polyacrylamide gel with a 5.2% polyacrylamide stacking gel on top. Gels were electroblotted onto PVDF Plus transfer membranes (Osmonics Inc., Westborough, MA) and the membranes were blocked and then incubated with antibodies. Primary antibodies used were Sfrp1 [ab4193] (Abcam plc, Cambridge, UK) diluted to 5.5 μg/ml, Dvl1 [06-939] diluted to 2.8 μg/ml (Upstate Biotechnology, Lake Placid, NY), β-catenin [C2206] diluted to 53 μg/ml (Sigma-Aldrich Inc, Saint Louis, Mo), GSK-3β [610202] diluted to 0.25 μg/ml, phospho-specific GSK-3β pY216 (Tyr216) [612312] diluted to 0.25 μg/ml (BD Transduction Laboratories, San Diego, CA) and phospho-GSK-3β (Ser9) [9336] diluted 1/1,000 (Cell Signaling Technology, Beverly, CA). Western blot membranes were re-incubated with a β-actin antibody (rabbit polyclonal to beta Actin-Loading Control [ab8227–50], Abcam plc, Cambridge, UK), diluted to 7 ng/ml. Antibodies were visualized with horseradish peroxidase conjugated secondary immunoglobulin diluted 1/1,000 (goat anti-rabbit IgG [P0448] or rabbit anti-mouse IgG [P0260], Dako, Glostrup, Denmark). Negative controls included membranes incubated in the absence of the primary antibodies. The bound immune complexes were detected using the ECL Plus Western blotting detection system and Hyperfilm ECL (Amersham International and Amersham Pharmacia Biotech, Buckinghamshire, England).

Immunohistochemistry

Immunohistochemical analysis was performed on innervated and 6, 13, or 21 days denervated hemidiaphragm and anterior tibial muscles from rat. Muscles were mounted, frozen, sectioned and incubated as described in Magnusson et al. (2003). Sections were blocked in 5% goat serum or 5% donkey serum in PBS depending on which species the secondary antibody was obtained from. Incubations were performed using a rabbit Sfrp1 antibody [sc-13939] (Santa Cruz Biotechnology Inc., Santa Cruz, CA) diluted to 0.4–1.0 μg/ml and two different Dvl1 antibodies ([06-939] Upstate Biotechnology, Lake Placid, NY; [sc-8025] Santa Cruz Biotechnology Inc., Santa Cruz, CA) in PBS with

0.1% bovine serum albumin. Negative controls included sections incubated in the absence of the primary antibody and sections incubated with a control IgG (normal rabbit IgG [sc-2027], 10 μg/ml, Santa Cruz Biotechnology Inc., Santa Cruz, CA). To compare the localization of unspecific and specific stainings separate sections were incubated with Sfrp1 antibody together with normal goat IgG [sc-2028], 10 μg/ml (Santa Cruz Biotechnology Inc., Santa Cruz, CA), and compared with sections incubated with normal rabbit IgG. AChRs were visualized with tetramethylrhodamine-conjugated α-bungarotoxin (Molecular Probes Inc., Eugene, OR). Secondary antibodies used were Alexa Fluor® 488 goat anti-rabbit IgG [A11034], Alexa Fluor® 488 donkey anti-goat IgG [A11055] (Molecular Probes Inc., Eugene, OR) and Rhodamine (TRITC)-conjugated AffiniPure donkey anti-rabbit IgG [711-025-152] (Jackson ImmunoResearch Laboratories Inc., West Grove, PA). Slides were mounted in Vectashield mounting medium (Vector Laboratories Inc., Burlingame, CA). Sections were examined using a Nikon Eclipse E600 microscope equipped with the C1 modular confocal microscope system (Nikon Instech Co., Ltd., Kanagawa, Japan).

Data analysis and statistics

Image analysis was performed on a Macintosh computer using the gel plotting macro of the public domain NIH Image program (1.62, developed at the US National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/). Results were obtained in uncalibrated optical density units.

For quantification of protein expression one Western blot representing each protein was analyzed. Each blot contained three different innervated and three different denervated perisynaptic hemidiaphragm muscle extracts. The image analysis data were normalized to give an average signal of 100.0 for each protein or phosphorylated protein in innervated muscle. Data are presented as mean values ± standard error of the mean (SEM). Student's *t*-test (two tailed) was used to determine whether or not the mean expression in denervated muscle was significantly different from that in innervated muscle.

Results

Sfrp1 mRNA expression

Northern blots showed Sfrp1 mRNA to be barely detectable in innervated hemidiaphragm muscle. A 4.4 kb transcript corresponding to the size of Sfrp1 mRNA (Finch et al. 1997; Rattner et al. 1997) was, however, clearly present in 6 days denervated hemidiaphragm muscle. The
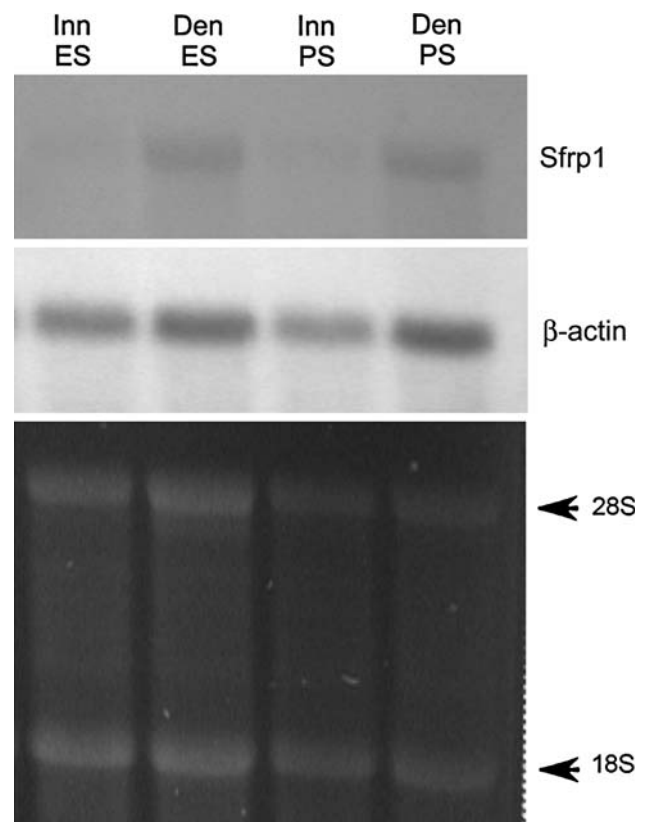

Fig. 1 Sfrp1 mRNA expression in innervated (Inn) and denervated (Den) mouse hemidiaphragm muscle. Sfrp1 mRNA expression is increased in extrasynaptic (ES) and perisynaptic (PS) regions of 6 days denervated hemidiaphragm muscle. Autoradiograms are shown for Sfrp1 (top) and for β-actin (middle) together with ethidium bromide fluorescence (bottom) for verification of RNA quality. Positions of 28S and 18S ribosomal RNA are indicated with arrows. The amount of total RNA loaded per lane was 15 μg. The Sfrp1 autoradiogram was exposed 8 days at −80°C with intensifying screen. Exposure time for β-actin was 7 days

expression was similar in perisynaptic and extrasynaptic regions (Fig. 1). Two different RNA preparations from 6 days denervated hemidiaphragm muscle showed similar results regarding Sfrp1 mRNA expression. Expression levels in denervated as well as in innervated hind-limb muscles were too weak for reliable detection on Northern blots.

Sfrp1 immunoreactivity

Examination of Sfrp1-like immunoreactivity on Western blots revealed a protein band of a size slightly above 30 kD in denervated hemidiaphragm muscle (Fig. 2), corresponding to the approximate size of Sfrp1 (Finch et al. 1997; Rattner et al. 1997). Weak or no expression was seen in innervated hemidiaphragm muscle (Fig. 2). The Sfrp1 antibody detected several protein bands on Western blots but only one band corresponded to the size of Sfrp1. Sfrp1-
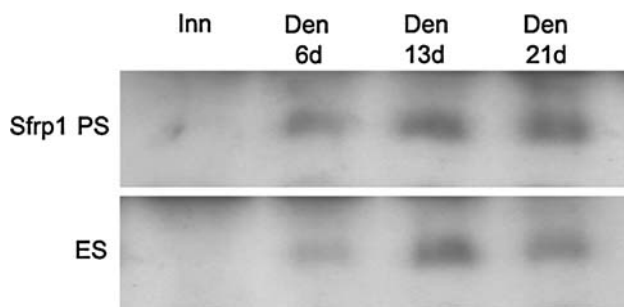
Fig. 2 Sfrp1-like immunoreactivity in innervated (Inn) and denervated (Den) mouse skeletal muscle. One protein band of a size slightly above 30 kD corresponds to the size of Sfrp1. Sfrp1-like immunoreactivity is increased in perisynaptic (PS) and extrasynaptic (ES) regions of 6, 13, and 21 days denervated hemidiaphragm muscle. The amount of protein loaded per lane was 28 μg

like immunoreactivity was detected in perisynaptic as well as in extrasynaptic regions of hemidiaphragm muscle (Fig. 2). Time course studies showed an up-regulation of the immunoreactivity in hemidiaphragm at all time points studied (6–21 days after denervation, Fig. 2). The expression of Sfrp1-like immunoreactivity was almost 10 times higher ($P < 0.01$) in 6 days denervated hemidiaphragm muscle compared to innervated muscle (100.0 ± 27.1 arbitrary units in innervated muscle, $n = 3$, and 1919.9 ± 296.8 arbitrary units in denervated muscle, $n = 3$). Negative controls using only secondary antibodies showed no bands on Western blots (results not shown).
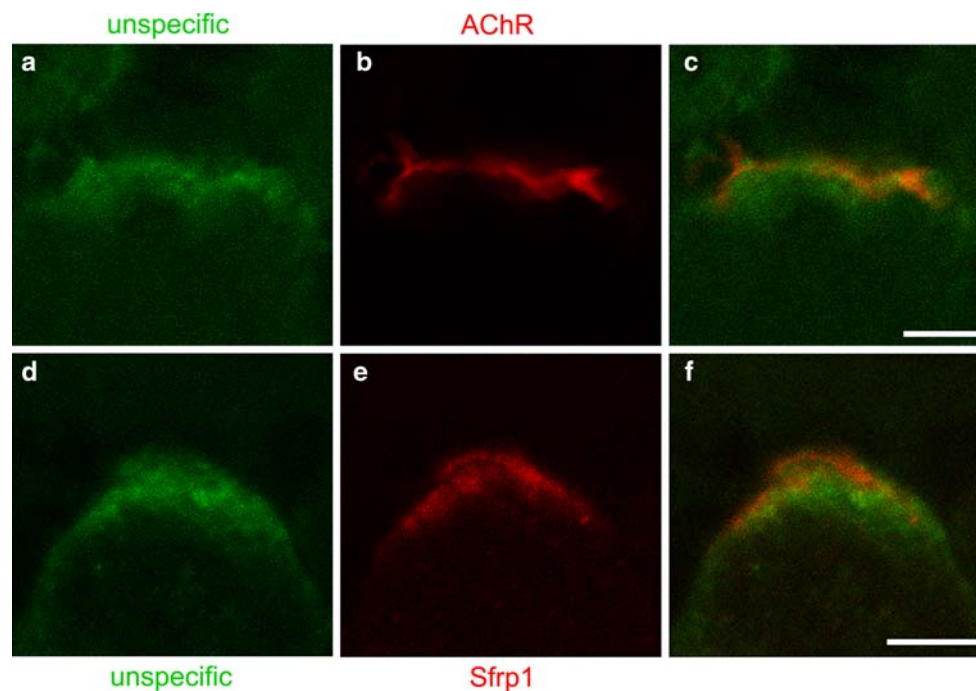
Sfrp1-like immunoreactivity was weak in denervated as well as in innervated anterior tibial muscle on Western blots (results not shown). The response to denervation, however, appeared to be similar to that of hemidiaphragm muscle.

Immunohistochemistry experiments with an anti-Sfrp1 (sc-13939) antibody on rat hemidiaphragm muscles showed a marked presence of Sfrp1-like immunoreactivity in the endplate region of muscle fibers (Figs. 3, 4). Co-labeling with fluorescence-labeled α-bungarotoxin showed the Sfrp1-like immunoreactivity to be concentrated in the vicinity of AchRs in innervated and in 21 days denervated hemidiaphragm muscle (Fig. 3). Immunoreactivity was also present in 6 days denervated hemidiaphragm muscle (results not shown). Similar results were obtained using anterior tibial muscle (results not shown). Staining of the endplate region was, however, also pronounced when a control IgG (normal rabbit IgG) was used instead of the Sfrp1 antibody (Fig. 4). To eliminate the possibility that the Sfrp1-like immunoreactivity was unspecific, sections were first incubated with normal control goat IgG together with normal control rabbit IgG. Normal control IgGs from both species stained muscle endplates in a similar manner and the staining obtained was clearly co-localized and overlapping (results not shown, see Svensson et al. 2008). Next, sections were incubated with the anti-Sfrp1 antibody together with normal goat IgG and the stainings observed, contrary to the case for the two
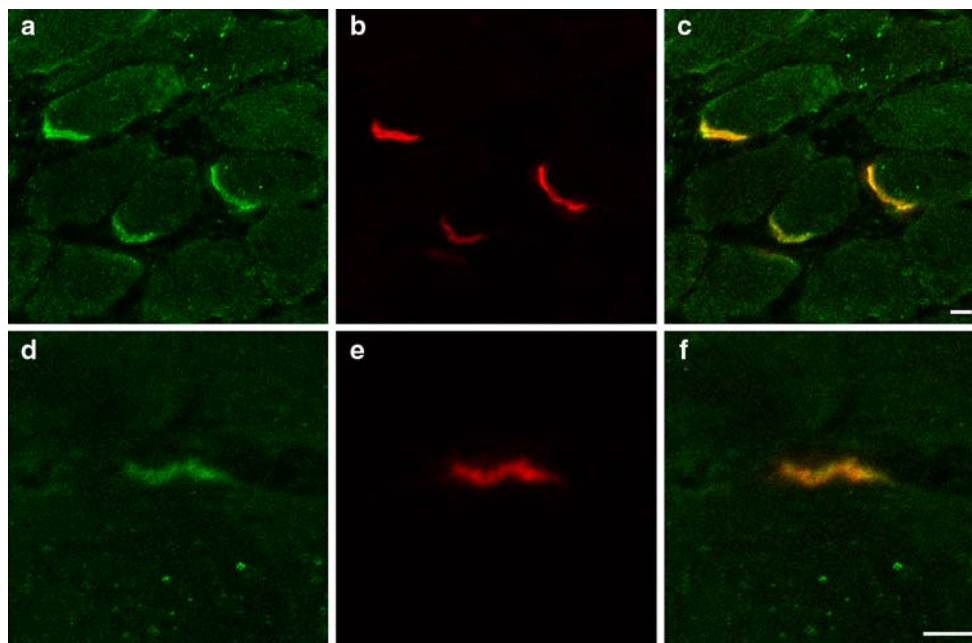
Fig. 3 Sfrp1-like immunoreactivity at the neuromuscular junction. The left column shows Sfrp1-like immunoreactivity (green) and the middle column shows acetylcholine receptors visualized by α-bungarotoxin (red). The right column shows the left and middle column pictures superimposed. (**a–c**) Cross section of 21 days denervated rat hemidiaphragm muscle. (**d–f**) Longitudinal section of innervated rat hemidiaphragm muscle. Antibody concentrations were 0.8 μg/ml (**a–c**) or 0.4 μg/ml (**d–f**). Bars = 10 μm

unspecific   AChR

unspecific   Sfrp1

Fig. 4 Sfrp1-like immunoreactivity does not co-localize with unspecific staining. (**a–c**) Negative control (normal rabbit IgG; green, **a**) showing unspecific staining of neuromuscular junctions in cross section of 21 days denervated rat hemidiaphragm muscle. Acetylcholine receptors are visualized by α-bungarotoxin (red, **b**) and the right column represents the left and middle column pictures superimposed (**c**). (**d–f**) The unspecific staining of normal goat IgG (green, **d**) does not co-localize with the Sfrp1-like immunoreactivity (red, **e** and superimposed picture, **f**) at muscle endplates in 21 days denervated rat hemidiaphragm muscle. Sfrp1 antibody and normal IgG concentrations were 1 and 10 μg/ml, respectively. Bars = 10 μm

normal control IgGs, did not completely overlap at the neuromuscular junction (Fig. 4). Sfrp1-like immunoreactivity seemed to co-localize more with staining for AchRs (Fig. 3), which is not the case for normal control IgGs (Fig. 4). Sections incubated in the presence of secondary antibodies only did not show any staining of the neuromuscular junction (results not shown).

Canonical Wnt signaling

Dvl1 mRNA expression was largely unaltered in denervated mouse hemidiaphragm and hind-limb muscles compared to innervated controls (results not shown). No obvious differences in expression between perisynaptic and extrasynaptic regions could be detected on Northern blots (results not shown). Dvl1-like immunoreactivity on Western blots showed a slight down-regulation in 6, 13 and 21 days denervated hemidiaphragm muscle (Fig. 5). Expression was at all time points similar in perisynaptic and extrasynaptic regions. Quantification of changes in expression in hemidiaphragm muscle revealed a 69% ($P < 0.05$) decrease in Dvl1-like immunoreactivity 6 days after denervation relative to expression in innervated muscle (100.0 ± 20.3 arbitrary units in innervated muscle, $n = 3$, and 30.7 ± 14.3 arbitrary units in denervated muscle, $n = 3$). No major

change in Dvl1-like immunoreactivity was detected between innervated and denervated anterior tibial muscle on Western blots (results not shown). Immunohistochemistry experiments with two different anti-Dvl1 antibodies showed no convincing specific Dvl1-like immunoreactivity at the neuromuscular junction neither in hemidiaphragm nor in anterior tibial muscle (results not shown).

GSK-3β expression was examined on Western blots using three different antibodies, one detecting total GSK-3β-like immunoreactivity and the other two detecting phosphorylated GSK-3β on Tyr216 or Ser9, respectively. Total GSK-3β-like immunoreactivity was slightly increased in 6, 13 and 21 days denervated hemidiaphragm muscle (Fig. 5). Total GSK-3β-like immunoreactivity showed an 83% ($P < 0.001$) increase in hemidiaphragm muscle 6 days after denervation (100.0 ± 3.4 arbitrary units in innervated muscle, $n = 3$, and 183.3 ± 6.0 arbitrary units in denervated muscle, $n = 3$). Western blots showed the levels of both Tyr216 and Ser9 phosphorylated forms of GSK-3β to be increased in hemidiaphragm muscle 6, 13 and 21 days after denervation (Fig. 5). In hemidiaphragm muscle the level of p-GSK-3β Tyr216 was 330% ($P < 0.01$) higher 6 days after denervation relative to the level in innervated controls (100.0 ± 30.4 arbitrary units in innervated muscle, $n = 3$, and 429.8 ± 48.0 arbitrary units
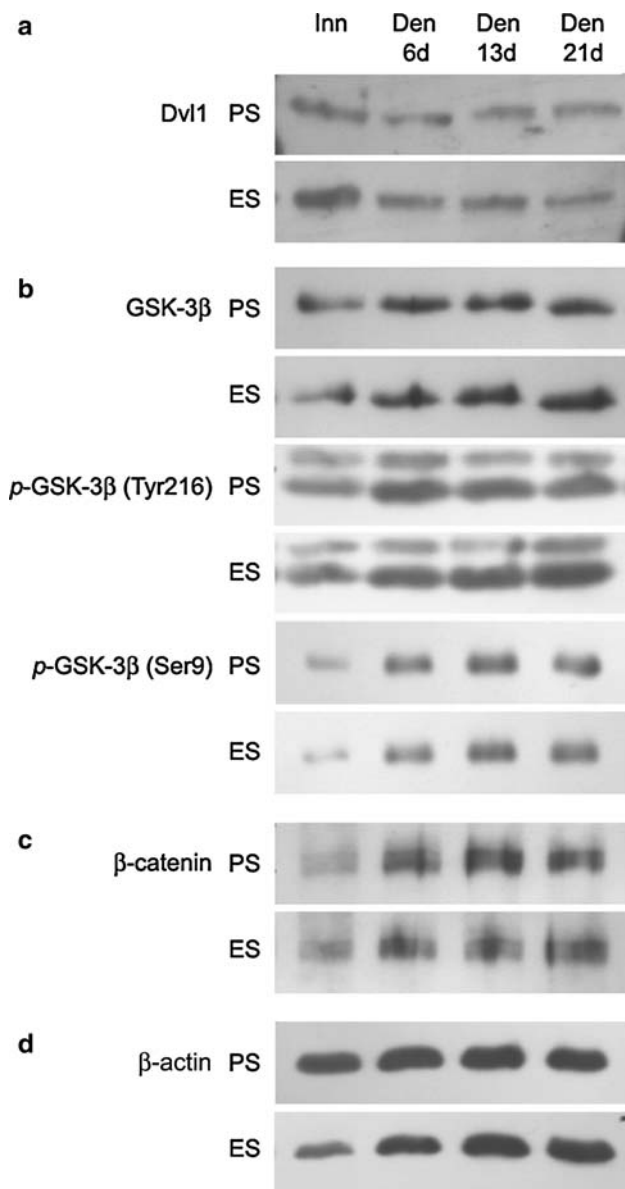
Fig. 5 Expression of key proteins involved in Wnt signaling in innervated (Inn) and denervated (Den) mouse hemidiaphragm muscle. (**a–c**) Dvl1 (**a**), GSK-3β (**b**) and β-catenin (**c**) immunoreactivities in perisynaptic (PS) and extrasynaptic (ES) regions of innervated and 6, 13, and 21 days denervated hemidiaphragm muscle. (**b**) Total GSK-3β-like immunoreactivity (top) is shown together with levels of phosphorylated GSK-3β on Tyr216 (middle) or Ser9 (bottom). The larger protein band stained by the p-GSK-3β Tyr216 antibody represents GSK-3α (middle, **b**). The amount of protein loaded per lane was 10 μg (p-GSK-3β Ser9, **b**) or 20 μg. Protein bands were of approximate sizes 85 kD (Dvl1, **a**), 46 kD (GSK-3β, **b**), and 94 kD (β-catenin, **c**). (**d**) β-actin expression

in denervated muscle, $n = 3$). The level of p-GSK-3β Ser9 was 260% ($P < 0.001$) higher in 6 days denervated hemidiaphragm muscle (100.0 ± 2.8 arbitrary units in innervated muscle, $n = 3$, and 363.6 ± 20.7 arbitrary units in denervated muscle, $n = 3$). For each of the GSK-3β antibodies used, similar levels of immunoreactivity were

observed in perisynaptic and extrasynaptic regions. No evident denervation-induced alteration in GSK-3β-like immunoreactivity or protein phosphorylation status could be detected in anterior tibial muscle on Western blots, using the three different antibodies (results not shown).

Western blots using a β-catenin antiserum showed two protein bands being up-regulated in denervated hemidiaphragm muscle (Fig. 5). Both bands were regulated similarly in response to denervation. Levels of β-catenin-like immunoreactivity were comparable in 6, 13 and 21 days denervated hemidiaphragm muscle (Fig. 5). No difference in expression was seen in perisynaptic and extrasynaptic regions. The expression of β-catenin-like immunoreactivity was 91% higher ($P < 0.01$) in 6 days denervated hemidiaphragm muscle compared to innervated muscle (100.0 ± 11.8 arbitrary units in innervated muscle, $n = 3$, and 191.2 ± 7.1 arbitrary units in denervated muscle, $n = 3$). β-catenin-like immunoreactivity was also increased in 6, 13 and 21 days denervated anterior tibial muscle on Western blots (results not shown).

Discussion

Expression levels of Sfrp1 and Sfrp2 mRNA have previously been reported to be up-regulated in muscle regeneration (Levin et al. 2001; Zhao and Hoffman 2004) and Sfrp2 mRNA expression was also shown to increase after denervation of rabbit skeletal muscle (Levin et al. 2001). The present study shows that Sfrp1 mRNA expression is also up-regulated in skeletal muscle after denervation. This was particularly evident in denervated hemidiaphragm muscle in which an up-regulation of Sfrp1-like immunoreactivity on Western blots was also observed. Increased expression of Sfrp1-like immunoreactivity was seen at all time points studied (6–21 days) after denervation. Expression of Sfrp1-like immunoreactivity, as well as mRNA, was observed in extrasynaptic as well as perisynaptic regions of denervated hemidiaphragm muscle. Immunohistochemically, however, Sfrp1-like immunoreactivity was found to be concentrated at neuromuscular junctions in the vicinity of AchRs (the endplate region) in hemidiaphragm muscle as well as in anterior tibial muscle. The strong Sfrp1-like immunoreactivity observed in endplate regions of 21 days denervated muscle suggests a postsynaptic location of Sfrp1 at the neuromuscular junction.

Specificity of Sfrp1-like immunoreactivity

Normal rabbit and goat IgGs (from non-immunized animals) can give a considerable staining of the endplate region of muscle fibers (Svensson et al. 2008). The specificity of the staining observed with the Sfrp1 antibody was

inferred by comparing the staining obtained with the Sfrp1 antibody and that observed with control IgGs (normal IgGs) from two different species (rabbit and goat). Whereas the stainings obtained with the two control IgGs overlapped well in hemidiaphragm muscle as well as in anterior tibial muscle (Svensson et al. 2008), the staining observed with the Sfrp1 antibody was to a large extent not overlapping with the non-specific staining observed with the control IgGs. The Sfrp1 immunoreactivity appeared to overlap more with the bungarotoxin staining of AchRs. Sfrp1 may therefore, like AchRs (Fertuck and Salpeter 1974), be concentrated at the top of the junctional folds.

Canonical Wnt signaling

Increased Sfrp1 expression in denervated skeletal muscle could be expected to result in decreased Wnt signaling as has been observed in other systems. Thus, Sfrp1 has been shown to antagonize Wnt signaling by forming complexes with both Wnt and Fzd proteins through the cysteine-rich ligand-binding domain (Bafico et al. 1999; Üren et al. 2000). A decrease in Wnt signaling is expected to result in decreased intracellular β-catenin levels (see "Introduction") and in human breast adenocarcinoma cells expression of Sfrp1 has, indeed, been shown to decrease the intracellular β-catenin concentration (Melkonyan et al. 1997). If Sfrp1 inhibits canonical Wnt signaling in denervated muscle one might expect a decrease in total β-catenin levels. The results of the present study, however, indicate an increase in β-catenin protein levels in skeletal muscle after denervation which is consistent with a previous report demonstrating up-regulation of β-catenin in denervated mouse skeletal muscle (Cifuentes-Diaz et al. 1998). A possible effect of Sfrp1 on canonical Wnt signaling in spatially restricted muscle regions cannot be excluded but the results may also suggest a role for Sfrp1 in denervated muscle other than interfering with Wnt signaling.

In the present study the expression of additional proteins involved in Wnt signaling, such as Dvl1 and GSK-3β, were also examined in innervated and denervated skeletal muscle. Dvl1 protein expression has previously been reported to localize at neuromuscular junctions (Luo et al. 2003) and to be up-regulated in skeletal muscle 6 days after denervation (Luo et al. 2002). In the present study no convincing Dvl1-like immunoreactivity was detected at neuromuscular junctions using two different anti-Dvl1 antibodies. Furthermore, Dvl1-like immunoreactivity on Western blot was found to be down-regulated in hemidiaphragm muscle after denervation. This was not, however, evident in denervated anterior tibial muscle. Activation or over-expression of Dvl leads to β-catenin accumulation (Lee et al. 1999). Since Dvl1-like immunoreactivity in the present study was found to be down-regulated in

denervated hemidiaphragm muscle, other mechanisms may be responsible for the increased β-catenin-like immunoreactivity observed in denervated hemidiaphragm muscle.

Other effects of Sfrp1

Sfrp1 has been shown to participate in neural differentiation of the retina (Esteve et al. 2003). This action does not involve β-catenin-dependent transcription, but seems to involve GSK-3β (Esteve et al. 2003). GSK-3β is constitutively active but can be inhibited either by forming complexes with other proteins (a Wnt-response, see Wodarz and Nusse 1998) or by phosphorylation at Ser9 residues (McManus et al. 2005). Phosphorylation at Ser9 is not the common way for Wnt to inhibit GSK-3β (Ding et al. 2000; McManus et al. 2005). Phosphorylation at Tyr216 activates GSK-3β (Wang et al. 1994). Overexpression of Sfrp1 increased the level of GSK-3β phosphorylated at Ser9 in chick retina cells, whereas the level of GSK-3β phosphorylated at Tyr216 was unaltered. Thus, Sfrp1 caused inhibition of GSK-3β activity (Esteve et al. 2003). Interestingly, this is similar to some of the results of the present study. Denervation induced increased levels of Sfrp1-like immunoreactivity and β-catenin-like immunoreactivity in hemidiaphragm muscle. In addition increased phosphorylation of GSK-3β at Ser9 was observed after denervation. The level of Tyr216 phosphorylation was, however, also increased after denervation in hemidiaphragm muscle.

In chicken and *Xenopus*, Sfrp1 has been shown to directly modify and reorient growth of retinal ganglion cell axons, without involvement of Wnt inhibition (Rodriguez et al. 2005). Acting as a chemoattractant, Sfrp1 was shown to promote neurite outgrowth in a dose-dependent manner in embryonic chick neural retina cultures. This function could be modulated by extracellular matrix molecules giving an attractive or repulsive effect (Rodriguez et al. 2005). If similar mechanisms operate in motor neurons increased expression of Sfrp1 in skeletal muscle following denervation may serve to promote reinnervation of denervated muscle fibers.

Conclusions

Expression levels of Sfrp1 mRNA and immunoreactivity increase after denervation of mouse hemidiaphragm muscle. Immunoreactivity, detected by Western blots, and mRNA, detected by Northern blots, are both expressed in extrasynaptic as well as perisynaptic parts of the denervated muscle. Immunoreactivity on tissue sections is, however, concentrated postsynaptically at neuromuscular junctions.

Using β-catenin-like immunoreactivity levels as a readout for canonical Wnt signaling no evidence for

decreased canonical Wnt signaling was obtained in denervated muscle. A possible effect of Sfrp1 on canonical Wnt signaling in spatially restricted muscle regions cannot be excluded but increased Sfrp1 expression in denervated muscle may also serve functions unrelated to canonical Wnt signaling. A role in communication between muscle and motor neurons is a possible such function.

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