

Short communication

## GALECTIN-1 EXPRESSION IN INNERVATED AND DENERVATED SKELETAL MUSCLE

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**Abstract:** Galectin-1 is a soluble carbohydrate-binding protein with a particularly high expression in skeletal muscle. Galectin-1 has been implicated in skeletal muscle development and in adult muscle regeneration, but also in the degeneration of neuronal processes and/or in peripheral nerve regeneration. Exogenously supplied oxidized galectin-1, which lacks carbohydrate-binding properties, has been shown to promote neurite outgrowth after sciatic nerve sectioning. In this study, we compared the expression of galectin-1 mRNA and immunoreactivity in innervated and denervated mouse and rat hind-limb and hemidiaphragm muscles. The results show that galectin-1 mRNA expression and immunoreactivity are up-regulated following denervation. The galectin-1 mRNA is expressed in the extrasynaptic and perisynaptic regions of the muscle, and its immunoreactivity can be detected in both regions by Western blot analysis. The results are compatible with a role for galectin-1 in facilitating reinnervation of denervated skeletal muscle.

**Key words:** Galectin-1, Skeletal muscle, Denervation

### INTRODUCTION

Galectin-1 (Gal1) is a soluble carbohydrate-binding protein expressed in various mammalian tissues, with a prominent expression in skeletal muscle [1]. Gal1 exists both in the cytosol and extracellularly [1, 2], and has been implicated in

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Abbreviations used: Gal1 – galectin-1; Inn – innervated; Den – denervated, PS – perisynaptic; ES – extrasynaptic

skeletal muscle development [3-6]. In the neuromuscular system, Gal1 may also play a role in neuronal degeneration and/or regeneration: Gal1 null mutant mice show a reduced rate of recovery of whisking movements after facial nerve crush [7] and delayed elimination of nerve endings following sciatic nerve section [8]. Besides the prominent occurrence in skeletal muscle, Gal1 is also expressed in motor neurons, and this expression is up-regulated after axotomy [7, 9]. Gal1 expression has also recently been reported to be up-regulated in Schwann cells in the anterior tibial muscle within 24 h of sciatic nerve sectioning [8]. Oxidized Gal1, which lacks carbohydrate-binding properties [10], has been shown to promote neurite outgrowth after sciatic nerve sectioning [11]. It is possible that reduced cytosolic Gal1 becomes oxidized extracellularly and acts indirectly by causing the release of neuronal growth-promoting factors from macrophages [11, 12].

With regard to a potential role for Gal1 in neuronal degeneration/regeneration, we examined the expression of Gal1 mRNA and Gal1-like immunoreactivity in innervated and denervated mouse and rat skeletal muscle, showing that, following denervation, the expression of Gal1 in adult skeletal muscle is up-regulated in the perisynaptic and extrasynaptic muscle regions. These results are compatible with a role for Gal1 in facilitating reinnervation of denervated skeletal muscle fibers.

## **MATERIALS AND METHODS**

### **Animals**

All the experiments were performed on adult male NMRI mice or SD rats (B & K Universal, Sollentuna, Sweden or Charles River, Uppsala, Sweden). 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. Six days (RNA extraction) or 6, 13 or 21 days (protein extraction) after denervation, muscles were harvested as described previously [13]. The experimental procedures were approved by the Ethical Committee for Animal Experiments at Linköping University, Sweden.

### **RNA extraction**

RNA extraction from the left hemidiaphragms of innervated or denervated mice was performed as described previously [13]. Muscle regions from 7 denervated (hypertrophic) or 16-20 innervated left hemidiaphragms were pooled. Gastrocnemius, soleus, anterior tibial and extensor digitorum longus muscles from innervated or denervated hind-limbs were pooled and processed for RNA extraction as described previously [13].

### **Cloning of a galectin-1 complementary DNA fragment**

A complementary DNA (cDNA) fragment to be used for the synthesis of a labelled complementary RNA probe was amplified and cloned as described previously [13]. Gal1 cDNA was obtained with primers (forward: 5'-TGT GTA

ACA CCA AGG AAG ATG G and reverse: 5'-CTG CCT TTA TTG AGG GCT ACA G) designed to amplify a 263-bp cDNA fragment corresponding to nucleotides 222-484 of the mouse Gal1 mRNA sequence (GenBank Accession number X15986, [14]). The cloned cDNA sequence was identical to the sequence expected from the reported mouse mRNA sequence.

#### **Transcription of the <sup>32</sup>P-labeled RNA probe and Northern blots**

The purified plasmid with the cloned cDNA fragment was linearized with Nco I, and an antisense RNA probe was transcribed with SP6 RNA polymerase. Transcription, RNA separation and hybridization were performed as described previously [13].

#### **Protein extraction**

Mouse and rat hemidiaphragm and mouse anterior tibial muscles were used for protein extraction. The perisynaptic (about the middle third of the muscle) and extrasynaptic regions were cut out from innervated or denervated hemidiaphragm muscle and processed separately. In the case of the mice, muscle regions from 3 left hemidiaphragms were pooled. Protein extraction was performed as described previously [15].

#### **Western blots**

Western blots were prepared essentially as described previously [16]. Rabbit antiserum against rat Gal1 (provided by Professor H. Leffler, Lund University, Sweden) diluted 1/1000 and a goat anti-mouse Gal1 antibody [AF1245] (R&D Systems Inc, Minneapolis, MN) diluted to 0.1 µg/ml were visualized with horseradish peroxidase-conjugated secondary immunoglobulins diluted 1/1000 (goat anti-rabbit IgG [P0448] or rabbit anti-goat IgG [P0449], Dako, Glostrup, Denmark). As a loading control, the 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 and visualized as described above.

#### **Data analysis and statistics**

Western blot 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/>) or ImageJ (Rasband, W.S., ImageJ, US National Institutes of Health, Bethesda, Maryland, USA, <http://rsb.info.nih.gov/ij/>, 1997-2007). The results were obtained in uncalibrated optical density units.

To quantify the protein expression, Western blots using the rabbit antiserum against rat Gal1 were analyzed. Each blot contained three different innervated and three different denervated perisynaptic mouse hemidiaphragm muscle extracts, or three different perisynaptic and three different extrasynaptic muscle extracts from innervated mouse hemidiaphragm muscle. Each extract used

contained muscle regions pooled from three left hemidiaphragms. The image analysis data was normalized to give an average signal of 100.0 for Gal1 in the perisynaptic region of the innervated muscle. The data is presented as mean values  $\pm$  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, or whether or not the mean expression in the extrasynaptic regions was significantly different from that in the perisynaptic region of the innervated muscle.

## RESULTS

### Galectin-1 mRNA expression

A transcript with an approximate size of 0.75 kb, corresponding to the size of Gal1 mRNA [17], was detected in hind-limb and hemidiaphragm muscles on Northern blots (Fig. 1). The mRNA expression in the hind-limb and hemidiaphragm muscles was up-regulated 6 days after denervation (Fig. 1). In the hemidiaphragm muscle, the expression was similar in the perisynaptic and extrasynaptic regions (Fig. 1B).

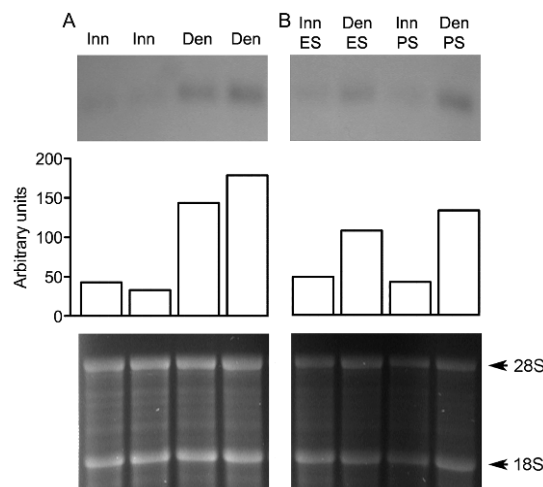


Fig. 1. Gal1 mRNA expression in innervated (Inn) and denervated (Den) mouse skeletal muscle. A – Gal1 mRNA expression in hind-limb muscle that was innervated or 6 days post-denervation (pooled samples containing gastrocnemius, soleus, anterior tibial and extensor digitorum longus muscles from individual animals). Lanes with identical labelling contain RNA preparations from different animals. B – Gal1 mRNA expression in the extrasynaptic (ES) and perisynaptic (PS) regions of hemidiaphragm muscle that was innervated or 6 days post-denervation (muscle regions pooled from 7 denervated or 16-20 innervated left hemidiaphragms). In A and B, autoradiograms are shown (top) together with densitometric quantification (middle) and ethidium bromide fluorescence (bottom) to verify the RNA quality. The positions of the 28S and 18S ribosomal RNA are indicated with arrows. The amount of total RNA loaded per lane was 15  $\mu$ g (A and B). The Gal1 autoradiograms were exposed at  $-80^{\circ}\text{C}$  with intensifying screens for 14 days.

### Galectin-1 immunoreactivity

Both the rabbit antiserum against rat Gal1 and the goat anti-mouse Gal1 antibody revealed a protein of 14.5 kD on Western blots, corresponding to the reported size of Gal1 [18]. The rabbit antiserum detected several protein bands, but only one band corresponded to the size of Gal1 (Fig. 2), whereas the goat antibody detected only one band (results not shown).

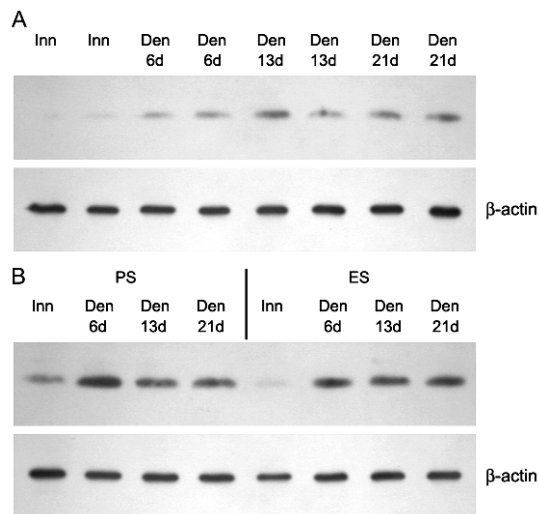


Fig. 2. Gal1-like immunoreactivity in innervated (Inn) and denervated (Den) mouse skeletal muscle. One protein band of 14.5 kD corresponds to the size of Gal1. A – Gal1-like immunoreactivity in anterior tibial muscle that was innervated, or 6, 13 or 21 days post-denervation. Lanes with identical labelling contain protein preparations from different animals. The picture below shows  $\beta$ -actin-like immunoreactivity as a loading control. B – Gal1-like immunoreactivity in the perisynaptic (PS) and extrasynaptic (ES) regions of hemidiaphragm muscle that was innervated, or 6, 13 or 21 days post-denervation (muscle regions pooled from 3 left hemidiaphragms for each time point). The picture below shows  $\beta$ -actin-like immunoreactivity as a loading control. The amount of protein loaded per lane was 3  $\mu$ g (A and B).

Time course studies showed an up-regulation of Gal1 immunoreactivity in the denervated muscles at all the time points studied (6-21 days after denervation) in both the anterior tibial (Fig. 2A) and hemidiaphragm (Fig. 2B) mouse skeletal muscles. Similar results were found for rat hemidiaphragm muscle (results not shown). The quantification of the expression in mouse hemidiaphragm muscle revealed a large increase ( $p < 0.01$ ) in the Gal1-like immunoreactivity 6 days after denervation relative to the expression in the innervated muscle (Fig. 3). The expression in innervated muscle was  $100.0 \pm 38.1$  arbitrary units, ( $n = 3$ ) and that in denervated muscle was  $1613.0 \pm 321.6$  arbitrary units ( $n = 3$ ). The Gal1-like immunoreactivity was detected in the perisynaptic and extrasynaptic regions of the hemidiaphragm muscle (Fig. 2B). Quantification of the expression in

innervated mouse hemidiaphragm muscle (Fig. 3) revealed no significant difference in the Gal1-like immunoreactivity in the extrasynaptic region relative to the expression in the perisynaptic region ( $100.0 \pm 9.6$  arbitrary units in the perisynaptic region,  $n = 3$ , and  $112.4 \pm 14.5$  arbitrary units in the extrasynaptic region,  $n = 3$ ). Negative controls using only secondary antibodies showed no bands on Western blots (results not shown).

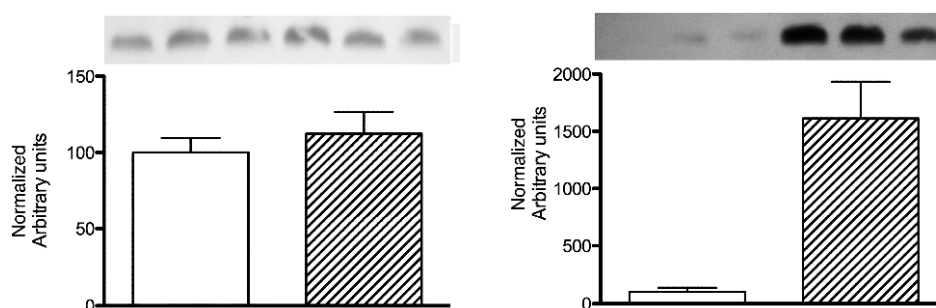


Fig. 3. Gal1-like immunoreactivity in mouse hemidiaphragm muscle. Left – Gal1-like immunoreactivity in the perisynaptic (left three lanes) and extrasynaptic (right three lanes) portions of innervated hemidiaphragm muscle (each lane contains muscle regions pooled from 3 left hemidiaphragms). The columns and bars represent the densitometric quantification of immunoreactivity expressed in arbitrary optical density units normalized to 100.0 in the perisynaptic region (error bars represent SEM,  $n = 3$ ). Right – Gal1-like immunoreactivity in the perisynaptic regions of hemidiaphragm muscle that was innervated (left three lanes) or 6 days post-denervation (right three lanes). Each lane contains muscle regions pooled from 3 left hemidiaphragms). The columns and bars represent the densitometric quantification of immunoreactivity expressed in arbitrary optical density units normalized to 100.0 in innervated muscle (error bars represent SEM,  $n = 3$ ). The amount of protein loaded per lane was 6  $\mu\text{g}$ .

## DISCUSSION

Previous studies have shown that Gal1 expression is up-regulated in motor neurons and Schwann cells after peripheral nerve lesion [7-9]. This study shows that both Gal1 mRNA expression and immunoreactivity, as determined by Northern blot and Western blot experiments, are also up-regulated in adult skeletal muscle within 6 days of denervation. The up-regulation of mRNA and the immunoreactivity on Western blots was observed for both hind-limb and hemidiaphragm muscles, and for extrasynaptic and perisynaptic muscle tissue. Similar results were obtained with mouse and rat hemidiaphragm muscle extracts using both a rabbit antiserum and a goat polyclonal antibody. These results are consistent with the previous microarray and proteomic analyses, which suggested that Gal1 mRNA expression is up-regulated in atrophic skeletal muscle in response to denervation [19, 20], and also in an amyotrophic lateral sclerosis transgenic mouse model [21].

Gal1 has been suggested to play a role in skeletal muscle development [3-6]. During muscle differentiation, Gal1 is secreted and interacts with laminin in the basement membrane [4]. This interaction has been suggested to inhibit myoblast attachment to laminin and hence fusion into myofibers [4]. However, other studies have shown a reduction in the fusion of Gal1 null myoblasts and an impaired regeneration of adult muscle fibers in Gal1 null mutant mice [5, 6]. Consequently, Gal1 has been suggested to enhance skeletal muscle growth during development and adult muscle regeneration [6, 22]. Hind-limb muscles become atrophic early after denervation, whereas hemidiaphragm muscle, as a whole, becomes transiently hypertrophic following denervation [23-25]. Although only some fiber types (oxidative) of the hemidiaphragm muscle become transiently hypertrophic, whereas other fiber types (glycolytic) only atrophy [24, 26], the overall weight of the muscle increases to a maximum 6 to 9 days after denervation [23]. Transient hypertrophy has been suggested to occur due to passive stretching as a result of continued activity in the contralateral innervated hemidiaphragm [23-25], although neurotrophic influences have also been implicated [27, 28]. This study shows increased Gal1 mRNA expression and immunoreactivity in both hind-limb muscle and hemidiaphragm muscle at early and late stages following denervation. The Gal1 response, therefore, would not seem to be strictly correlated to muscle atrophy or hypertrophy. In adult skeletal muscle, Gal1 is highly expressed in the extracellular matrix [3] and our immunohistochemical experiments showed a prominent staining of the extracellular matrix in innervated rat hemidiaphragm and anterior tibial muscles, and in those 6 and 21 days post-denervation (results not shown). This is consistent with previous results obtained for innervated muscle with the same rabbit antiserum against rat Gal1 [3].

In the neuromuscular system, Gal1 has been suggested to play a role in the degeneration of neuronal processes and/or in peripheral nerve regeneration. Thus, in Gal1 null mutant mice, elimination of nerve endings was delayed at neuromuscular junctions following sciatic nerve section [8], and the rate of functional recovery of whisking movement was reduced after facial nerve crush [7]. Oxidized Gal1 has also been shown to promote neurite outgrowth after sciatic nerve axotomy [11], and injecting oxidized Gal1 into skeletal muscle in an amyotrophic lateral sclerosis transgenic mouse model was shown to delay the onset of disease and to prolong survival [29]. Gal1 may act on neuronal processes in a number of different ways, and some of the effects may also be concentration dependent. Gal1 exists in a reversible monomer-dimer equilibrium with a dissociation constant of about 7  $\mu\text{M}$ , and both forms can bind carbohydrate ligands [30]. Under non-reducing conditions, free Gal1 becomes oxidized and loses its carbohydrate-binding properties with a half-life of 6 to 10 hours. However, Gal1 bound to glycoprotein ligands is highly stable also in the absence of reducing agents [30]. The effects of exogenously applied Gal1 on various cell types vary with concentration and oxidation status. Thus, high concentrations ( $\mu\text{M}$ ) inhibit the proliferation of certain cells, while low

concentrations (nM) are mitogenic [31, 32]. With regard to neuronal degeneration/regeneration, relatively high concentrations of reduced Gal1 (about 0.2  $\mu$ M) cause the degeneration of neuronal processes [8], whereas much lower concentrations (pM) of oxidized Gal1 promote neurite outgrowth after sciatic nerve axotomy [11]. A promotion of axonal regeneration was also shown with low concentrations of Gal1 $\beta$ , a natural monomeric form of Gal1 lacking six amino-terminal residues [33]. Oxidized Gal1 has been suggested to bind to macrophages and stimulate them to release some factor which promotes axon outgrowth and Schwann cell migration [11, 12]. However, recent studies have also identified Gal1 as a novel ligand of neuropilin-1 [34]. Neuropilin-1 acts as a receptor for both class 3 semaphorins and vascular endothelial growth factor [35], two factors of known importance for motor neuron guidance and survival [36, 37]. Thus, Gal1 may act on motor neurons in a number of different ways. The results of this study, showing increased Gal1 expression in the extrasynaptic and perisynaptic regions of denervated skeletal muscle, are compatible with a role for Gal1 in facilitating reinnervation of denervated skeletal muscle.

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