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Heparan sulfates in skeletal muscle development and physiology
Cover illustration: False color light micrograph of a differentiated skeletal muscle culture (depicted in blue), merged with a 488 nm image of the same area (depicted in purple), indicating the expression of enhanced green fluorescent protein (EGFP) in transfected myotubes (9).

Flap front: Possible modifications to the heparosan backbone.

Flap back: Cloning of the scFv antibody-encoding DNA in a prokaryotic expression vector for the production of antibodies (upper panels) or in a eukaryotic expression vector for endogenous production of intrabodies (lower panels).

Heparan sulfates in skeletal muscle development and physiology
G.J. Jenniskens. Thesis University of Nijmegen
-With Ref. -With summary in Dutch

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Heparan sulfates in skeletal muscle development and physiology

Een wetenschappelijke proeve
op het gebied van de
Medische Wetenschappen

Proefschrift

ter verkrijging van de graad van doctor aan de Katholieke Universiteit Nijmegen,
on gezag van de Rector Magnificus Prof. Dr. C.W.P.M. Blom,
volgens besluit van het College van Decanen in het openbaar te verdedigen
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te Roermond
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Chapter 1

General Introduction

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Submitted.
For centuries, it has been recognized that skeletal muscle effectuates positioning and movement in multicellular organisms. Initial studies focused on tissue structure and histology, later studies on metabolism and electrophysiology. Nowadays, sophisticated research tools such as NMR, video speed confocal imaging of intracellular ion fluxes, and recombinant DNA techniques enable scientists to intervene in the cellular physiology and learn about muscle functioning at the cellular and subcellular level. In recent years, there has been an emerging interest in the composition of the extracellular matrix (ECM) and in the developmental and physiological roles of its constituents. In this review, we will focus on a single class of ECM-resident molecules and their emerging roles in muscle development and physiology: heparan sulfate proteoglycans. The emphasis will be on the structure and significance of the heparan sulfate moiety.

**Skeletal muscle tissue**

In higher organisms, three distinct types of muscle tissue can be distinguished: 1) **smooth muscle**, which is involuntary, 2) **cardiac muscle**, which is also involuntary and forms most of the heart, and 3) **skeletal muscle**, which owes its name to its attachment to bones and its important role in the positioning and the voluntary (somatic) movement of the skeleton and which is also an important player in breathing (involuntary; diaphragm).

**Morphology and physiology**

Attachment of skeletal muscle to bony structures is achieved by tendons, which are continuous with a sheet of connective tissue surrounding the muscle, the epimysium. Skeletal muscle is build up of hundreds to thousands of long cylindrical cells, called muscle fibers or myofibers, which are parallel ordered in fascicles (Fig. 1). A sheet of connective tissue, called the perimysium, which is continuous with the epimysium, surrounds the fascicles. The endomysium surrounds the separate myofibers. The sum of the connective tissue located between myofibers is often called interstitium, whereas the endomysial component that lies closest to the myofiber surface is referred to as basal lamina (BL; Sanes, 1986; see further). The cell membrane surrounding a myofiber is called the sarcolemma.
Figure 1. Connective tissue of skeletal muscle. (a) Tendons that are connected to bones are continuous with the connective tissue of skeletal muscle. (b) The connective tissue is organized in three continuous layers: the epimysium encircles the whole muscle, the perimysium surrounds bundles of 10-100 individual myofibers, so called fascicles, and the endomysium separates individual myofibers from one another. Blood vessels and motor neurons are located within the perimysium and divide into a network of capillaries and nerve branches extending into the endomysium (Adapted from Torotora GJ and Reynolds Garabowski S (1996), Principles of anatomy and physiology, Harper Collins, New York. (with permission)).

Myofibrils

Myofibrils, the contractile elements of striated muscle, are 1-2 μm in diameter and are build up of thin filaments (actin complexed with troponin and tropomyosin) and thick filaments (myosin), which are arranged in sarcomeres, the basic functional units of muscle contraction (Fig. 2). Sarcomeres are bordered by Z discs, narrow areas of dense material, in which actin filaments are anchored in a regular pattern. Myosin filaments are orderly located between the actin filaments, overlapping with two opposite arrays of actin filaments to a greater or lesser extent (depending on the contractile state of the muscle). The regular arrangement of both types of filaments is causal for the striated appearance of skeletal muscle in microscopy. A darker area within each sarcomere, called the A band, consists mostly of the myosin filaments but also includes portions of the actin filaments. Alternating with A bands are the I bands, regions of lesser density which contain the rest of the actin filaments as well as the Z disc. In the center of the A band, the H zone represents the region where only myosin but no actin filaments are located. The M line is formed by proteins that connect adjacent myosin fibers and divides the H zone in two.
Figure 2. Organization of skeletal muscle. (a) Individual myofibers (muscle cells) are composed of multiple myofibrils and surrounded by the sarcolemma. (b) Myofibrils contain thin (actin) filaments and thick (myosin) filaments, arranged in sarcomeres, the basic contractile elements of skeletal muscle. (c) Narrow regions of dense material, called Z disks, separate sarcomeres. Actin filaments are anchored into the Z discs in a regular pattern, and overlap partially with myosin filaments. The alternating density of the highly arranged molecules is causal for the striated appearance of skeletal muscle. Dark A bands, consisting of the myosin filaments and parts of the actin filaments, alternate with I bands, which are composed of actin filaments and the Z disc. The H zone within the A band covers the region where only myosin filaments are located, and the M line comprises of proteins that connect adjacent myosin filaments. (Adapted from Torotora GJ and Reynolds Garabowski S (1996), Principles of anatomy and physiology, Harper Collins, New York. (with permission)).
Muscle contraction

Actin and myosin filaments function by shortening the distance between the Z disks (i.e. contraction) of a sarcomere, according to the sliding filament theory, first described in the mid-1950s (Huxley, 2000). In short, an increase in the sarcoplasmic calcium (Ca²⁺) concentration causes a change of shape in the troponin-tropomyosin complex, enabling myosin to bind to actin. Under the release of ADP, myosin undergoes a conformational change drawing the thick filament towards the H zone (power stroke). Next, ATP binds to myosin, causing it to detach from the actin filament. The subsequent hydrolysis of ATP to ADP reorients myosin to its original state, enabling a second power stroke if ATP is available and the sarcoplasmic Ca²⁺ concentration remains high. The sliding of actin and myosin filaments causes the shortening of sarcomeres, which in turn leads to the shortening of the muscle fibers and ultimately to the contraction of the muscle.

Neuromuscular junction

Originally, the trigger for muscle contraction comes from motor neurons, which communicate with muscle fibers through specialized regions called neuromuscular junctions (NMJs; Fig. 3). At NMJs, the axon terminal of a single branch of an innervating motor neuron contacts a single myofiber. Both cells show specializations at these sites of synaptic contact (Engel, 1994). The axon terminal is divided into a cluster of synaptic end bulbs, which contain large numbers of membrane vesicles filled with the neurotransmitter acetylcholine (ACh). When a nerve action potential reaches the synaptic end bulbs, ACh is secreted by fusion of the synaptic vesicles with the plasma membrane. ACh diffuses over the 50 nm wide synaptic cleft, which is filled with the synaptic BL (sBL; see below), between the nerve terminal and the facing muscle sarcolemma where it binds to acetylcholine receptors (AChRs). AChRs are large ACh-binding cation channels inserted in the postsynaptic sarcolemma on top of surface-increasing invaginations, the junctional folds. Upon binding of ACh, the AChR channel opens which leads to an influx of Na⁺ ions along its electrochemical gradient, creating an endplate potential (EPP; Fig. 4). Voltage gated Na⁺ channels (vgNa⁺Chs), present in the postsynaptic sarcolemma at the troughs of the junctional folds, sense the EPP and open to increase the Na⁺ influx thus causing an action potential (AP). Next, the AP travels along the sarcolemma. The ACh released in the synaptic cleft is quickly degraded by acetylcholine esterase (AChE), an enzyme located specifically within the sBL.
**Figure 3.** Skeletal muscle innervation and the neuromuscular junction. (a) Axon terminals of innervating motor neurons contact individual myofibers in specialized regions called synapses. (b) Here, synaptic end bulbs, filled with acetylcholine (ACh)-containing synaptic vesicles, which can fuse with the presynaptic membrane, lay in shallow gutters formed by the muscle membrane. (c) The postsynaptic membrane contains many invaginations, junctional folds, on top of which acetylcholine receptors (AChRs) are located. Upon arrival of a neural action potential, ACh is released by exocytosis and diffuses over the synaptic cleft, where it binds to AChRs in the motor end plate. (Adapted from Torotora GJ and Reynolds Garabowski S (1996), Principles of anatomy and physiology, Harper Collins, New York. (with permission)).

**Sarcoplasmic reticulum and transverse tubules**

Each myofibril is encircled by an elaborate tubular membrane system called the sarcoplasmic reticulum (SR; Fig. 5; Franzini-Armstrong, 1972). The SR is similar to the smooth endoplasmic reticulum of nonmuscle cells, and primarily functions as a Ca$^{2+}$ store. Highly specialized ion pumps and ion channels in the SR membrane maintain a Ca$^{2+}$ gradient such, that the cytosolic Ca$^{2+}$ concentration ($[\text{Ca}^{2+}]_c$) is low (less than 10$^{-7}$M) when the muscle is in its relaxed state (Berchtold et al., 2000). In
Figure 4. Excitation-contraction coupling in skeletal muscle. Upon binding of ACh, AChRs open which leads to an influx of Na⁺ ions, creating a local endplate potential (EPP). Subsequently, voltage-gated Na⁺ channels (VgNa⁺Chs), present in the postsynaptic sarcolemma at the bottoms of the junctional folds, open which increases the Na⁺ influx to form an action potential (AP). The AP travels along the sarcolemma to trigger dihydropyridine receptors (DHPRs) present in the T-tubule system, which in turn activate ryanodin receptors (RyRs) in the sarcoplasmic reticulum (SR). This activation leads to the release of Ca²⁺ into the cytosol, which causes muscle contraction through the sliding of myosin filaments over actin filaments. Sarcoplasmic reticulum calcium ATPases (SERCAs), present in the SR membrane, pump Ca²⁺ back into the SR, which restores the muscle resting potential.

In mammalian muscles, two spatially distinct forms of SR are present: the longitudinal SR extends the full length of the A band, whereas the fenestrated SR is oriented along the I band. At each A-I band junction, tubular invaginations of the sarcolemma, called transverse tubules (T-tubules), penetrate transversely towards the center of the myofiber. The inside of T-tubules is continuous with the extracellular environment and filled with extracellular fluid and ECM (Davis and Carlson, 1994).
Figure 5. Skeletal muscle architecture: the sarcoplasmic reticulum, T-tubules, and the triad. Myofibrils (1) are encircled by the SR (2), an elaborate membrane system that connects to tubular invaginations in the sarcolemma present at the A-I band junction, the transverse tubules (T-tubules; 5, T). The terminal cisternae of the SR (3) together with the T-tubule membrane system are called triads (4), two of which are present per sarcomere. The SR over the I band is called the fenestrated SR, whereas the SR over the A band is called the longitudinal SR. 6, sarcolemma; 7, basal lamina; 8, collagen fibrils (From Krstic RV (1979), General histology of the mammal, Springer-Verlag, New York. (with permission)).

Excitation-contraction coupling

Although both membrane systems are physically separated, highly specialized proteins enable the conversion of a sarcolemmal AP to SR Ca\(^{2+}\) release into the cytosol (Fig. 4). Dihydropyridine receptors (DHPRs) that are present in the T-tubular membrane are L-type Ca\(^{2+}\) channels containing a voltage sensor. Opposite to DHPRs, ryanodine receptors (RyRs, large tetrameric Ca\(^{2+}\) channels) are located in the SR membrane. When an AP travels along the sarcolemma of the T-tubule system, the...
voltage sensor in the DHPR is activated, which causes the protein to undergo a conformational change. The sarcoplasmic loop between DHPR transmembrane domains 2 and 3 interacts with the large sarcoplasmic foot-domain of the RyR (Tanabe et al., 1990; El-Hayek et al., 1995; Nakai et al., 1998), causing this Ca\textsuperscript{2+} channel to open and release large amounts of Ca\textsuperscript{2+} into the cytosol, leading to muscle contraction. Upon release, Ca\textsuperscript{2+} is rapidly pumped back into the SR by sarcoplasmic reticulum Ca\textsuperscript{2+} ATPases (SERCA\textsubscript{s}). This cascade of AP-induced Ca\textsuperscript{2+} release and re-uptake, which leads to temporal muscle contraction, is called excitation-contraction (e-c) coupling.

Skeletal muscle: development

Skeletal muscle tissue is derived from progenitor cells present in the embryonic mesenchyme of the somites (Fig. 6; Grinnel, 1995; Buckingham 2001). Somites arise at an early stage of development by segmentation of the paraxial mesoderm, lateral to the neural groove and develop to form the myotome. Myotome cells, or presumptive myoblasts, are vigorously proliferating spindle-shaped cells, which have the capacity to differentiate into myoblasts. Myoblasts are capable of fusion to form syncytial myotubes, the precursors of myofibers.

**Figure 6.** Early events in myogenesis and synaptogenesis. (a) Myogenic precursor cells arise from the somite, motor axons from somata in the neural tube, and Schwann cells from the neural crest. (b) Once these cells reach sites of muscles, myoblasts fuse to form myotubes that are innervated by motor axons, which are then surrounded by Schwann cells. (c) Initial contacts are unspecialized yet capable of rudimentary transmission. (d) As development proceeds, growth cones differentiate into nerve terminals, and AChRs accumulate in the postsynaptic membrane. (e) By birth, the NMJ is fully functional. (From Sanes and Lichtman, 1999 (with permission)).
Myogenesis

During embryogenesis in mouse (Mus musculus) and rat (Rattus norvegicus), somites develop from approximately embryonic day 8 (E8; Hogan, 1994). Organogenesis takes place from E10 until E14, followed by fetal growth and development until birth at approximately E20. At E12, muscle primordia already contain some multinucleate primary myotubes surrounded by myoblasts. Primary myotubes are elongated by myoblast fusion during two days, after which secondary myotubes are formed on the scaffold of primary myotubes between E14 and E16 (Kelly and Zacks, 1969; Rubinstein and Kelly, 1981). Extracellular material is deposited on the myotube surface, beginning on E14, to form a continuous layer by E19 (Nakajima et al., 1980; Chiu and Sanes, 1984). The systems that specify myogenic identity are only beginning to be unraveled (Buckingham, 2001). Spatiotemporal expression of myogenic determination factors such as myogenin, Myf5, and MRF5 and family members of the MyoD, Wnt, and Pax families, together with the presence of signaling molecules such as growth factors (e.g. IGF, FGF-2, FGF-4, and TGF-β), coordinate the proliferation and differentiation of myogenic cells (Rudnicki and Jaenisch, 1995; Buckingham, 2001).

Synaptogenesis

Motor nerves reach muscle primordia during the formation of primary myotubes, and innervate most of them, at about E12. Spontaneous quantal release of ACh precedes synaptic differentiation and the resulting electrical activity is shown to be important in differentiation and mononeuronal innervation of the myofiber and NMJ morphology (Grinnell, 1995; Busetto et al., 2000; Bezakova and Lømo, 2001). The nerve and the muscle each contribute to the synaptic differentiation on both the presynaptic and the postsynaptic side (Sanes et al., 1998b; Lin et al., 2001). Specializations of the synaptic region include: 1) the clustering of AChRs in the motor endplate, which can be detected from E14, and replacement of the embryonic γ-subunit of the AChR by the mature ε-subunit 2) the enlargement of the nerve-muscle contact area, 3) the coverage of the nerve terminal by Schwann cells, 4) the widening of the synaptic cleft and the formation of a sBL, 5) the formation of dense zones, 6) the accumulation of AChE in the sBL, soon after the onset of AChR clustering and upon synaptic activity, and 7) the development of 1 μm deep junctional folds in the postsynaptic membrane. Together these changes culminate in functional neuromuscular junctions with all pre- and postsynaptic specializations a few days postnatal. (Chiu and Sanes, 1984; Hall and Sanes, 1993; Grinnell, 1995; Burden, 1998; Meier and Wallace, 1998; Sanes and Lichtman, 1999; 2001).
Satellite cells

Of special interest is the regenerative capacity of muscle satellite cells that are present between the BL and the sarcolemma of myofibers. These undifferentiated multipotent muscle precursor cells mediate the postnatal growth and regeneration of muscle and are also activated in response to muscle exercise or trauma. Upon activation, satellite cells undergo multiple rounds of division prior to fusing with existing myofibers or regeneration of injured muscle. Although the molecular mechanisms that regulate the activation and function of satellite cells are elusive, it is clear that factors, which are also involved in myogenesis, play important roles (Bischoff, 1994; Seale and Rudnicki, 2000; Hawke and Garry, 2001).

Extracellular matrix

The extracellular connective tissue of muscle is organized into three discrete but mutually continuous sheets: the epimysium, the perimysium, and the endomysium. Muscle spindles, blood vessels, nerves, and fat cells are located within the perimysium. Here, blood vessels and nerves divide into a rich network of capillaries and nerve branches extending into the endomysium, which also contains fibroblasts, mast cells, and macrophages. The BL (see below) is a specialized sub-structure of the ECM, which lies close to the cell membrane. A sheet-like 50-100 nm thick BL, into which the extracellular domains of cell surface proteins may extend, completely encompasses muscle fibers.

The BL is a macromolecular network, which provides a structural and regulatory environment for cells. The BL is composed of several collagenous glycoproteins, noncollagenous glycoproteins, and proteoglycans (Table 1; Wight, 1991; Hall and Sanes, 1993; Kannus et al., 1998; Meier and Wallace, 1998; Sanes and Lichtman, 1999). Whereas collagens are the major structural components of BL, noncollagenous glycoproteins, e.g. laminins, fibronectin, and tenascin mediate the adhesion of several cell types to collagenous substrates. Proteoglycans are major structural and regulatory components of the BL but also of the cell surface. These components will be elaborated upon further in this text.

Basal lamina

The BL of skeletal muscle contains significant amounts of collagen and laminin, that self-assemble to form supramolecular networks. These networks are interconnected by a large number of interactions with BL-resident proteins, notably entactin (nidogen) and proteoglycans. Cells
### Table 1. Components of the basal lamina and cell surface of muscle cells and their location

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Location</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetylcholinesterase</td>
<td>s, BL</td>
<td>McMahan et al., 1978</td>
</tr>
<tr>
<td>Acetylcholine receptor</td>
<td>s, Cell S</td>
<td>Salpeter and Loring, 1985</td>
</tr>
<tr>
<td>Carbohydrates (Lectin specific)</td>
<td>s, Cell S</td>
<td>Sanes and Cheny, 1982</td>
</tr>
<tr>
<td>Chondroitin sulfate proteoglycan</td>
<td>c, BL</td>
<td>Carrio and Caplan, 1982</td>
</tr>
<tr>
<td>Collagen IV (α1 and α2)</td>
<td>c, BL</td>
<td>Sanes et al., 1990</td>
</tr>
<tr>
<td>Collagen IV (α3, α4, and α5)</td>
<td>s, BL</td>
<td>Miner and Sanes, 1994</td>
</tr>
<tr>
<td>Collagen V</td>
<td>e, BL</td>
<td>Sanes and Cheny, 1982</td>
</tr>
<tr>
<td>Collagen XIII</td>
<td>c, BL</td>
<td>Sund et al., 2001</td>
</tr>
<tr>
<td>Collagen XVIII*</td>
<td>c, BL</td>
<td>Halfter et al., 1998</td>
</tr>
<tr>
<td>Decorin</td>
<td>c, BL</td>
<td>Andrade and Brandon, 1991</td>
</tr>
<tr>
<td>Dermatan sulfate proteoglycan</td>
<td>c, BL</td>
<td>Eggen et al., 1994</td>
</tr>
<tr>
<td>Dystrophin-glycoprotein complex</td>
<td>c/s, Cell S</td>
<td>Grady et al., 2000</td>
</tr>
<tr>
<td>ErbB2, 3, and 4</td>
<td>s, Cell S</td>
<td>Zhu et al., 1995</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>c, BL</td>
<td>Sanes and Cheny, 1982</td>
</tr>
<tr>
<td>Glypicans*</td>
<td>c, Cell S</td>
<td>Brandan et al., 1996</td>
</tr>
<tr>
<td>Heparan-binding growth associated molecule</td>
<td>s, BL</td>
<td>Szabat and Rauvala, 1996</td>
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<tr>
<td>Heparan sulfate epitopes</td>
<td>c/s, BL</td>
<td>Jenniskens et al., 2000</td>
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<td>Heparan sulfate proteoglycan</td>
<td>s, BL</td>
<td>Anderson and Fambrough, 1983</td>
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<td>Integrin-α3b1</td>
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<td>Cohen et al., 2000</td>
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<td>Integrin-α7(αV 8 β3)</td>
<td>s, Cell S</td>
<td>von der Mark et al., 1991</td>
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<td>Laminin-2 (merosin)</td>
<td>e,n, BL</td>
<td>Patton et al., 1997</td>
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<tr>
<td>Laminin-3 (s-laminin)</td>
<td>s, BL</td>
<td>Hunter et al., 1989</td>
</tr>
<tr>
<td>Laminins-4,-9, and -11</td>
<td>s, BL</td>
<td>Patton et al., 1997</td>
</tr>
<tr>
<td>Laminin-8</td>
<td>n, BL</td>
<td>Patton et al., 1997</td>
</tr>
<tr>
<td>M-cadherin</td>
<td>c/s, Cell S</td>
<td>Cifuentes-Diaz et al., 1996</td>
</tr>
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<td>Muscle-specific kinase (MuSK)</td>
<td>s, Cell S</td>
<td>Valenzuela et al., 1995</td>
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<td>N-acetylgalactosaminyl-terminated carbohydrate</td>
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<td>Scott et al., 1986</td>
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<tr>
<td>N-acetylgalactosaminyl-terminated transferase</td>
<td>s, Cell S</td>
<td>Scott et al., 1990</td>
</tr>
<tr>
<td>Neural agrin*</td>
<td>s, BL</td>
<td>McMahan, 1990</td>
</tr>
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<td>Neural cell adhesion molecule (N-CAM)</td>
<td>s, Cell S</td>
<td>Rieger et al., 1985</td>
</tr>
<tr>
<td>Neurogulin-β3</td>
<td>s, BL</td>
<td>Rimer et al., 1998</td>
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<tr>
<td>Neurogulin-α</td>
<td>s, Coll S</td>
<td>Rimer et al., 1998</td>
</tr>
<tr>
<td>Nexin 1</td>
<td>s, BL</td>
<td>Festoff et al., 1991</td>
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<tr>
<td>Nidogen</td>
<td>c, BL</td>
<td>Lerner and Torchia, 1986</td>
</tr>
<tr>
<td>Porlecain*</td>
<td>c, BL</td>
<td>Iozzo, 1994</td>
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<tr>
<td>S-nidogen</td>
<td>s, BL</td>
<td>Chiu and Ko, 1994</td>
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<td>Syndecan-2*</td>
<td>s, Cell S</td>
<td>Hsueh et al., 1998</td>
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<td>Syndecans*</td>
<td>c, Cell S</td>
<td>Bernfield et al., 1992</td>
</tr>
<tr>
<td>Tenascin-C</td>
<td>c, BL</td>
<td>Matsumoto et al., 1994</td>
</tr>
<tr>
<td>Tenascin-X</td>
<td>c, BL</td>
<td>Matsumoto et al., 1994</td>
</tr>
<tr>
<td>Tenascin-Y</td>
<td>c, BL</td>
<td>Hagiok et al., 1996</td>
</tr>
<tr>
<td>Voltage gated sodium channels</td>
<td>c, Cell S</td>
<td>Lupa and Caldwell, 1991</td>
</tr>
</tbody>
</table>

Due to limitations of space, only a single reference has been cited for each entry.

* Heparan sulfate proteoglycan

Abbreviations: BL, basal lamina; Cell S, cell surface; c, common; c/s, common but concentrated (or different constitution) in synaptic; e, extrasynaptic; n, neural; s, synaptic
are firmly anchored to adjacent BL by many receptor-ligand interactions, like the binding of collagen IV and laminins to integrins, laminin interactions with the dystrophin-glycoprotein complex, and the binding of laminins to cell surface proteoglycans (Fig. 7; Timpl and Brown, 1995; Erickson and Coughman, 2000).

The BL shows structural and functional specializations at sites of interaction with other cells. In the NMJ a sBL, which is continuous with the myofiber BL and with the Schwann cell BL, traverses the synaptic cleft and extends into the junctional folds (Hall and Sanes, 1993). Although morphologically indistinguishable from the extrasynaptic BL to which it is fused, the sBL is biochemically specialized to a high degree. Despite the fact that it only encompasses 0.1% of the myofiber surface, several BL-resident proteins or protein isoforms are concentrated in or are specific for the sBL. These include: neural agrin (McMahan, 1990), specific collagen IV isoforms (Sanes et al., 1990), several laminin isoforms (Hunter et al., 1989; Patton et al., 1997), AChE (McMahan et al., 1978), heparan sulfate proteoglycan (HSPG; Anderson and Fambrough, 1983), a N-acetylglactosaminyl-terminated carbohydrate (Scott et al., 1988), nexin I (Festoff et al., 1991), and entactin (Chiu and Ko, 1994). Other proteins, like some collagen types, are totally absent from the sBL (Sanes and Cheney, 1982a). Only fibronectin and some other isoforms of collagen and laminin are shared (Sanes, 1982; Sanes and Chiu, 1983; Sanes et al., 1990; Miner and Sanes, 1994; Patton et al., 1997). Staining with lectins or with monoclonal antibodies identified several carbohydrates specifically located on the myofiber surface (Sanes and Cheney, 1982b; Iglesias et al., 1992; Martin et al., 1999). Using phage display-derived antibodies against BL-resident heparan sulfate (HS) epitopes, it was shown that HS epitopes are also differentially distributed between synaptic and extrasynaptic BL (Jenniskens et al., 2000).

In recent years, it has become apparent that the BL plays many developmental and physiological roles. The highly regulated spatiotemporal distribution of specific BL-components, contributed by muscle or by the innervating nerve, is thought to create a specific microenvironment. The focal sequestering of biologically active proteins that bind to BL components, like e.g. growth factors, may be causal for processes such as myogenesis (Ontell and Kozeka, 1984; Kardami et al., 1988; Jansen and Fladby, 1990), synaptogenesis (Anderson and Fambrough, 1983; Burden, 1998), and regeneration (Gulati et al., 1983; Caldwell et al., 1990; Husmann et al., 1996; Grounds et al., 1998). Moreover, during regeneration of the NMJ, pre- and postsynaptic specializations re-occur at the site of the original sBL even in absence of the muscle or nerve cell, respectively (Sanes, 1986). The structural integrity of the NMJ is lost when the BL is degraded (Salpeter et al., 1992).
**Figure 7.** Complex interactions between the cell surface and the basal lamina. (a) At the neuromuscular junction, the nerve terminal of a motor neuron lies in a shallow gutter formed by the postsynaptic (muscle) membrane. The BL of the muscle and the nerve are continuous, though different in composition. The postsynaptic membrane contains clusters of AChRs on top of the junctional folds (adapted from Hall and Sanes, 1993 (with permission)). (b) In extrasynaptic areas the dystrophin glycoprotein complex (composed of dystrophin, α-dystroglycan (α), β-dystroglycan (β), sarcospan, sarcoglycans, and MuSK (M)) interacts with laminin(-2 or -8), which in turn interact with e.g. nidogen and collagens. (c) In synaptic areas the dystrophin glycoprotein complex contains utrophin instead of dystrophin and connects with AChRs through rapsyn (R). Agrin (A), a HSPG, interacts with α-dystroglycan, MuSK, and laminin(-3, -4, -9, or -11), which in turn interact with e.g. nidogen, collagens, and the HSPG perlecan, to which AChE is bound (Adapted from Jacobson et al., 2001 (with permission)).

**Collagens**

Collagens form a family of closely related proteins that distinguish themselves from other glycoproteins by a triple-helical segment. In skeletal muscle ECM, several collagen types and isoforms are demonstrated *in situ* (Mayne and Sanderson, 1985; Sanes, 1986; Sund et al., 2001) as well as in cultured myocytes (Beach et al., 1985). Of these proteins, collagen XVIII bares HS chains (Halfter et al., 1998), whereas
collagen types IX and XII are part-time proteoglycans, sometimes having GAG side chains (Erickson and Couchman, 2000). Collagens I and III are major components of the epimysium and the perimysium, whereas collagen V is present in lesser amounts (Mayne and Sanderson, 1985). In the BL, collagens I, III, IV, and V are detected, of which collagen IV is most prominent. Collagen IV isoforms show a differential BL-distribution: α1 and α2 chains are less abundant in the sBL (Sanes et al., 1990), whereas α3 and α4 are unique for, and α5 is strongly enriched in the sBL (Miner and Sanes, 1994). Some proteins, like AChE, contain a collagenous subunit that is responsible for the anchoring of this enzyme in the BL as well as for its synaptic localization (Hall, 1973; Vigny et al., 1983; Inestrosa and Perelman, 1989), and which interacts with heparin (Rossi and Rotundo, 1996; Deprez et al., 2000). Mice that lack perlecans do not cluster AChE in the sBL (Arikawa-Hirasawa et al., 2002). Collagens are indispensable for cell adhesion and play important roles in developmental processes (Lai and Chu, 1996; Velleman, 1999; Sumiyoshi et al., 2001).

Down regulation of collagen isoforms or genetic mutations leading to defective proteins may be causal for muscular dystrophies and congenital myopathies as shown for collagen types I, III, IV, VI, and XIII (Hantai et al., 1985; Lamandé et al., 1998; Kvist et al., 2001).

Laminins

Laminins are a major class of BL proteins, composed of three disulfide-linked subunits, α, β, and γ. Several laminin isoforms, composed of different subunits, show a restricted distribution in BL, especially with regard to the NMJ. Whereas laminin-2 (merosin) is located in the extrasynaptic neural BL, the isoforms -3 (s-laminin), -4, -9, and -11 are specific for the synaptic portion of the BL (Hunter et al., 1989; Patton et al., 1997). During myogenesis and synaptogenesis as well as after denervation, the expression of laminin isoforms is tightly regulated (Bayne et al., 1984; Olwin and Hall, 1985; Sanes et al., 1986; Gullberg et al., 1999). Laminins have been experimentally implicated in myofiber formation (Blanco-Bose and Blau, 2001) and are involved in the adhesion of collagenous substrates to cells through integrins (Gu et al., 1994; Crawley et al., 1997) and through α-dystroglycan (Pall et al., 1996).

Decreased expression of, deficiency for, mutations in, and blocking of laminin isoforms induce several muscular dystrophies and congenital myopathies (Angoli et al., 1997; Miyagoe et al., 1997; Brown et al., 1999; Cohn et al., 1999; Merlín et al., 1999). Moreover, aberrant distribution of several BL-proteins, notably laminin isoforms, is indicative for the diagnosis of several congenital muscular dystrophies in mice and men (Hantai et al., 1985; Tome et al., 1994; Herrmann et al., 1996; Li et al., 1997; Ringelmann et al., 1999; Patton et al., 2001).
**Tenascins**

Another component of BL is the class of tenascins, hexameric proteins that are involved in cell adhesion (Yokosaki et al., 1994) and are present in NMJ regions (Pedrosa-Domellöf et al., 1995). The isoforms tenascin-C and tenascin-X are reciprocally distributed in skeletal muscle (Matsumoto et al., 1994). Whereas tenascin-X and tenascin-Y are shown to be involved in myogenesis (Burch et al., 1995; Hagios et al., 1996; 1999), tenascin-C expression is modulated during muscle healing (Jarvinen et al., 2000) and in muscular dystrophy and inflammatory and congenital myopathies (Settles et al., 1996; Muller-Felber et al., 1998; Ringelmann et al., 1999).

The expression of tenascin-C is correlated with macrophage invasion in Duchenne muscular dystrophy (Gullberg et al., 1997), and its deficiency results in abnormal NMJ formation and reinnervation (Cifuentes-Diaz et al., 1998; 2002).

**Proteoglycans**

Proteoglycans are complex macromolecules consisting of a protein core to which one or more glycosaminoglycan (GAG) moieties are covalently attached. GAGs are linear polymers of repeating disaccharides that contain a hexosamine and either a hexuronic acid or a galactose unit. Different types of GAGs exist, depending on the composition of the basic disaccharide unit (Table 2; Kjellén and Lindahl, 1991; Wright et al., 1991): **Hyaluronic acid** (HA) is the simplest GAG structure, consisting of N-acetyl-D-glucosamine (GlcNAc) and glucuronic acid (GlcA) linked by β1,3 and β1,4 bonds, which can have up to 25,000 disaccharides. HA is the only GAG that is not linked to a protein core.

The **galactosaminoglycans** consist of two types: **Chondroitin sulfates** (CS) have a backbone consisting of N-acetyl-D-galactosamine (GalNAc) and GlcA, linked by β1,3 and β1,4 bonds, of up to 250 disaccharides. Depending on the positions of sulfation of particular GalNAc residues, CS is referred to as chondroitin-4-sulfate or chondroitin-6-sulfate. **Dermatan sulfates** (DS) have a backbone similar to CS, in which individual D-GlcA residues are epimerized to L-iduronic acid (IduA), which in turn may be sulfated at position 2. By convention, the designation of the β1,3 glycoside bond changes to α1,3 upon epimerization.

The **glucosaminoglycans** consist of three types: **Keratan sulfates** (KS) consist of a backbone of alternating GlcNAc and galactose (gal), bound by β1,3 and β1,4 bonds, of up to 80 disaccharides. Sulfation can occur on the 6 position of either sugar residues. **Heparan sulfates** (HS) have a backbone structure that consists of GlcNAc and GlcA, bound by α1,4 and β1,4 linkage, which can be up to 150 disaccharides in length. Upon chain elongation, a large number of modifications occur in the backbone, including N-deacetylation and N-sulfation, C-5 epimerization, 2-O-
sulfation, 3-O-sulfation, and 6-O-sulfation. Due to these modifications, highly sulfated domains alternate with poorly sulfated domains within one HS chain. Heparin (Hep) is structurally similar to HS and has a degree of N-sulfation of usually more than 70% as compared to less than 50% in HS. The term ‘heparin’ is reserved for the GAG component of the small proteoglycan serglycin, which is synthesized by mast cells; all other structurally related GAGs are called ‘heparan sulfate’ (Kjellén and Lindahl, 1991).

Several GAGs are present in skeletal muscle, of which CS, DS, and HS are the most common. The CS/DS proteoglycan decorin, a large DS proteoglycan, and a CS proteoglycan are present throughout the perimysium and endomysium (Carrino and Caplan, 1982; Andrade and Brandan, 1991; Eggen et al., 1994), whereas a specific CS proteoglycan is located within the T-tubular network (Davis and Carlson, 1994). Changes in quantity and intra-muscular distribution of all types of GAGs, most notably of CS, have been reported during myogenesis, regeneration, and in pathological conditions (Watanabe et al., 1986; Bertolotto et al., 1987; Miller et al., 1987; Parthasarathy and Tanzer, 1987; Miller et al., 1991).

Table 2. Various types of glycosaminoglycans

<table>
<thead>
<tr>
<th>GAG</th>
<th>Disaccharide composition</th>
<th># Units</th>
<th>Possible modifications</th>
</tr>
</thead>
<tbody>
<tr>
<td>CS</td>
<td>GlcA(β1,3)GalNAc(β1,4)</td>
<td>250</td>
<td>4- or 6-O-sulfation</td>
</tr>
<tr>
<td>DS</td>
<td>IduA(α1,3)GalNAc(β1,4)</td>
<td>250</td>
<td>2-, 4-, and 6-O-sulfation</td>
</tr>
<tr>
<td></td>
<td>GlcA(β1,3)GalNAc(β1,4)</td>
<td>250</td>
<td>2-, 4-, and 6-O-sulfation</td>
</tr>
<tr>
<td>HA</td>
<td>GlcA(β1,4)GlcNAc(α1,4)</td>
<td>150</td>
<td>N-deacetylation/ N-sulfation (&lt;50%/&gt;70%), CS-epimerization, 2-, 3-, and 6-O-sulfation</td>
</tr>
<tr>
<td>HS/Hep</td>
<td>IduA(α1,4)GlcNAc(α1,4)</td>
<td>150</td>
<td>N-deacetylation/ N-sulfation (&lt;50%/&gt;70%), CS-epimerization, 2-, 3-, and 6-O-sulfation</td>
</tr>
<tr>
<td>KS</td>
<td>gal(β1,4)GlcNAc(β1,3)</td>
<td>80</td>
<td>6-O-sulfation</td>
</tr>
</tbody>
</table>

Gal, D-galactose; GalNAc, N-acetyl-D-galactosamine; GlcA, glucuronic acid; GlcNAc, N-acetyl-D-glucosamine; IduA, L-iduronic acid.

Heparan sulfate proteoglycans

Heparan sulfate proteoglycans (HSPGs) constitute the major portion of PGs in the BL and on the cellular surface of skeletal muscle (Sanes, 1986; Brandan and Inestrosa, 1987) and of cultured myotubes (Noonan et al., 1986; van Kuppevelt et al., 1992). A variety of core proteins exist, to which one or more HS moieties are attached (Fig. 8). Both the protein core and the HS moiety define the structure and function of HSPGs. Binding of various extracellular matrix components or of growth factors can be effectuated by mechanisms depending on the protein core, the HS sequence, or both. Based on the protein core, muscle BL proteoglycans include collagen XVIII (Halfter et al., 1998), perlecanc (Iozzo, 1994), and
agrin (McMahan, 1990). Cell surface proteoglycans include the families of syndecans (Bernfield et al., 1992) and glypicans (Campos et al., 1993). The identification of agrin, a protein indispensable for AChR clustering, as a HSPG (Tsen et al., 1995), the role for perlecan in AChE clustering (Torres and Inestrosa, 1983; Brandan et al., 1985; Arikawa-Hirasawa et al., 2002), and their involvement in muscular diseases showed HSPGs to be major players in developmental and regulatory processes in muscle.

Agrin

The BL-resident HSPG agrin encompasses a core protein of 220-250 kDa, containing two GAG attachment sites (Tsen et al., 1995). Neural agrin is a key factor in the clustering and expression of AChRs in the postsynaptic membrane (Fallon et al., 1985; Lupa and Caldwell, 1991; Ferns et al., 1993; Hoch et al., 1994a; 1994b; Gesemann et al., 1995; Denzer et al., 1997b; Pun et al., 1997; Meier et al., 1998a; 1998b). Agrin is also involved in AChE clustering (Wallace et al., 1985; Ruegg and Bixby, 1998) and in the clustering of vgNa' Chs (Lupa and Cadwell, 1991; Sharp and Cadwell, 1996). Several alternatively spliced isoforms of the protein are described (Ferns et al., 1992, 1993; McMahan et al., 1992; Ruegg et al., 1992). The expression of these isoforms is developmentally regulated (Hoch et al., 1993) and their distribution is highly specific, especially with respect to...
the synaptic site (Groffen et al., 1998; Sanes et al., 1998a; Burgess et al., 1999; Hagiwara and Fallon, 2001). Certain domains in neural agrin isoforms are capable of inducing postsynaptic differentiation (Reist et al., 1992; Cohen et al., 1997; Meier et al., 1997; 1998a; Godfrey et al., 2000). Neural agrin also controls the stability of postsynaptic specializations (Bezakova et al., 2001), whereas muscle-derived agrin regulates the organization of cytoskeletal proteins and AChR aggregates (Wallace, 1995; Bezakova and Lømo, 2001).

Agrin interacts with growth factors and with several BL and cell surface proteins, among which collagen type IV, laminin isoforms, tenascin, and the dystrophin-glycoprotein complex (Campanelli et al., 1994; Gee et al., 1994; O'Toole et al., 1996; Cotman et al., 1999). AChRs are bound to this complex through rapsyn (Apel et al., 1995; Cartaud et al., 1998; Fuhrer et al., 1999; Ramarao et al., 2001), a protein that inhibits agrin-induced AChR clustering when it is over expressed (Han et al., 1999). At the NMJ, the interaction of agrin with the MuSK receptor tyrosine kinase complex is necessary for its functioning (Glass et al., 1996; Glass and Yancopoulos, 1997; Herbst and Burden, 2000). The importance of these interactions is shown in mice knock out for agrin, MuSK, or rapsyn, which all have severely disrupted NMJs (Gautam et al., 1996; 1999). Laminin and integrin are also involved in the regulation of AChR clustering by agrin (Denzer et al., 1997a; Martin and Sanes, 1997; Burkin et al., 2000), whereas heparin and HS inhibit agrin-induced AChR clustering (Wallace, 1990; Hopf and Hoch, 1997; Sanes et al., 1998a).

Perlecan

Perlecan was the first BL-HSPG identified (Hassell et al., 1980). Its 270-467 kDa core protein carries three HS chains at its N-terminal domain that may contribute up to approximately 50% of the total molecular mass (Costell et al., 1997; Noonan et al., 1991). Perlecan is present in virtually all BLs, and its interactions with several BL-resident proteins such as type IV collagen (Laurie et al., 1986; Villar et al., 1999), fibronectin (Here-mans et al., 1990), and laminins (Battaglia et al., 1992; Henry et al., 2001) play an important role in the assembly and integrity of the BL (Costell et al., 1999). During myogenesis, the expression of perlecan is down regulated (Larrafn et al., 1997a). At the NMJ, perlecan is involved in the anchorage of AChE in the sBL via its collagen tail (Torres and Inestrosa, 1983; Brandan et al., 1985; von Bernhardi and Inestrosa, 1990; Peng et al., 1999; Arikawa-Hirasawa et al., 2002), in association with dystroglycan (Peng et al., 1998; 1999). Splice variants, in which multiple modules may be absent, have been detected in human, mouse, and in the nematode Caenorhabditis elegans (Iozzo, 1994).
Collagen XVIII

Collagen XVIII is the only collagen type shown to be a HSPG (Halfter et al., 1998). The 180 kDa protein contains ten triple-helical domains, which are variable in length. A C-terminal endostatin domain, which may have anti-angiogenic and tumour-suppressor activity, interacts with several BL proteins among which laminin-1 and perlecan (Sasaki et al., 1998). Three variant forms of collagen XVIII are described in mice and two in human, all of which are expressed in a tissue specific manner (Muragaki et al., 1995; Rehn and Pihlajaniemi, 1995; Saarela et al., 1998).

Glypicans

Glypicans are cell surface HSPGs that are anchored to the extracellular face of the plasma membrane via glycosyl phosphatidylinositol. The core proteins have a largely globular structure with HS chains attached proximal to the plasma membrane (Filmus and Selleck, 2001). Originally found in intestine and lung, glypican was shown to be present at the surface of skeletal muscle cells (Campos et al., 1993). Changes in the expression of various glypican isoforms during development were reported in various organs among which skeletal muscle (Brandan et al., 1996; Saunders et al., 1997; Litwack et al., 1998). Although a role in myogenesis is not yet reported, it is well established that glypicans can regulate the activity of growth and survival factors (Lander and Selleck, 2000; Filmus and Selleck, 2001). Six isoforms of glypican are reported in mammals, whereas only one ortholog (dally) has been described in Drosophila melanogaster (Bernfield et al., 1999; Tumova et al., 2000).

Syndecans

Syndecans are transmembrane proteins with three HS chains attached to the N-terminus, distal from the plasma membrane. Whereas some syndecans also contain CS chains, the optimal biological activity of syndecan-1 is dependent on the presence of all three HS chains (Langford et al., 1998). The four members of this family (syndecan 1-4) have highly related cytoplasmic and transmembrane domains, but differ significantly in their extracellular domains, except for the location and number of HS attachment sites (Bernfield et al., 1992). Syndecan isoforms are differentially expressed during development and in mature tissues (Bernfield et al., 1993; Larraín et al., 1997b; Sogos et al., 1998; Olguín and Brandan, 2001). By binding of components of the cellular microenvironment to their GAG chains, and because of their attachment to the cytoskeleton, syndecans are major players in the control of cell morphology, adhesion and migration (Chernousov and Carey 1993; Liu et al., 1998; Woods et al., 1998). The regulatory roles of syndecans, by binding of growth factors, are well documented (Carey, 1997). In skeletal...
muscle, roles of syndecans include the inhibition of myoblast differentiation by syndecan-1 through a basic fibroblast growth factor-dependent mechanism (Larrain et al., 1998), the involvement of syndecan-3 and -4 in satellite cell maintenance and muscle regeneration (Cornelison et al., 2001), the involvement of syndecan-2 in neural synaptogenesis (Hsueh et al., 1998), and the acceleration of myogenesis after syndecan-3 inhibition (Fuentealba et al., 1999).

Figure 9. Biosynthesis of heparosan and modification to HS. (a) The biosynthesis of heparosan occurs on a GAG linker tetrasaccharide (GlcA(β1,3)Gal(β1,3)Gal(β1,4)Xylβ1-O-), which is attached to specific serine residues in the protein. The linker tetrasaccharide is synthesized by the stepwise addition by the respective glycosyltransferases of xylose, two galactose molecules, and glucuronic acid, followed by the addition of the first N-acetylglucosamine residue by GlcNAc transferase I. Next, HS co-polymerase, which has both GlcA transferase and GlcNAc transferase activity, synthesizes

N-Deacylation/ N-Sulfation

C5-Epimerization

2-O-Sulfation

6-O-Sulfation

3-O-Sulfation
the repeating disaccharides \([-4\text{GlcNAc}(\alpha 1-4)\text{GlcA}\beta 1-1, n]\) by alternative addition of \(\beta\)-GlcA and \(\alpha\)-GlcNAc molecules. XylT, xylosyltransferase; GalT-1, galactosyltransferase I; GalT-2, galactosyltransferase II; GlcAT-1, glucuronyltransferase I; GlcNAcT-1, GlcNAc transferase I (adapted from Sugahara and Kitagawa, 2000 (with permission)). (b) The bare HS-backbone can subsequently be modified by a series of enzymes. \(N\)-deacetylation and \(N\)-sulfation is performed by glucosaminyl \(N\)-deacetylase/\(N\)-sulfotransferase, C5-epimerization by D-glucuronyl C5-epimerase, 2-O-sulfation by heparan sulfate 2-sulfotransferase, 3-O-sulfation by 3-O-sulfotransferase, and 6-O-sulfation by heparan sulfate 6-O-sulfotransferase (adapted from Lindahl et al., 1994 (with permission)).

**Biosynthesis of HS**

The proposed pathway of HS biosynthesis consists of a series of reactions catalyzed by various enzymes, each responsible for a single step (Fig. 9; Lindahl et al., 1998; Selleck, 2000; Sugahara and Kitagawa, 2000).

**Biosynthesis of the HS backbone (heparosan)**

First, a linker tetrasaccharide is \(O\)-linked to the serine residue of a consensus linkage region, consisting of Ser-Gly-X-Gly (\(X\) represents any amino acid; Bourdon et al., 1987; Dolan et al., 1997) or Ser-Gly-Asp (Dolan et al., 1997; Costell et al., 1997). It was shown that the proximal occurrence of Ser-Gly repeats (Zhang et al., 1995) as well as clusters of acidic amino acids (Zhang and Esko, 1994) favors glycosylation of a core protein with HS. The linker tetrasaccharide \((\text{GlcA}(\beta 1,3)\text{Gal}(\beta 1,3)\text{Gal}(\beta 1,4)\text{Xyl}\beta 1-O-Ser})\) is formed through the stepwise addition of each monosaccharide residue by the respective specific glycosyltransferase, and is followed by the addition of the first GlcNAc residue by GlcNAc transferase I (Sugahara and Kitagawa, 2000; Selleck, 2000). Next, the repeating disaccharides \([-4\text{GlcA}\beta 1-4\text{GlcNAc}\alpha 1-1, n]\) are added by HS copolymerase, an enzyme with both \(\alpha\)-GlcNAc transferase and \(\beta\)-GlcA transferase activity, which alternatively adds GlcNAc and GlcA.

**Modification of heparosan to HS**

The bare HS backbone (heparosan) is subsequently modified by a series of enzymes that convert it into an active HS molecule with functional domains, containing unique sequences of GlcA and IduA, and of \(N\)-acetyl, \(N\)-sulfate, and \(O\)-sulfate groups at various positions in various combinations (Habuchi, 2000; Selleck, 2000; Esko and Lindahl, 2001).
1) The first modifications of the polysaccharide are catalysed by the bifunctional enzyme glucosaminyl N-deacetylase/N-sulfotransferase (NDST). NDST functions by N-deacetylation and N-sulfation of selected GlcNAc residues, which is a prerequisite for all other modifications. Four isoforms of NDST have been cloned (Hashimoto et al., 1992; Eriksson et al., 1994; Orellana et al., 1994; Aikawa and Esko, 1999). NDST-1 and NDST-2 have a wide tissue distribution (Humphries et al., 1997; 1998; Kusche-Gullberg et al., 1998; Aikawa et al., 2001), whereas NDST-3 and NDST-4 display a more restricted expression. Disruption (Fan et al., 2000; Ringvall et al., 2000) or decreased expression (Aikawa and Esko, 1999) of the gene encoding NDST-1 results in significantly lower N-sulfation levels of HS. Mice defective for NDST-1 die neonatally because of respiratory distress. NDST-2-deficient mice show no obvious HS defects, but instead are unable to synthesize fully sulfated heparin (Humphries et al., 1999; Forsberg et al., 1999). These results indicate the importance of NDST-1 and NDST-2 in HS and heparin synthesis, respectively.

2) C-5 epimerization of D-GlcA to L-IduA, performed by the enzyme D-glucuronyl C5-epimerase, is the second modification of the HS backbone (Crawford et al., 2001; Li et al., 2001a). No isoforms are known for this enzyme.

3) 2-O-sulfation of uronic acid residues (IduA or GlcA) is conducted by heparan sulfate 2-sulfotransferase (HS2ST), as was shown by studies of the purified (Kobayashi et al., 1996) and cloned (Kobayashi et al., 1997) enzyme, as well as in a CHO cell mutant, pgsF-17, defective in 2-O-sulfation (Bai and Esko, 1996). Only one gene encoding HS2ST has been identified in mammals. Mice defective for this enzyme show renal agenesis and die neonatally (Bullock et al., 1998).

4) 3-O-sulfation of glucosamine residues is catalyzed by heparan sulfate glucosaminyl 3-O-sulfotransferase (3-OST), as can be concluded from experiments with the purified enzyme (Liu et al., 1996) and the cloned 3-OST-1 (Schworak et al., 1997). Four additionally cloned isoforms (3-OST-2, -3A, -3B, and -4; Schworak et al., 1999) are shown to be differentially expressed in various tissues.

5) 6-O-sulfation of glucosamine residues is performed by heparan sulfate 6-O-sulfotransferase (HS6ST), an enzyme that has been purified (Habuchi et al., 1995) and cloned (Habuchi et al., 1998), and of which three isoforms are reported (HS6ST-1, -2, and -3; Habuchi et al., 2000). The expression of these isoforms is tissue-specific and each isoform has different substrate specificity, suggesting an important role for this enzyme in the synthesis of tissue-specific HS epitopes.

The large number of modifications in its backbone potentially makes HS a very information-dense biopolymer, to which several proteins may bind in a monosaccharide sequence-specific way (Salmivirta et al., 1996; Lindahl et al., 1998; Bernfield et al., 1999; Tumova et al., 2000; Turnbull et al., 2001; Gallagher, 2001).
Physiological roles of HS

A large number of unique HS sequences are formed due to the variety in tissue-expression profile and substrate specificity of the various glycosyltransferase and sulfotransferase isoforms. The inter- and intra-organ distribution of HS epitopes has been shown to differ in various organs in different stages of development (David et al., 1992; van den Born et al., 1992; van Kuppevelt et al., 1998; Jenniskens et al., 2000; Denissen et al., 2002; Jenniskens et al., 2002a; van de Westerlo et al., 2002). Consensus sequences of some of these HS epitopes, as well as HS sequences involved in the binding of growth factors and antithrombin III have thus far been elucidated (Kjellén and Lindahl, 1991; Habuchi et al., 1992; Turnbull et al., 1992; Guimond et al., 1993; Lyon et al., 1994; Ashikari et al., 1995; Kreuger et al., 2001). Critical roles of HS in developmental processes and specific signaling pathways have recently been demonstrated by the identification of various mutations in biosynthetic enzymes of HS in Drosophila and Caenorhabditis, and in CHO cell lines (Sugahara and Kitagawa, 2000; Filmus and Selleck, 2001; Lander and Selleck, 2001). Indeed, HSPGs and especially the sulfation patterns of the HS moiety are considered to be regulators of developmental signaling (Lander, 1998; Selleck, 2000; Dhoot et al., 2001). Moreover, the generation of knock out mice for various proteoglycan core proteins or proteins involved in HS biosynthesis have significantly added to our understanding of the physiological roles of HS (Forsberg and Kjellén, 2001). Due to their binding capacities for a large number of proteins (Table 3), HS or heparin present in the ECM or on cellular surfaces have been implicated in many cellular processes such as cell proliferation, cell differentiation, angiogenesis, metastasis, tumor growth, tissue repair, tissue modeling, cell adhesion, microbial invasion, viral infection, endocytosis, lipoprotein metabolism, and protein expression (Jackson et al., 1991; Williams and Fuki, 1997; Iozzo, 1998; Lyon and Gallagher, 1998; Bernfield et al., 1999; Park et al., 2000; Perrimon and Bernfield, 2000; Tumova et al., 2000).

HS in myogenesis

Prior to the attribution of specific functions to individual proteoglycans present in the ECM, several reports recognized the involvement of ECM components in skeletal muscle development (Ontell and Kozeka, 1984; Jansen and Fladby, 1990). Especially for HSPGs, roles in development are envisaged (Perrimon and Bernfield, 2000). The developmental regulation of proteoglycan and HS synthesis during myogenesis in vitro is suggestive for possible roles in underlying processes (Noonan et al., 1986; Miller et al., 1987; Larraín et al., 1997a; 1997b; Jenniskens et al., 2000).
Similar regulation of expression patterns in vivo strengthens the involvement of individual HSPGs or HS epitopes in myogenesis (Velleman, 1999; Velleman et al., 1999; Charbonnier et al., 2000; Cifuentes-Diaz et al., 2000; Gallagher, 2001; Olguin and Brandan, 2001; Jenniskens et al., 2002a). Heparin was shown to inhibit skeletal muscle growth (Kardami et al., 1988), whereas the addition of heparin, HS, or HS mimetics to satellite cell cultures increases differentiation (Rapraeger et al., 1991; Olwin and Rapraeger, 1992; Stockholm et al., 1999). Satellite cells from mdx mice display elevated levels of HSPG receptors for FGF (Crisona et al., 1998). Moreover, Unc-52, the C. elegans ortholog of perlecan (Rogalski et al., 1993), is shown to be involved in body wall muscle integrity (Rogalski et al., 1995). The current view is that the concert of regulatory proteins and HS epitopes, present in highly specific spatiotemporal distribution patterns, provides an extracellular microenvironment that is causal for the events of myogenesis.

Table 3. Various groups of glycosaminoglycan-binding proteins

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Extracellular matrix proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipolytic enzymes</td>
<td>Collagens</td>
</tr>
<tr>
<td>Kinases</td>
<td>Fibronectin</td>
</tr>
<tr>
<td>Phosphatases</td>
<td>Laminin</td>
</tr>
<tr>
<td>Carbohydrate hydrolases, eliminases, transferases</td>
<td>Thrombospondin</td>
</tr>
<tr>
<td>Proteases and esterases</td>
<td>Vitronectin</td>
</tr>
<tr>
<td>Nucleases, polymerases, topoisomerases</td>
<td>Fibrillin</td>
</tr>
<tr>
<td>Other enzymes, oxidases, synthetases, dismutases</td>
<td>Receptor proteins</td>
</tr>
<tr>
<td>Enzyme inhibitors</td>
<td>Steroid receptors</td>
</tr>
<tr>
<td>Serine protease inhibitors (serpins)</td>
<td>Growth factor receptors</td>
</tr>
<tr>
<td>Lipoproteins</td>
<td>Channel proteins</td>
</tr>
<tr>
<td>Low and very low density lipoproteins</td>
<td>Viral coat proteins</td>
</tr>
<tr>
<td>Apolipoproteins</td>
<td>HIV</td>
</tr>
<tr>
<td>Growth factors</td>
<td>HSV</td>
</tr>
<tr>
<td>Fibroblast growth factors</td>
<td>Dengue</td>
</tr>
<tr>
<td>Epidermal growth factors</td>
<td>Nuclear proteins</td>
</tr>
<tr>
<td>Hepatocyte growth factors</td>
<td>Histones</td>
</tr>
<tr>
<td>Platelet-derived growth factors</td>
<td>Transcription factors</td>
</tr>
<tr>
<td>Transforming growth factors</td>
<td>Others</td>
</tr>
<tr>
<td>Vascular endothelial growth factors</td>
<td>Other proteins</td>
</tr>
<tr>
<td>Chemokines</td>
<td>Prion proteins</td>
</tr>
<tr>
<td>CXC chemokines</td>
<td>Amyloid proteins</td>
</tr>
<tr>
<td>CC chemokines</td>
<td>Fibrin</td>
</tr>
<tr>
<td>Selectins</td>
<td>Ion channels</td>
</tr>
</tbody>
</table>

Modified from Gunay and Linhardt, 1999

**HS in synaptogenesis**

Parallel to the recognition of the importance of HSPGs in muscle proliferation and differentiation, roles emerged for this class of proteoglycans in neural differentiation (Nurcombe et al., 1993; Brickman et al., 1998) and in the genesis of specialized cellular structures, such as
the NMJ (Sanes and Lichtman, 2001). Axonal pathfinding towards and through muscle primordia is very accurate and thought to be guided by molecules of the interstitium (Letourneau et al., 1994; Burden, 1998). BL-HSPGs or associated proteins have been implicated in the induction of postsynaptic differentiation (Anderson and Fambrough, 1983; Chiu and Sanes, 1984; Bayne et al., 1984; Peng et al., 1995; 1998). A good example is the well-described role of agrin in AChR clustering (Gordon and Hall, 1989; Ferns et al., 1993; Gordon et al., 1993; Bowen et al., 1996; Mook-Jung and Gordon, 1996; Li and Loeb, 2001). The sequestering of AChE and certain laminin isoforms in the sBL is also attributed to HS (Vigny et al., 1983; Brandan et al., 1985; San Antonio et al., 1993; Rossi and Rotundo 1996; Patton et al., 1997; Peng et al., 1999). Moreover, HSPGs were shown to be necessary for sBL formation and stabilization of AChR clusters (Grady et al., 2000; Jacobson et al., 2001). In vitro studies confirmed the direct involvement of HS and heparin in synaptogenesis by the demonstration of their ability to block AChR accumulation (Hirano and Kidokoro, 1989; Wallace, 1990; Hopf and Hoch, 1997; Hoch, 1999) and myotube innervation (Mars et al., 2000).

HS in excitation-contraction coupling

Little information is available about possible roles of HSPGs or their HS moieties in muscle physiology and e-c coupling, let alone about their mechanism of action. Extracellular heparin was shown to affect the kinetic properties of the DHPR (Knaus et al., 1990; 1992; Lacinova et al., 1993; Martinez et al., 1996). Whereas the RyR is activated by heparin in vitro (Ritov et al., 1985; Bezprozvannyy et al., 1993; Ehrlich et al., 1994), the structurally similar inositol trisphosphate receptor is inhibited (Gosh et al., 1988; Kobayashi et al., 1988). Intracellular application of heparin blocks e-c coupling in an activation-dependent manner in toad, but not in rat muscle fibers, an effect most likely caused by a direct action of heparin on the DHPR voltage sensor (Lamb et al., 1994). The loss of anion-buffering capacity of the strongly negatively charged HS polymers near ion channels has been proposed as a mechanism with regard to ion channel modulation by HS or heparin (Bezprozvanny et al., 1993).

Direct evidence for the involvement of extracellular HS in muscle calcium kinetics has recently been obtained by our group in C2C12 myoblast cultures in which HS epitopes were physiologically knocked out (Jenniskens et al., 2002b) and in primary myoblast cultures from mice carrying targeted disruptions in the gene encoding NDST-1 (Jenniskens et al., 2002c). Moreover, a unique GAG was discovered in the terminal cisternae of skeletal muscle SR, which is involved in calcium housekeeping (Jenniskens et al., 2002d).
HSPGs and HS in muscle pathology

In recent years, there has been an increasing number of reports on inherited or artificially induced defects in proteins of the skeletal muscle ECM, and on HSPGs present on the cell surface or in the BL, that are involved in (interstitial) myopathies (Table 4). In mdx mice, an animal model for Duchenne muscular dystrophy, an overdeposition of various ECM molecules was reported (Quirico-Santos et al., 1995).

The perlecan gene is mutated in chondrodystrophic myotonia (Schwartz-Jampel syndrome; Nicole et al., 2000) and in dyssegmental dysplasia (Silverman-Handmaker type; Arikawa-Hirasawa et al., 2001). In Schwartz-Jampel syndrome a significantly reduced number of truncated perlecan molecules is present in the skeletal muscle BL. The resulting partial impairment of perlecan function causes myotonic myopathy, which is consistent with the finding that perlecan is crucial for clustering of AChE at the NMJ (Arikawa-Hirasawa et al., 2002). Moreover, mutations in the Unc-52 gene of C. elegans are reported to be lethal or lead to paralysis (Rogalski et al., 1993). Mice knock out for agrin show aberrant synaptogenesis (Gautam et al., 1996; 1999), whereas the expression of agrin from an artificial minigene can rescue congenital muscular dystrophy in a mouse model (Moll et al., 2001). The Simpson-Golabi-Behmel syndrome has been attributed to defects in glypican-3 (Pilia et al., 1996; Cano-Gauci et al., 1999).

Table 4. Inherited or artificially induced defects in cell surface and BL components and the resulting interstitial myopathy

<table>
<thead>
<tr>
<th>Affected</th>
<th>Cause*</th>
<th>Interstitial myopathy</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agrin*</td>
<td>A</td>
<td>Congenital muscular dystrophy</td>
<td>Moll et al., 2001</td>
</tr>
<tr>
<td>Collagen VI</td>
<td>A</td>
<td>Defective neuromuscular synaptogenesis</td>
<td>Gautam et al., 1999</td>
</tr>
<tr>
<td>Collagen XIII</td>
<td>I</td>
<td>Bethilehem myopathy</td>
<td>Lamande et al., 1998</td>
</tr>
<tr>
<td>Glypican-3*</td>
<td>I</td>
<td>Simpson-Golabi-Behmel syndrome</td>
<td>Pilia et al., 1998</td>
</tr>
<tr>
<td>Laminin-2</td>
<td>I</td>
<td>Merosin-negative congenital muscular dystrophy</td>
<td>Tome et al., 1994</td>
</tr>
<tr>
<td>Laminin-a2</td>
<td>I</td>
<td>Congenital muscular dystrophy</td>
<td>Hermann et al., 1996</td>
</tr>
<tr>
<td>Laminin-b1</td>
<td>I</td>
<td>Limb-girdle muscular dystrophy</td>
<td>Li et al., 1997</td>
</tr>
<tr>
<td>MuSK</td>
<td>A</td>
<td>Defective neuromuscular synaptogenesis</td>
<td>Gautam et al., 1999</td>
</tr>
<tr>
<td>Perlecan*</td>
<td>I</td>
<td>Schwartz-Jampel syndrome</td>
<td>Nicole et al., 2000</td>
</tr>
<tr>
<td></td>
<td>I</td>
<td>Silverman-Handmaker dyssegmental dysplasia</td>
<td>Arikawa-Hirasawa et al., 2001</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>AChE clustering defects</td>
<td>Arikawa-Hirasawa et al., 2002</td>
</tr>
<tr>
<td>Rapsyn</td>
<td>A</td>
<td>Defective neuromuscular synaptogenesis</td>
<td>Gautam et al., 1999</td>
</tr>
<tr>
<td>Sarcoglycans</td>
<td>I</td>
<td>Limb-girdle muscular dystrophy</td>
<td>Sewry et al., 1996</td>
</tr>
<tr>
<td>Tenascin-C</td>
<td>A</td>
<td>Neuromuscular junction defects</td>
<td>Cifuentes-Diaz et al., 1998</td>
</tr>
<tr>
<td>Unc-52**</td>
<td>I</td>
<td>Body wall muscle integrity and paralysis</td>
<td>Rogalski et al., 1995</td>
</tr>
</tbody>
</table>

Due to limitations of space, only a single reference has been cited for each entry.

* A, artificially induced; I, inherited
* Heparan sulfate proteoglycan
** Perlecan ortholog in C. elegans.
Thus, recent data indicate the involvement of several HSPGs in myopathies and muscular dystrophies. Mice knock out for NDST-1 die neonatally due to respiratory distress, whereas NDST-2 deficient mice show no defects. Therefore, the exact functions of the HS moieties, and possibly of individual HS epitopes, in muscle remain to be investigated.

**Aim of the study and scope of this thesis**

A growing number of physiological functions are attributed to the BL surrounding skeletal muscle fibers. Biochemical evidence is available for active roles of BL-resident HSPGs in myogenesis and muscle physiology. However, most research has focused on their protein cores, thus ignoring the HS moiety. The scope of this project was to investigate the distribution of HS and possible HS subspecies in skeletal muscle and to elucidate their role in muscle development and in e-c coupling. In 1997, when the research described in this thesis was initiated, only few antibodies that recognize HS had been described, but the recent demonstration that antibodies against specific HS epitopes could be raised by means of phage display included a promise for progress.

First, antibodies were selected by means of phage display against HS isolated from skeletal muscle (chapter 2). These antibodies present new tools in the study on the structure and role of HS. Several different antibodies were shown to define distinct HS epitopes that have a mutually different distribution in skeletal muscle. The unique distribution pattern of distinct HS epitopes, especially with regard to the NMJ, raises important issues as to their roles in muscle physiology.

In chapter 3 the HS epitopes that are selectively recognized by anti-HS antibodies are characterized. It is shown that all ten antibodies studied, have a different inter- and intra-tissue distribution and that they recognize different structural modifications of HS.

Chapter 4 describes studies on the spatiotemporal distribution of HS epitopes during myogenesis and synaptogenesis in situ in the mouse. Differences in their location and in the onset of their expression were noticed. These results may indicate an active role for specific HS epitopes in muscle development.

As described in chapter 5, the participation of HS in muscle calcium kinetics was investigated in primary myotube cultures of mice carrying a targeted disruption of genes involved in HS biosynthesis. Mice knock out for NDST-1 are severely affected in their HS composition, and a significant change in the kinetics of electrically induced calcium spikes was observed.

In chapter 6 various newly devided eukaryotic expression systems are presented, in which anti-HS antibodies are expressed as intrabodies in a myoblast cell line (C2C12). The expression of these intrabodies results
in the phenotypic knock out of HS at the cellular surface. Myotubes devoid of HS were studied with regard to their excitation-induced calcium spiking, thus for the first time uncovering a role for HS epitopes in muscle calcium housekeeping.

A rare HS-like GAG epitope with a unique distribution in the terminal cisternae of the SR is described in chapter 7. Calcium kinetics were studied in C2C12 myotubes in which this epitope was knocked out by expression of its antibody. The singular location and the novel action of this unique GAG epitope further strengthen the involvement of GAGs in muscle e-c coupling.

Chapter 8 concludes this thesis with a survey and indications for future research.

References


Heparan Sulfate Heterogeneity in Skeletal Muscle Basal Lamina: Demonstration by Phage Display-Derived Antibodies

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Toin H. van Kuppevelt

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The basal lamina enveloping skeletal muscle fibers contains different glycoproteins, including proteoglycans. To obtain more information on the glycosaminoglycan moiety of proteoglycans, we have selected a panel of anti-heparan sulfate antibodies from a semisynthetic antibody phage display library by panning against glycosaminoglycan preparations derived from skeletal muscle. Epitope recognition by the antibodies is strongly dependent on O- and N-sulfation of the heparan sulfate. Immunostaining with these antibodies showed a distinct distribution of heparan sulfate epitopes in muscle basal lamina of various species. Clear differences in staining intensity were observed between neural, synaptic, and extrasynaptic basal laminae. Moreover, temporal and regional changes in abundance of heparan sulfate epitopes were observed during muscle development both in vitro and in vivo. Taken together, these data suggest a role for specific heparan sulfate domains/species in myogenesis and synaptogenesis. Detailed analysis of the functions of heparan sulfate epitopes in muscle morphogenesis has now become feasible with the isolation of antibodies specific for distinct heparan sulfate epitopes.

Introduction

The basal lamina (BL) enveloping skeletal muscle fibers plays various roles in muscle development and regeneration (Sanes, 1986; Wright et al., 1991). Several BL molecules have been identified as specifically synaptic, extrasynaptic, or common (Sanes, 1982; Hall and Sanes, 1993). On the protein level, these include isoforms of laminin, collagen, and entactin (Sanes et al., 1990; Chiu and Ko, 1994; Patton et al., 1997). Lectin staining (Sanes and Cheney, 1982; Iglesias et al., 1992) and a recent report on synapse-specific carbohydrates (Martin et al., 1999) indicate a spatial heterogeneity for carbohydrates as well.

Heparan sulfate proteoglycans (HSPGs), consisting of a core protein and a carbohydrate moiety (heparan sulfate (HS)), are main components of muscle BLs. So far, three BL HSPGs have been identified: perlecan, agrin, and type XVIII collagen (Noonan et al., 1991; Tsen et al., 1995; Halfter et al., 1998). HSPGs are implicated in developmental processes underlying myogenesis and synaptogenesis (Anderson and Fambrough, 1983; Anderson et al., 1984; Bayne et al., 1984; Dmytrenko et al., 1990). Perlecan may effect neuromuscular junction (NMJ) formation by binding growth factors (Peng et al., 1998). Agrin, a major HSPG of the synaptic BL (sBL), orchestrates acetylcholine receptor (AChR) clustering (Campanelli et al., 1994; Ruegg and Bixby, 1998). Heparin and HS are involved in AChR clustering induced by nerve (Hirano and Kidokoro,
1989) and agrin (Wallace, 1990). In muscle cell lines defective in glycosaminoglycan (GAG) synthesis, a causal relationship between GAGs and AChR clustering is demonstrated (Ferns et al., 1993; Gordon et al., 1993; Mook-Jung and Gordon, 1996). HS binding may also mediate the synapse-specific anchoring of BL-resident proteins such as acetylcholine esterase and possibly certain laminin isoforms (Brandan et al., 1985; San Antonio et al., 1993; Patton et al., 1997).

Another major characteristic of HS is the binding of growth factors such as neuregulin (Fischbach and Rosen, 1997), midkine (Zhou et al., 1997), heparin-binding growth-associated molecule (Peng et al., 1995; Szabat and Rauvala, 1996), heparin-binding epidermal growth factor-like growth factor (Chen et al., 1995), and basic fibroblast growth factor. The latter protein is involved in postsynaptic differentiation (Peng et al., 1991) and maintenance of the proliferative state of satellite cells (Rapraeger et al., 1991; Olwin and Rapraeger, 1992; Olwin et al., 1994; Crisona et al., 1998). Considering the diversity of proteins that bind HS, HS molecules may contain unique domains (epitopes) that are specific for these interactions.

Studies of HSPGs have mainly been focused on the protein core. The structure and the role of the HS moiety are difficult to investigate because of a lack of appropriate tools. Only a few antibodies that recognize HS epitopes have been described (David et al., 1992; van den Born et al., 1992). Recently, we adapted phage display technology to obtain epitope-specific antibodies against HS (van Kuppevelt et al., 1998). Here we report on the isolation, characterization, and application of antibodies selected against HS-containing GAG preparations from skeletal muscle. We provide evidence for the existence of several specific, differentially distributed HS epitopes in (synaptic and extrasynaptic) muscle and nerve BLs. Moreover, we found a shift in abundance of these epitopes in BLs of developing muscle both in vitro and in vivo. These data suggest an involvement of specific HS epitopes in myogenesis and synaptogenesis.

**Materials and Methods**

**Materials**

Synthetic single-chain variable fragment (scFv) library #1 (Nissim et al., 1994) was generously provided by Dr. G. Winter (Cambridge University, Cambridge, United Kingdom). Human skeletal muscle samples were generously provided by Prof. Dr. D. Ruiter (Department of Pathology, University of Nijmegen, Nijmegen, The Netherlands). *Torpedo marmorata* electric organ was generously provided by Dr. M.H. De Baets (Department of Immunology, University of Maastricht, Maastricht, The
Netherlands. Mice (C3H, male, 70 days), rats (Wistar, male, six weeks), and rat embryos (Wistar, 10, 13, 16, and 19 d after conception) were obtained from the University of Nijmegen Central Animal Laboratory. C3H mouse-derived skeletal muscle (C2C12) cell line was purchased from American Type Culture Collection (Rockville, MD). Glycosaminoglycan-deficient myoblast (S27) cell line was a generous gift of Dr. Z. Hall (Department of Physiology, University of California, San Francisco, CA); mutant Chinese hamster ovary (CHO) cell lines were kindly provided by Dr. J. Esko (Department of Biochemistry, University of Alabama, Birmingham, AL).

For phage display, two Escherichia coli strains were used: suppressor strain TG1 [K12, supE, hsdA5, thi Δ(lac-proAB), F'(traD36, proAB+, lacIq, lac ZΔM15)] and non-suppressor strain HB2151 [K12, ara, thi Δ(lac-pro), F'(proAB+, lacIq, ZΔM15)]. Helper phage VCS-M13 was from Stratagene (La Jolla, CA).

All chemicals used were purchased from Merck (Darmstadt, Germany), unless stated otherwise. Bacterial media (2xTY and LB) and cell culture media were from Life Technologies (Paisley, Scotland). Chondroitinase ABC (from *Proteus vulgaris*, EC 4.2.2.4), chemically modified heparin kit, anti-chondroitin sulfate (CS) "stub" antibody (2B6), and anti-heparan sulfate stub antibody (3G10) were from Seikagaku Kogyo Co. (Tokyo, Japan). Heparinase III (from *Flavobacterium heparinum*, EC 4.2.2.8), heparan sulfate from bovine kidney and from porcine intestinal mucosa, heparin from porcine intestinal mucosa, chondroitin 4-sulfate and chondroitin 6-sulfate from bovine trachea, dermatan sulfate (DS) from porcine intestinal mucosa, keratan sulfate from bovine cornea, hyaluronic acid from human umbilical cord, DNA from calf thymus, phenylmethylsulfonyl fluoride (PMSF), N-ethylmaleimide, aprotinin, sodium azide, pepstatin A, and bovine serum albumin (fraction V) were from Sigma (St. Louis, MO). Microlon 96-wells microtiter plates and immunotubes were from Greiner (Frickenhausen, Germany). Anti-c-Myc tag mouse monoclonal IgG (clone 9E10) was from Boehringer Mannheim (Mannheim, Germany). Anti-c-Myc tag rabbit polyclonal IgG (A-14) was from Santa Cruz Biotechnology (Santa Cruz, CA). Alkaline phosphatase-conjugated rabbit anti-mouse IgG was from Dakopatts (Glostrup, Denmark). Alexa 488-conjugated goat anti-rabbit IgG and tetramethylrhodamine isothiocyanate (TRITC)-conjugated α-bungarotoxin were from Molecular Probes (Eugene, OR). Mowiol (4-88) was from Calbiochem (La Jolla, CA). PCR chemicals and *Taq* polymerase (DNA polymerase from *Thermus aquaticus*) were from Promega (Madison, WI). PCR primers were from Biolegio (Malden, The Netherlands), and restriction enzyme *BstNI* was from New England Biolabs (Beverly, MA). ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit was from PE Applied Biosystems (Norwalk, CT).

All experiments were performed at ambient temperature (21°C), unless stated otherwise.
**Isolation of glycosaminoglycans from skeletal muscle**

Mouse and human skeletal muscle specimens were homogenized, defatted in 20 vol of acetone at -20°C for 16 h, and dried in a desiccator. Per gram of muscle tissue, 4 ml 50 mM sodium phosphate buffer, pH 6.5, containing 2 mM EDTA, 2 mM cysteine, and 10 units papain were added. Papain digestion was performed for 16 h at 65°C and the remaining debris was pelleted. Residual protein fragments were removed from the glycosaminoglycans by mild alkaline borohydride digestion in 0.5 M NaOH/0.05 M NaBH₄ at 4°C. After overnight digestion, the mixture was neutralized by addition of 6 M HCl. Residual protein fragments were precipitated by addition of 100% (w/v) trichloroacetic acid to a final concentration of 6% and precipitation at 0°C for 1 h. Precipitated proteins were removed by centrifugation (10,000 xg for 20 min at 4°C) and glycosaminoglycans were isolated by addition of 5 vol. of 100% ethanol to the supernatant and overnight precipitation at -20°C. After centrifugation (10,000 xg for 30 min at 4°C), the pelleted glycosaminoglycans were washed with 70% ethanol, dried, and dissolved in 10 mM Tris-HCl, pH 6.8. This crude glycosaminoglycan preparation was further deprived of protein contamination by DEAE Sepharose column chromatography, eluting glycosaminoglycans at 0.5 M and 1.0 M NaCl in 10 mM Tris-HCl, pH 6.8. GAG-containing eluates were pooled, and after ethanol precipitation the residual salt was removed by a 70% (v/v) ethanol wash. The resulting glycosaminoglycan preparations were dissolved in MilliQ water and stored at 4°C.

**Phage display**

Phage display was essentially performed as described (Van Kuppevelt et al., 1998). Synthetic scFv library #1 was subjected to four rounds of panning against mouse or human skeletal muscle glycosaminoglycan preparations. The library contains approximately 10⁸ different scFv antibody clones, composed of 50 different heavy (VH) chain V segments with synthetic (randomly synthesized) complementarity-determining region 3 (CDR3) fragments and one light (VL) segment. This library was in vitro-synthesized from V gene segments, derived from human lymphocytes, using PCR (Tomlinson et al., 1992; Nissim et al., 1994). After the last round of selection, single colonies were picked and the antibodies expressed by these clones were evaluated for reactivity by ELISA. Clones displaying reactive antibodies were further analyzed by colony-PCR amplification of the antibody coding region and restriction digestion of the full-length PCR products with BstNI (CC↓A/GGG). Unique clones were grown at a larger scale and individual plasmid DNAs were sequenced using PelB-seq (5'GGAGATGGTGATTACTC-3') as a primer (located within the PelB leader sequence).
Large scale preparation of antibodies

To produce large quantities of scFv antibodies, plasmid DNA from selected clones was used to transform non-suppressor *E. coli* strain HB2151. Five hundred milliliters of prewarmed 2xTY medium containing 0.1% (w/v) glucose and 100 mg/ml ampicillin were inoculated with an overnight culture of transformed HB2151 and grown with vigorous shaking at 37°C until an OD₆₀₀ of 0.3 was reached. Induction was effectuated by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. After 3 h incubation at 30°C the culture was cooled on ice for 20 min and cells were pelleted (3000 xg for 10 min at 4°C). One-tenth volume of 10x protease inhibitor mix (0.1 M EDTA, 250 mM iodoacetamine, 1 M N-ethylmaleimide, 1% (w/v) NaN₃, 1.5 mTIU/ml aprotinin, 0.1% (w/v) pepstatin A, 1 mM PMSF) was added to the supernatant, which was subsequently divided into aliquots and stored at 4°C. The cells were resuspended by vigorous vortexing in 5 ml ice-cold 200 mM sodium-borate buffer, pH 8.0, containing 160 mM NaCl, 1 mM EDTA and protease inhibitors. After centrifugation (5000 xg for 30 min at 4°C) the supernatant (the periplasmic fraction containing the scFv antibodies) was filtered through a 0.45 μm filter, dialyzed overnight at 4°C against PBS, divided into aliquots, and stored at -20°C.

Evaluation of antibody specificity by ELISA

Unless stated otherwise, supernatants of IPTG-induced HB2151 cultures were used for ELISA. Affinity of the antibodies to various molecules was evaluated by ELISA in two ways: scFv antibodies were applied to wells of Microlon microtiter plates, coated with the molecule concerned (10 mg/ml coating solution), and allowed to bind for 90 min. Alternatively, scFv antibodies were pre-incubated overnight with the test molecule (10 mg/ml) in PBS/0.1% (w/v) Marvel, followed by transfer to and 90 min incubation in wells previously coated with heparin. Test molecules included glycosaminoglycan preparations from mouse and human skeletal muscle, HS preparations from bovine kidney and human lung, prepared as described above, commercially available heparan sulfate from bovine kidney and from porcine intestinal mucosa, heparin, chemically and enzymatically modified heparin, chondroitin 4-sulfate, chondroitin 6-sulfate, dermatan sulfate, keratan sulfate, hyaluronic acid, DNA, Marvel and bovine serum albumin (fraction V). Bound scFv antibodies were detected using anti-c-Myc mouse monoclonal antibody 9E10, followed by alkaline phosphatase-conjugated rabbit anti-mouse IgG (60 min each). Plates were washed three times with PBS containing 0.1% (v/v) Tween-20 (PBST) after each incubation. Enzyme activity was detected using 1 mg/ml *p*-nitrophenyl phosphate in 1 M diethanolamine/0.5 mM MgCl₂, pH 9.8, and absorbance was read at 405 nm.
**Immunohistochemistry**

Periplasmic fractions of IPTG-induced HB2151 cultures were used for immunohistochemistry, unless stated otherwise. Location of the epitopes recognized by the antibodies in several tissues was assessed both on cryosections of tissue specimens and on monolayers of cultured cell lines. Tissues included human skeletal muscle and diaphragm, rat and C3H mouse skeletal muscle, rat denervated skeletal muscle, rat embryos, and *T. marmorata* electric organ. Tissue specimens were snap-frozen in liquid nitrogen-cooled isopentane and stored at -80°C. Wild-type and glycosylation-deficient CHO cell lines were studied at confluency, whereas myoblast (C2C12 and S27) cell lines were analyzed at various stages of differentiation. Cells were grown as previously described (Portiér et al., 1999), differentiated in Ultroser brain extract medium, washed three times with PBS, dried overnight, and stored at -80°C. Cryosections (3 or 5 μm) or tissue cultures were rehydrated for 10 min in PBS, blocked with PBS containing 0.1% (w/v) BSA for 20 min, and incubated with scFv antibodies for 90 min. Bound antibodies were detected using anti-c-Myc rabbit polyclonal antibody A-14 and Alexa 488-conjugated goat anti-rabbit IgG (60 min each). For visualization of AChR clusters, TRITC-conjugated alpha-bungarotoxin was included in the final incubation. Cryosections or tissue cultures were washed three times with PBS after each incubation. Finally, the cryosections or tissue cultures were fixed in 100% methanol, dried and embedded in Mowiol (10% (w/v) in 0.1 M Tris-HCl, pH 8.5/ 25% (v/v) glycerol/ 2.5% (w/v) NaN3). As a control, primary, secondary, or conjugated antibodies were omitted.

**Evaluation of antibody specificity by immunohistochemistry**

To assess the heparan sulfate specificity of the scFv antibodies, cryosections or tissue cultures were pre-incubated with heparinase III to digest heparan sulfate (0.02 U/ml in 50 mM NaAc/ 50 mM Ca(Ac)2, pH 7.0) overnight at 37°C, or with chondroitinase ABC, which digests chondroitin and dermatan sulfate (1 U/ml in 25 mM Tris-HCl, pH 8.0) for 30 min at 37°C. As a control, cryosections or tissue cultures were incubated in the reaction buffer without enzyme. After washing three times with PBS and blocking with PBS/ 0.1% (w/v) BSA, cryosections and tissue cultures were incubated with antibodies and processed for immunofluorescence as described above. The efficiency of chondroitinase ABC treatment was evaluated by incubation of cryosections with an antibody (2B6) against chondroitin sulfate “stubs”, generated by chondroitinase. Heparan sulfate stubs were visualized using anti-Δ-heparan sulfate antibody (3G10).
Denervation of rat skeletal muscle

The musculus gastrocnemius and the musculus soleus of the left legs of young adult rats were denervated by cutting the efferent motor nerves innervating these muscles. The ends of these nerves were fastened to the musculus biceps femoris to prevent reinnervation (Degens et al., 1992). After 11 d, rats were killed, and the calves of both the left (denervated) and right (control) legs were isolated and processed as described in immunohistochemistry.

Results

Selection of antibodies against skeletal muscle GAGs

To select scFv antibodies against skeletal muscle GAG epitopes, GAGs were isolated from human and from C3H mouse skeletal muscle. Typically, 10 μg GAG could be purified from 1 g muscle tissue (wet weight). All GAG preparations contained approximately equal amounts of CS and HS and were approximately 4-fold richer in DS (Fig. 1).

Four rounds of panning were performed against mouse skeletal muscle-derived GAG preparations, resulting in antibodies that bear the prefix AO. Antibodies with the prefix RB were obtained after panning against human skeletal muscle-derived GAGs. This approach yielded a set of unique anti-HS antibodies, based on the amino acid sequence of their heavy chain CDR3, a major determinant in antigen specificity (Table 1).

Figure 1. Silver-stained 1% agarose gel of a muscle glycosaminoglycan preparation. Lane 1, sample buffer (control); lane 2, 5 ng GAG standard; lane 3, 20 ng GAG standard; lane 4, Typical glycosaminoglycan preparation of mouse skeletal muscle. Dermatan sulfate (DS) is present at approximately 4-fold higher concentration compared with chondroitin sulfate (CS) and heparan sulfate (HS).
Table 1. **CDR3 sequences and germline \( V_n \) gene segments of anti-HS scFv antibodies**

<table>
<thead>
<tr>
<th>Clone</th>
<th>CDR3 sequence</th>
<th>( V_n ) family</th>
<th>DP segment</th>
</tr>
</thead>
<tbody>
<tr>
<td>AO4B05</td>
<td>LKQQGIS</td>
<td>3</td>
<td>53</td>
</tr>
<tr>
<td>AO4B08</td>
<td>SLRMNGWRAHQ</td>
<td>3</td>
<td>47</td>
</tr>
<tr>
<td>AO4F12</td>
<td>AMTQKKPRKLSL</td>
<td>1</td>
<td>7</td>
</tr>
<tr>
<td>RB4CB9</td>
<td>HAPLANTRNT</td>
<td>3</td>
<td>38</td>
</tr>
<tr>
<td>RB4CD12</td>
<td>GMRPRL</td>
<td>3</td>
<td>38</td>
</tr>
<tr>
<td>RB4EA12</td>
<td>RRYALDY</td>
<td>3</td>
<td>32</td>
</tr>
<tr>
<td>RB4EG12</td>
<td>SGRKYFRADMN</td>
<td>1</td>
<td>4</td>
</tr>
</tbody>
</table>

Antibodies with the prefix AO were obtained after panning against GAGs from mouse skeletal muscle, whereas antibodies with the prefix RB were selected by panning against GAGs isolated from human skeletal muscle. CDR3 sequences are shown in single-letter amino acid code. \( V_n \) families and DP segments were deduced from the Sanger Centre Germline Quiry (http://www.sanger.ac.uk/DataSearch/gq_search.shtml) by applying the full length \( V_n \) sequences of the anti-HS scFv antibody clones (nomenclature according to Tomlinson et al., 1992).

Table 2. **Evaluation of anti-HS antibody specificity by ELISA**

<table>
<thead>
<tr>
<th>GAG preparation</th>
<th>AO4B05</th>
<th>AO4B08</th>
<th>AO4F12</th>
<th>RB4CB9</th>
<th>RB4CD12</th>
<th>RB4EA12</th>
<th>RB4EG12</th>
</tr>
</thead>
<tbody>
<tr>
<td>K5 capsular polysaccharide (E. coli)</td>
<td>++</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HS (bovine kidney)</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>+/-</td>
<td>+</td>
<td>+/-</td>
</tr>
<tr>
<td>HS (porcine intestinal mucosa)</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>+/-</td>
<td>+</td>
<td>+/-</td>
</tr>
<tr>
<td>HS (human lung, 0.5 M NaCl fraction)</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>HS (human lung, 1.0 M NaCl fraction)</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Heparin (porcine intestinal mucosa)</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Heparin, desulfated and N-acetylated</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Heparin, desulfated and N-sulfated</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Antibody-containing supernatants of IPTG-induced E. coli HB2151 cultures were applied to various GAG preparations immobilized on microtiter plates. Bound antibodies were detected using anti-c-Myc mouse monoclonal antibody 9E10, followed by alkaline phosphatase-conjugated rabbit anti-mouse IgG, after which enzymatic activity was measured using p-nitrophenyl phosphate as a substrate. Substrate affinity: +++, very strong; +++, strong; +++, moderate; +/-, weak; -, absent (n=5).

Characterization of antibodies

All antibodies showed a high reactivity in ELISA for the GAG preparation against which they were selected, whereas the reactivity for various GAG species derived from other tissues varied significantly. Despite the fact that the antibodies were selected against a GAG mixture that consisted predominantly of DS, antibodies showed affinity only for HS and heparin. No reactivity was observed with chondroitin 4-sulfate, chondroitin 6-sulfate, dermatan sulfate, keratan sulfate, hyaluronic acid, DNA, Marvel (blocking agent) and Microlon (data not shown). Antibodies reacted to various extents with a highly sulfated HS fraction (eluting at 1.0 M NaCl in ion exchange chromatography) and with a low-sulfated fraction (eluting at 0.5 M NaCl) of human lung (Table 2). All antibodies showed a major cross-reactivity with heparin, which is highly sulfated. Antibodies
Figure 2. Staining of heparinase III-treated skeletal muscle cryosections with anti-HS scFv and anti-HS stub antibodies. Non-treated (a) and heparinase III-treated (b, c) cryosections of mouse skeletal muscle tissue were incubated with periplasmic fraction of anti-HS antibody AO4F12 (a, b) or anti-heparan sulfate stub antibody (3G10) (c). Bound scFv antibodies were visualized by incubation with rabbit polyclonal anti-c-Myc IgG (a1, b1), followed by Alexa 488-conjugated goat anti-rabbit or anti-mouse IgG (a and b, or c, respectively). AChR clusters present in the neuromuscular junction were visualized using TRITC-conjugated alpha-bungarotoxin (a2-c2). Although in untreated tissue, the AO4F12 epitope is clearly present in the muscle BL (a1), staining disappeared during heparinase treatment (b1), indicating the HS nature of the epitope. Staining of heparan sulfate stubs in heparinase-treated tissue showed HS to be present throughout the muscle BL (c1). Note the higher staining intensity of AO4F12 at NMJs (a1, a2, arrows), regardless of the overall quantity of HS in the NMJ (c1, c2, arrows). Scale bar, 50 μm.

AO4B05, AO4B08, and (to a somewhat lesser extent) RB4CD12 showed a high reactivity for HS from bovine kidney and porcine intestinal mucosa, whereas all other antibodies interacted only moderately or weakly. K5 capsular polysaccharide from E. coli, which is similar to the HS precursor, was not bound by any of the antibodies.

To investigate which chemical groups are recognized by the different antibodies, we determined the reactivity of the antibodies towards modified heparin preparations (Table 2). Completely desulfated and N-acetylated heparin as well as completely desulfated and N-sulfated heparin were not recognized by any of the antibodies. Heparin that was N-desulfated and N-acetylated also was not recognized by the antibodies, except for AO4F12, which showed a weak binding.
To ascertain the HS specificity of the antibodies, immunofluorescence studies were performed on cryosections of skeletal muscle tissue that were treated with heparinase III before incubation. Heparinase treatment of cryosections resulted in a total loss of staining for all antibodies (Fig. 2), whereas treatment with chondroitinase ABC did not (data not shown). Staining of heparinase-treated cryosections with anti-HS stub antibody 3G10 (which reveals all HS that is present) showed HS to be equally distributed in synaptic and extrasynaptic BL (Fig. 2c).

### Table 3. Immunostaining patterns of anti-HS antibodies

<table>
<thead>
<tr>
<th>Tissue</th>
<th>AO4B05</th>
<th>AO4B08</th>
<th>AO6F12</th>
<th>RB4CB9</th>
<th>RB4CD12</th>
<th>RB4EA12</th>
<th>RB4EG12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mature human, rat, and mouse skeletal muscle</td>
<td>++</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>+/-</td>
<td>-</td>
</tr>
<tr>
<td>Extrasynaptic basal lamina</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>Synaptic basal lamina</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Endo- and perineurium</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>Capillary basal lamina</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>Arterial basal lamina</td>
<td>+</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>Electric organ (Torpedo marmarota)</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
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<tr>
<td>Electrocyte, non-innervated face</td>
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<td>++</td>
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<td>+</td>
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<tr>
<td>Electrocyte, innervated face</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>Endoneurium</td>
<td>++</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+/-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Perineurium</td>
<td>+/-</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>+</td>
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<td>+</td>
</tr>
<tr>
<td>Cell surface (on contact places)</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>Basal lamina (during differentiation)</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
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<tr>
<td>Smooth muscle basal lamina (artery)</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
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<tr>
<td>Cell surface (on contact places)</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>Basal lamina (during differentiation)</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
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<tr>
<td>S27 cell line (defective in proteoglycan synthesis)</td>
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<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td></td>
</tr>
<tr>
<td>Cell surface (on contact places)</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>Basal lamina (during differentiation)</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td></td>
</tr>
<tr>
<td>CHO cell line (wild-type)</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>Cell surface</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>CHO cell line (677 mutant)</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
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</tr>
<tr>
<td>Cell surface</td>
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<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td></td>
</tr>
<tr>
<td>CHO cell line (F17 mutant)</td>
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<td>+++</td>
<td>+++</td>
<td>+++</td>
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<tr>
<td>Cell surface</td>
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<td>+++</td>
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</tr>
<tr>
<td>CHO cell line (745 mutant)</td>
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<tr>
<td>Cell surface</td>
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<td>+++</td>
<td>+++</td>
<td>+++</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Cryosections of human, rat, and mouse skeletal muscle, T. marmarota electric organ, and rat embryo, as well as fixed monolayers of CHO (wild-type and glycosylation-deficient mutants, grown to confluency), and C2C12 and S27 cells (differentiated for 0-15 d) were incubated with periplasmic fractions containing anti-HS scFv antibodies. Bound antibodies were visualized by incubation with rabbit polyclonal anti-c-Myc IgG followed by Alexa 488-conjugated goat anti-rabbit IgG. AChR clusters present in muscle neuromuscular junction, T. marmarota electrocytes, and C2C12 myotubes were visualized using TRITC-conjugated α-bungarotoxin.

Staining intensity: ++++, very strong; ++, strong; +, moderate; +/-, weak; -, absent (n=3 (cryosections), n=2 (tissue cultures)).

- Staining intensity decreased with differentiation level.
- Staining intensity increased with differentiation level.
- Small granules within the cytosol: ++.
- Small granules around the nucleus: ++.

**Cell lines that are defective in GAG synthesis are not surface-stained by anti-HS antibodies**

To further establish the anti-HS nature of the scFv antibodies, we investigated cell lines that are defective in GAG synthesis. Developmental stages from half-confluent to 8 d of differentiation of the S27 cell line

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**CHAPTER TWO**
(Gordon and Hall, 1989) and confluent cultures of different CHO cell lines (wild type, N-acetylglucosaminyl- and glucuronosyltransferase-deficient; pgsD-677 (Lidholt et al., 1992), heparan sulfate uronic acid 2-O-sulfotransferase deficient; pgsF-17 (Dr. J. Esko, personal communication), and xylosyl transferase deficient; pgsA-745 (Esko et al., 1985)) were analyzed by immunofluorescence.

In contrast to wild-type myoblast cell line C2C12 (see below), the surface of S27 myoblasts was not immunoreactive for any of the antibodies, nor were places of cell-cell contact. On alignment and fusion, and at day 8 of differentiation, myotubes were not stained either, indicating that the BL of this mutant cell line does not contain any of the HS epitopes recognized by any of the antibodies (Table 3, Fig. 3). A noteworthy observation was the distinct staining of perinuclear and cytosolic granules by some antibodies (Table 3).

**Figure 3.** Staining of S27 muscle cell line with anti-HS scFv antibodies. S27 cultures were grown to confluency and subsequently differentiated up to 8 days. Cultures of different developmental stages (half confluent (a1-c1), 1 day (a2-c2), and 8 days (a3-c3) of differentiation) were fixed and subsequently incubated with the periplasmic fraction of scFv antibodies AO4B05 (a), RB4CD12 (b), and RB4EA12 (c), respectively. Bound antibodies were visualized by incubation with rabbit polyclonal anti-c-Myc IgG followed by Alexa 488-conjugated goat anti-rabbit IgG. None of the epitopes recognized by any of the antibodies can be visualized at the surface of myoblasts (a1-c1). For AO4B05, staining is not visible in aligning myoblasts (a2), or during myotube formation (a3). The epitope recognized by RB4CD12 is present in perinuclear granules in myoblasts (b1, arrows). During the alignment of myoblasts, the granular staining around the nucleus persists (b2, arrows), to change into a predominant cytosolic granular staining during myotube formation (b3). ScFv antibody RB4EA12 strongly stains perinuclear granules in myoblasts (c1, arrows). In aligning myoblasts and during myotube formation, the granular staining around the nucleus persists (c2, c3, arrows). Scale bar, 25 µm.
Figure 4. Staining of wild-type and glycosylation-deficient CHO cell lines with anti-HS scFv antibodies. CHO cultures (wild type (a1 to c1), N-acetylglucosaminyl- and glucuronosyltransferase-deficient pgsD-677 (a2-c2), heparan sulfate uronic acid 2-O-sulfotransferase-deficient pgsF-17 (a3-c3), and xylosyl transferase-deficient pgsA-745 (a4-c4)) were grown to confluency and subsequently fixed and incubated with the periplasmic fraction of scFv antibodies AO4B05 (a), RB4CD12 (b), and RB4EA12 (c), respectively. Bound antibodies were visualized as in figure 3. The AO4B05 epitope is present to a high degree at the surface of wild-type CHO cells where cell-cell contacts are made (a1, arrowhead). Staining is not visible in any of the CHO mutant cell lines (a2-a4). Wild-type CHO cells are moderately stained at the surface by antibody RB4CD12 at places of cell-cell contact (b1, arrowhead). In CHO mutant cell line pgsD-677 a faint granular perinuclear staining is visible (b2, arrow), whereas cell lines pgsF-17 and pgsA-745 show a slightly elevated background staining (b3 and b4, respectively). The epitope recognized by RB4EA12 does not appear at the surface in any of the CHO cell lines but shows a distinct, perinuclear, and granular staining in all CHO cell lines used. In wild-type, pgsD-677, and pgsF-17 cells, these granules are predominantly located at the perinuclear region on one side of the cell (c1-c3, arrows). In pgsA-745 cells, the granular staining is present around the entire nucleus (c4). Scale bar, 25 μm.

Wild-type CHO cells showed a clear surface staining at sites of cell-cell contact when incubated with antibodies AO4B05, AO4B08, AO4F12, RB4CB9, and RB4CD12 (Table 3, Fig. 4a,b), whereas incubation with RB4EA12 and RB4EG12 did not (Table 3, Fig. 4c). None of the glycosylation-defective CHO mutant cell lines showed any surface staining (Fig. 4). As in the S27 cell line, some antibodies showed a distinct staining of perinuclear and cytosolic granules (Table 3, Fig. 4).
**Anti-HS antibodies bind distinct HS epitopes in skeletal muscle basal lamina**

Incubation of cryosections of human, rat, and mouse skeletal muscle with each of the anti-HS antibodies yielded a clear staining of the muscle BL, which was similar in the species examined (Table 3, Fig. 5). Staining patterns of the antibodies on muscle BL were mutually distinct, ranging from a strong staining of the entire BL (AO4B05, AO4B08, AO4F12, RB4CB9, and RB4CD12), to a staining concentrated in (RB4EA12), or almost exclusive for (RB4EG12) the sBL. Antibodies AO4B05, AO4F12, RB4CD12, RB4EA12, and RB4EG12 stained the sBL more intensely than the extrasynaptic BL. The BL of neural tissues showed very strong (AO4F12, RB4CD12, and RB4EA12), strong (AO4B05), or moderate (AO4B08, RB4CB9, and RB4EG12) staining. BLs of blood vessels showed strong to moderate (AO4B05, AO4B08, AO4F12, RB4CB9, and RB4CD12) or no (RB4EA12 and RB4EG12) staining. The latter two antibodies hardly stain muscle BL extrasynaptically and appear to be neuron- and synapse-specific. Most antibodies that stain blood vessels showed differences in staining intensity between arteries, large blood vessels, and capillaries.

The staining patterns of anti-HS antibodies provided convincing evidence for the existence and unique distribution of multiple HS epitopes within the skeletal muscle BL. To investigate the distribution of these HS epitopes with regard to the sBL, cryosections containing NMJs were incubated both with the antibodies and with TRITC-conjugated α-bungarotoxin. α-Bungarotoxin exclusively binds AChRs, thus allowing identification of NMJs. The AO4F12 epitope does not fully colocalize with AChR clusters, yet there is considerable overlap between the distribution of the AO4F12 epitope in the sBL and the presence of dense patches of AChR on the postsynaptic membrane (Fig. 5a1-a3). RB4CD12, on the other hand, showed an almost complete colocalization with AChR clusters (Fig. 5b1-b3). RB4EA12 showed a strong preference for neural and synaptic BL, thus completely colocalizing with AChR clusters in NMJs (Fig. 5c1-c3). Finally, the RB4EG12 epitope showed a moderate staining that was limited to neural and synaptic BLs only (Fig. 5d1-d3).

**HS epitopes recognized by anti-HS antibodies abound in T. marmorota electric organ**

Because the anti-HS antibodies showed differential staining patterns with regard to nerve- and muscle-derived (extrasynaptic and synaptic) BLs, and to investigate whether the HS epitopes are present in non-mammalian species as well, we tested the antibodies for BL staining in the electric organ of the electric ray (T. marmorota). The electric organ is evolutionary derived from muscle tissue and shows dense patches of AChR clustering on the innervated face of the electrocytes. The various anti-HS antibodies showed a distinct staining of the electric organ (Table...
Figure 5. Staining of mouse skeletal muscle basal lamina with anti-HS scFv antibodies. Cryosections of C3H skeletal muscle were incubated with periplasmic fractions of anti-HS antibodies AO4F12 (a), RB4CD12 (b), RB4EA12 (c), and RB4EG12 (d), respectively. Bound antibodies were visualized by incubation with rabbit polyclonal anti-c-Myc IgG followed by Alexa 488-conjugated goat anti-rabbit IgG (a1-d1). AChR clusters present in the neuromuscular junction were visualized using TRITC-conjugated alpha-bungarotoxin (a2-d2). Double-label micrographs (a3-d3) show in yellow the colocalization of the HS epitopes bound by the scFvs and AChR clusters. The epitope recognized by AO4F12 is present in endoneural and perineural, as well as in endomysial BLs, but is clearly more abundant in synaptic versus extrasynaptic BL (a1). Note that this epitope does not fully colocalize with AChR clusters; there is a clear overlap from the BL epitope recognition (green) via a zone in which both epitopes are present (yellow) to the dense patches of AChR (red) (a3). The RB4CD12 epitope is also present throughout neural and endomysial BLs and is slightly more abundant at NMJs (b1), but covers the entire region of AChR clustering (b3). Antibody RB4EA12 stains epitopes present in neural BL to a larger extent than those present in endomysial BL (c1, c3, arrows), shows a high abundancy in sBL (c1), and covers areas of AChR clustering entirely (c3). The epitope recognized by RB4EG12 hardly stains endomysial BL but resides in neural BL and at NMJs (d1), where it does not completely cover areas of AChR clustering (d3). Scale bar, 50 μm.
Figure 6. Staining of *Torpedo* electric organ with anti-HS scFv antibodies. Cryosections of *Torpedo* electric organ were incubated with periplasmic fractions of anti-HS scFv antibodies AO4B05 (a), AO4F12 (b), and RB4CB9 (c), respectively. Bound antibodies (a1-c1 and a2-c2) and AChR clusters (a3-c3) were visualized as in figure 5. Double-label micrographs (a4 to c4) show in yellow the colocalization of AChR clusters and the HS epitopes bound by the scFvs. The AO4B05 epitope is present in large quantities in the endoneurium (a1, asterisks) and in electrocyte BLs (a2) but hardly at all in the perineurium (a1, arrows). Double staining of electrocytes reveals that this epitope is present not only on the innervated face but throughout the electrocyte BL (a4, arrows).

The epitope recognized by AO4F12 is present in the endoneurium (b1, asterisk), but is more abundant in the perineural BLs (b1, arrows). In electrocytes, the presence of this epitope on the innervated face is less pronounced, whereas specific regions of the non-innervated membrane accommodate the epitope in large amounts (b2). Double staining shows this epitope to be partially colocalized with AChR clusters on the electrocyte-innervated membrane (b4). The endoneurium is not stained by antibody RB4CB9 (c1, asterisk), whereas this epitope is very abundant in the perineurium (c1, arrows). In electrocytes, its presence is restricted to the innervated membrane (c2-c4). Scale bar, 50 μm.

3, Fig. 6), ranging from a strong staining of both electrocyte and endoneural BLs (AO4B05 (Fig. 6a1-a4), AO4B08, AO4F12 (Fig. 6b1-b4), and RB4CD12), to a predominantly strong (RB4CB9 (Fig. 6c2-c4)), moderate (RB4EG12), or weak (RB4EA12) staining of the electrocyte BL. AO4B08, AO4F12 (Fig. 6b2-b4), and RB4CD12 stained specific regions of the electrocyte with a higher intensity. AO4F12 (Fig. 6b1), RB4CB9
(Fig. 6c1), and, to a lesser extent, AO4B08 and RB4CD12 stained the perineural BL. The endoneural BL was strongly stained by AO4B05 (Fig. 6a1) and to a lesser extent by AO4B08, AO4F12 (Fig. 6b1), RB4CD12, and RB4EG12.

Double-label micrographs of the epitope recognized by AO4B05 with alpha-bungarotoxin showed that the AO4B05 epitope partially colocalizes with the dense patches of AChR clusters on the innervated face of the electrocytes (Fig. 6a4). Double labeling of the AO4F12 epitope showed a slightly lower staining intensity of the non-innervated membrane, as compared with the innervated membrane, except for some brightly stained regions (Fig. 6b4). The RB4CB9 epitope was almost exclusively located at the electrocyte-innervated face, virtually completely colocalizing with the AChR clusters (Fig. 6c4).

Anti-HS antibodies show a developmental occurrence of HS epitopes in skeletal muscle basal lamina

The diversity of staining patterns obtained with the antibodies in mature skeletal muscle prompted us to investigate the occurrence of HS epitopes during muscle development. Special attention was paid to changes in the occurrence of specific HS epitopes within the endomysial, neural, and synaptic BL. This study was performed in three ways. First, cryosections of rat embryos at various developmental stages (days 10, 13, 16, and 19 in utero) were studied. In this way, the occurrence of and possible changes in BL-HS epitopes during muscular development and synaptogenesis could be studied in the presence of both muscular and neural tissue. Second, cultures of the mouse skeletal muscle cell line C2C12 at developmental stages ranging from half-confluent to 15 d of differentiation were analyzed. In doing so, we could monitor the presence of and changes in HS epitopes during myogenesis, as well as during the clustering of AChRs in the presence of muscular tissue only. Third, cryosections of denervated skeletal muscle of rat were studied. In denervated muscle cells, we looked at a possible upregulation or downregulation of HS epitopes as a result of the regeneration process.

In early embryonic stages of the rat (days 10-16), strong staining of the endomysial as well as a distinct interaction with neural BL was observed on immunostaining with AO4B05, AO4B08, AO4F12, RB4CB9, and RB4CD12. Antibody RB4EA12 predominantly stained neural tissue, whereas RB4EG12 showed an amorphous staining in developing muscle regions (data not shown). Rat embryos at day 19 in utero showed a more defined organ texture in cryosections, which enabled us to examine the presence of the HS epitopes in greater detail (Table 3, Fig. 7). Although RB4CD12 showed a strong staining of the entire neural and endomysial BL, the staining intensity was markedly lower in the sBL (Fig. 7a). RB4CB9, on the other hand, stained the sBL considerably stronger than the extrasynaptic BL (Fig. 7b). RB4EG12, binding HS epitopes present in
neural and synaptic BL in fully developed skeletal muscle, strongly interacted with HS epitopes within the sBL and showed a faint, although definite staining of the extrasynaptic BL (Fig. 7c). The epitope recognized by RB4EA12, preferentially staining neural tissue and sBL in mature muscle, could hardly be visualized in BL of skeletal muscle tissue at day 19 of rat embryogenesis. However, this antibody did stain large cytosolic granules (Fig. 7d). Staining of BL in tissues other than skeletal muscle was also observed (data not shown).
Figure 8. Staining of C2C12 muscle cell line with scFv antibody AO4B05 during differentiation. C2C12 cultures were grown to confluency and subsequently differentiated up to 15 days. Cultures of different developmental stages (half confluent (a), 1 d (b), 8 d (c1, c2), and 15 d (d1, d2) of differentiation) were fixed and subsequently incubated with the periplasmic fraction of scFv antibody AO4B05. Bound scFv antibodies (a, b, c1, d1) and AChR clusters present on the surface of multinucleated myotubes (c2, d2) were visualized as in figure 5. The epitope recognized by AO4B05 is present to a high degree at the surface of myoblasts in regions where cell-cell contacts are made (a). In aligning myoblasts (b), a clear surface staining is visible that becomes more pronounced after myotube formation at sites where AChR clusters develop (c1, c2, arrows). As differentiation proceeds, both the staining of the BL and the staining at AChR clusters decrease (d1, d2, arrows). Scale bar, 25 μm.

Cultures of mouse skeletal muscle cell line C2C12 were incubated with antibodies at stages ranging from half-confluent to 15 d of differentiation (Table 3, Fig. 8-10). Immunostaining with AO4B05, AO4B08, AO4F12, RB4CB9, and RB4CD12 resulted in a strong staining of the myoblast surface. An intense staining was observed at places where myoblasts made contact. However, upon alignment and fusion (processes that trigger BL formation), the entire myotube surface was stained. AChR clusters, which develop on the surface of multi-nucleated myotubes at approximately day 3 of differentiation, were also stained by these antibodies. AO4B05 (Fig. 8), RB4CB9, and RB4CD12 (Fig. 9), especially, showed an enhanced staining of the myotube BL at sites of AChR
Figure 9. Staining of C₂C₁₂ muscle cell line with scFv antibody RB4CD12 during differentiation. C₂C₁₂ cultures were grown to confluency and subsequently differentiated up to 15 days. Cultures of different developmental stages (half confluent (a), 1 d (b), 8 d (c₁, c₂), and 15 d (d₁, d₂) of differentiation) were fixed and subsequently incubated with the periplasmic fraction of scFv antibody RB4CD12. Bound scFv antibodies (a, b, c₁, d₁) and AChR clusters present on the surface of multinucleated myotubes (c₂, d₂) were visualized as in figure 5. The RB4CD12 epitope can be visualized at the myoblast surface at sites where cells have made mutual contacts (a). Note the perinuclear staining of the myoblasts at half-confluent stage (a, arrowheads). After alignment, a strong surface staining is visible (b). During myotube formation, this staining intensifies, especially at sites of AChR clustering (c₁, c₂, arrows). Ongoing differentiation does not lead to reduced AChR cluster staining, whereas the overall staining of the BL decreases slightly (d₁, d₂, arrows). Scale bar, 25 μm.

clustering. A striking feature was that the overall staining intensity decreased strongly during differentiation for antibodies AO4B05 (Fig. 8), AO4B08, and AO4F12. Antibodies RB4EA12 and RB4EG12 were not able to stain the surface of C₂C₁₂ cells at any stage of differentiation. Both antibodies stained small cytosolic granules which were predominantly present near the nuclei (Fig. 10).

Incubation of cryosections of rat skeletal muscle 11 days after denervation with various anti-HS antibodies did not result in staining patterns that were any different from control muscle. After heparinase treatment, no differences in staining intensity with anti-HS stub antibody 3G10 could be seen in BLs of denervated, versus control muscle (data not shown).
Figure 10. Staining of C2C12 muscle cell line with scFv antibody RB4EA12 during differentiation. C2C12 cultures were grown to confluency and subsequently differentiated up to 15 days. Cultures of different developmental stages (half confluent (a), 1 d (b), 8 d (c1, c2), and 15 d (d1, d2) of differentiation) were fixed and subsequently incubated with the periplasmic fraction of scFv antibody RB4EA12. Bound scFv antibodies (a, b, c1, d1) and AChR clusters present on the surface of multinucleated myotubes (c2, d2) were visualized as in figure 5. The epitope recognized by RB4EA12 is not present at the surface of myoblasts in regions where cell-cell contacts have been made (a). Note the faint staining in the perinuclear region of myoblasts at the half-confluent stage (a, arrowheads). A granular staining within the cytosol is visible in early stages of alignment (b) but disappears during myotube formation (c1). During further differentiation, no BL staining is seen at AChR clusters (d1, d2, arrows). Nevertheless, a faint perinuclear staining occurs in some myotubes displaying AChR clusters (d1, d2, arrows). Scale bar, 25 µm.

Discussion

In this paper, we report the selection of a set of unique anti-HS scFv antibodies. The HS epitopes recognized by these antibodies are shown to be differentially distributed in BLs of both developing and mature skeletal muscle. GAG preparations isolated from mouse and human skeletal muscle specimens were used to select a series of anti-HS antibodies by phage display. Despite the enrichment of the muscle GAG preparations for DS, only anti-HS antibodies were selected. To our knowledge, no anti-DS antibodies have been described so far.
In ELISA, all anti-HS scFv antibodies showed a differential reactivity with several HS preparations and with heparin, reflecting the epitope specificity of each antibody. The requirement of both N- and O-sulfate groups for proper epitope recognition was shown by desulfation of heparin, which is known for its high number of disaccharide units and high levels of N-sulfation. Desulfation completely abolished recognition by all antibodies, and N-resulfation could not restore the heparin-antibody interaction. Because CS and DS are not bound by any of the antibodies, sulfation patterns specific for HS are likely to be important in the structure of the epitopes involved.

In our experiments, CHO cells showed a distinct HS staining for most antibodies, which was less intense than the staining of C2C12 cells. This is probably because HS from CHO cells is relatively poorly sulfated (40-45% N-sulfation and ~0.8 sulfate/disaccharide (Bame et al., 1991)). None of the cell-surface HS epitopes recognized by any of the antibodies described here could be detected in cell lines that are defective in GAG synthesis. This was the case with the S2, cell line, a genetic variant of the C2 mouse skeletal muscle cell line, which is severely hampered in GAG synthesis but does align and fuse to form myotubes during differentiation (Gordon and Hall, 1989). Several CHO mutants defective in GAG synthesis caused by the loss or impaired functioning of enzymes involved in glycosylation (Esko et al., 1985; Lidholt et al., 1992) failed to show any cell surface staining, which indicates their inability to properly synthesize the HS epitopes involved. The granular staining seen with some antibodies in many cells may reflect the staining of certain cellular compartments like the Golgi apparatus or lysosomes. Because of the defective cellular machinery for the correct synthesis of GAGs, immature HS epitopes or degradation products of HS molecules may be confined to these organelles.

All anti-HS scFv antibodies showed distinct reactivity in immunofluorescence with the BL of mature skeletal muscle. Staining patterns of the antibodies on human and rat muscle were consistent with those obtained on mice, reflecting an interspecies conservation of the epitopes involved. Most antibodies stained the entire muscular BL, but some antibodies showed a more intense staining in synaptic regions. Because of the presence of junctional folds in the postsynaptic membrane, BL is two- to threefold more abundant at NMJs than extrasynaptically (Sanes and Chiu, 1983). This local concentration of BL might explain the higher staining intensity of some antibodies at the NMJ, but we did not observe a higher abundance of HS in the synaptic cleft by heparitinase III digestion and anti-stub staining. A more appealing explanation is the possibility that certain HS epitopes are specifically concentrated in the sBL. The incomplete overlap of the AO4F12 epitope with AChR clusters, in contrast with e.g. RB4CD12 and RB4EA12 (Fig. 5), suggests differences in location of these epitopes within the sBL. Antibodies that predominantly recognize epitopes present in neural and synaptic BL,
such as RB4EA12 and RB4EG12, may indicate the neural origin of the epitopes involved. Results obtained on aneurally cultured skeletal muscle cells support this view, because these antibodies did not stain BLs at sites of AChR clustering (see further). The synapse-specific occurrence of distinct HS epitopes may prove to be causal for the restricted location of NMJ-resident, HS-binding proteins such as agrin, acetylcholine esterase, growth factors, and certain laminin isoforms.

Most HS epitopes recognized by the antibodies proved to be located close to AChR clusters, present on the innervated face of electrocytes, in the electric organ of the electric ray (*T. marmorata*). Anti-HS antibodies recognized their epitopes, which were embedded in mutually distinct patterns and quantities within neural BLs and in BLs on both the innervated and non-innervated side of the electrocytes. Despite the conserved distribution of the epitopes with regard to neural, synaptic, and extrasynaptic BL among the mammals tested, the distribution within the elasmobranch electric organ appeared to differ. Extracellular matrix isolated from *Torpedo* electric organ can induce AChR clustering in fibroblasts (Hartman et al., 1991). The heavily glycosylated HSPG agrin appears to be involved in the clustering of AChR in *Torpedo* electrocytes (Cartaud et al. 1996). The staining patterns of our anti-HS antibodies on cryosections of the electric organ add proof to the mutually distinct HS epitopes involved and raise curiosity about their function in organ morphogenesis.

During myogenesis in developing rat embryos, some of the HS epitopes were present in endomysial and synaptic BL in a pattern different from that seen in mature muscular tissue. Because NMJs appear between day 14 and 16 of embryonic life (Engel, 1994), the occurrence of HS epitopes during synaptogenesis was investigated on cryosections of embryos at days 10-19 in utero. Most antibodies stained endomysial as well as neural BLs during embryonic muscular development, as may be expected on the basis of their staining patterns in mature skeletal muscle tissue. However, clear differences in developmental appearance could be distinguished for epitopes recognized by some antibodies (RB4CB9, RB4CD12, RB4EA12, and RB4EG12) especially at sites of synaptogenesis. Local binding of growth factors and cytokines to specific HS sequences, as reviewed recently by Lyon and Gallagher (1998), may prove to be elemental in tissue morphogenesis. The distinct distribution in both time and space of these HS epitopes argues for such a regulatory mechanism.

The HS epitopes recognized by our antibodies were present in *C2C12* skeletal muscle cells at various stages of differentiation. Aneurally grown *C2C12* myoblasts start aligning when they reach confluency. When culture medium is changed to differentiation medium containing 10% rat brain extract, AChR clusters appear at approximately day 3 of differentiation (Portiér et al., 1999). On mutual contact, *C2C12* myoblasts expressed most of the HS epitopes described in this paper in large quantities on their surface. Alignment and fusion resulted in a complete
staining of the newly formed BL by corresponding antibodies. These observations are in accordance with the 3-fold increase in HS synthesis in myotube cultures, compared with proliferating or aligning cultures (Noonan et al., 1986). Some antibodies showed steady levels or even a marked increase in overall staining intensity of the BL during further differentiation, consistent with the upregulation of the HSPG glypican during C2C12 differentiation (Brandan et al., 1996). Overall BL staining intensity of other antibodies decreased during later stages of differentiation. These results may be related to observations of Larraín et al. (1997a,b) on downregulation of the HSPGs perlecan and syndecan-1 during C2C12 cell differentiation. AChR cluster formation was accompanied by a strong local increase of certain HS epitopes, arguing for a possible role of these epitopes in the clustering of this ion channel. Antibodies RB4EA12 and RB4EG12 were not capable of BL staining at any stage of C2C12 cell differentiation, in accordance with their supposed neural origin.

Attempts to detect possible changes in the abundance of HS epitopes in denervated skeletal muscle proved to be elusive. Endomysial and neural BLs persist after damage or degeneration of either muscle or nerve cells, or both (Hall and Sanes, 1993). Synaptic and extrasynaptic proteoglycan deposits are conserved in both size and morphology in denervated skeletal muscle (Anderson et al., 1984), serving as scaffolds for the regeneration of both muscle and nerve tissue, thus causing NMJs to develop at sites where they were present before the degeneration. Moreover, Fadic and co-workers (1990) reported proteoglycan synthesis to be upregulated after denervation. Recently, GAGs were shown to be potent stimulants of insulin-like growth factor-1-mediated muscle reinnervation (Gorio et al., 1998). Because HS binds several growth factors involved in tissue morphogenesis and because of the unique distribution of certain HS epitopes, we suspect certain roles for HS epitopes in this regeneration process.

In conclusion, we show that it is possible to select for highly specific anti-HS antibodies against GAG preparations from skeletal muscle. The antibody-defined HS epitopes have distinct distribution characteristics in skeletal muscle BL and are similarly distributed in humans, rats, and mice. Obvious differences in extrasynaptic and synaptic BL staining were observed in mature versus developing skeletal muscle. The unique distribution patterns in skeletal muscle of the HS epitopes recognized by the scFv antibodies described in this article, both in time and in space, raise questions as to the biological roles of these HS epitopes. Of special interest are their roles in myogenesis, more specifically in synaptogenesis and the accompanying postsynaptic specializations such as the clustering of AChRs and other ion channels. The occurrence of these HS epitopes in HSPGs that have already been implicated in developmental processes awaits further investigation. Tools are now available to study more accurately the role of HS epitopes separate from their core protein.
References


Chapter 3

Large, Tissue-Regulated, Domain Diversity of Heparan Sulfates Demonstrated by Phage Display Antibodies

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Heparan sulfates (HS) are long, linear polysaccharides with a high degree of variability. They bind to a vast number of proteins such as growth factors and cytokines, and these interactions are likely to be mediated by specific HS domains. To investigate the structural diversity and topological distribution of HS domains in tissues, we selected a panel of 10 unique anti-HS antibodies using phage display technology. All 10 antibodies recognize a specific HS epitope as demonstrated by ELISA using defined synthetic HS oligosaccharides, modified HS/heparin molecules, and HS isolated from a variety of organs. The chemical groups involved in the epitopes could be indicated, the position of sulfate groups being of major importance. All HS epitopes have a defined tissue distribution as shown by immunohistochemistry using rat organs.

Taken together, data show that in vivo a large number of defined HS epitopes exists, which do not occur randomly, but are tightly, topologically, regulated.

Introduction

Heparan sulfates (HS), a class of glycosaminoglycans, are long linear complex polysaccharides covalently bound to a protein core. They have an ubiquitous distribution in the extracellular matrix and on cell surfaces and have been implicated in many basic biological phenomena such as cell migration, adhesion and differentiation. They play a role in such diverse processes as growth factor/cytokine handling, enzyme regulation, lipid metabolism, blood coagulation, and viral entry (Kjellén and Lindahl, 1991; Ruoslahti and Yamaguchi, 1991; Salmivirta et al., 1996; Iozzo, 1998). This involvement is mediated by interactions of HS with a vast number of proteins such as growth factors/ cytokines, enzymes, protease inhibitors, extracellular matrix molecules and viral coat proteins (Conrad, 1998; David, 1993). The large number of interactions suggests an extensive structural variation within HS. Chemical analysis of HS-derived disaccharides indeed indicates a large structural diversity (Lyon et al., 1994; Sanderson et al., 1994; Maccarana et al., 1996), which is brought about by specific chain modifications during HS biosynthesis. The importance of defined monosaccharide sequences for specific interactions with proteins has been demonstrated for the binding and activation of anti-thrombin III by HS/heparin (Atha et al., 1984; Lindahl et al., 1984). In addition, specific structural requirements for binding to basic fibroblast growth factor and hepatocyte growth factor have been defined (Ashikari et al., 1995; Faham et al., 1996). These observations indicate that HS modifications do not occur randomly, but that a controlled expression
of specific domains/sequences in HS exists. To investigate if indeed a large number of different HS domains in tissues occurs, we selected a panel of epitope-specific anti-HS antibodies using phage display technology. Using these antibodies we chemically and topologically characterized the HS epitopes involved. We chose antibody phage display because it allows for the generation of antibodies against poorly immunogenic molecules such as HS.

Materials and Methods

Materials

A human semi-synthetic antibody phage display library (Nissim et al., 1994, now officially named synthetic scFv Library #1) was generously provided by Dr. G. Winter, Cambridge University, Cambridge, U.K. This library contains 50 different VH genes with synthetic random complementarity determining region 3 segments, which are 4-12 amino acid residues in length. The heavy chains are combined with a single light chain gene (DPL 16). The library contains > 10^6 different clones. All antibodies contain a c-Myc tag.

Heparan sulfate from bovine kidney, chondroitinase ABC (Proteus vulgaris, EC 4.2.2.4), chemically modified heparan sulfate and heparin kits, anti-chondroitin sulfate "stub" antibody (2B6), and anti-heparan sulfate "stub" antibody (3G10) were from Seikagaku Kogyo Co., Tokyo, Japan. Heparin from porcine intestinal mucosa, chondroitin 4-sulfate from whale cartilage, chondroitin 6-sulfate from shark cartilage, dermatan sulfate from porcine skin, keratan sulfate from bovine cornea, hyaluronate from human umbilical cord, DNA from calf thymus, dextran sulfate, bovine serum albumin (fraction V), heparinase I (from Flavobacterium heparinum, EC 4.2.2.7), heparinase II (from Flavobacterium heparinum), and heparinase III (heparitinase, from Flavobacterium heparinum, EC 4.2.2.8), were from Sigma (St Louis, MO). Alkaline phosphatase-conjugated rabbit anti-mouse IgG was from Dakopatts (Glostrup, Denmark). Mouse anti-c-Myc tag IgG (clone 9E10) was from Boehringer Mannheim (Mannheim, Germany). Alexa 488-conjugated goat anti-mouse IgG was from Molecular Probes (Eugene, OR). PCR kit was from Promega (Madison, WI). ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit was from PE Applied Biosystems (Norwalk, CT). Mowiol (4-88) was from Calbiochem (La Jolla, CA). Restriction enzyme BstNI was from New England Biolabs (Beverly, MA). Polystyrene Maxisorp Immunotubes were from Nunc (Roskilde, Denmark). Various HS preparations were a generous gift from Dr. Keiichi Yoshida, Seikagaku Corp. (Japan). Synthetic HS oligosaccharides were all from
Selection of antibodies against heparan sulfate by library panning

Phage display-derived antibodies were obtained as described (van Kuppevelt et al., 1998), using 4 rounds of panning against bovine kidney HS, human lung HS (HS isolated using papain/alkaline borohydride digestion followed by anion exchange chromatography), and mouse and human skeletal muscle HS (Jenniskens et al., 2000). Briefly, antibody-expressing phages were added to HS-coated tubes and bound phages were eluted at high pH in order to allow for infection of E. coli TG1 cells. After overnight amplification, phage were rescued by addition of helper phage and used for further rounds of selections. After the last two rounds, single bacterial colonies were picked and the antibodies expressed were evaluated.

Screening for bacteria expressing antibodies against heparan sulfate

Screening for bacteria expressing anti-HS antibodies was performed as described (van Kuppevelt et al., 1998). Briefly, single colonies picked from the last 2 rounds of selection were grown in 96-well polystyrene round bottom plates until bacterial growth was visible. Antibody production was induced by adding isopropyl-β-D-thiogalactopyranoside. Plates were centrifuged and the supernatant containing soluble antibodies was applied to wells of polystyrene microtiter plates previously coated with HS. Bound antibodies were detected using a mouse monoclonal antibody (9E10) directed against the c-Myc tag, followed by incubation with alkaline phosphatase-conjugated rabbit anti-mouse IgG. Alkaline phosphatase activity was detected using p-nitrophenyl phosphate as a substrate. Absorbance was measured at 405 nm.

Screening bacterial clones for unique antibody inserts using PCR, fingerprinting, and sequencing

Bacteria expressing anti-HS antibodies were analyzed for the presence of full length inserts by 25 cycles of PCR using plasmid DNA as template (van Kuppevelt et al., 1998). Fingerprinting was performed using BstNI (CC<sup>AA</sup>/GG) as the restriction enzyme. PCR and fingerprinting analysis were performed using 1% (w/v) and 4% (w/v) agarose gels respectively. To establish the complementarity determining region 3, and the germ line V<sub>H</sub> gene DNA segments, unique clones were sequenced using FOR LINK SEQ RIC (5'-GCCACCTCCGCTGAACC-3') as the
primer (located in the linker region between the \( V_H \) and the \( V_L \) gene). For this purpose, dsDNA was isolated using standard procedures.

**Source of antibodies**

To obtain optimal amounts of soluble anti-HS antibodies, phages were allowed to infect the non-suppressor *E. coli* strain HB2151. The periplasmic fraction of bacteria was used as the source of antibodies (van Kuppevelt *et al.*, 1998). Briefly, bacteria were grown at 37°C until an optical density of 0.5-0.8 was reached. Induction was obtained by addition of isopropyl-\( \beta \)-D-thiogalactopyranoside. After incubation at 30°C for 3h, the culture was harvested, and the pellet resuspended in 200 mM Na-borate buffer (pH 8.0) containing 160 mM NaCl and 1 mM EDTA. After centrifugation the supernatant was filtered and dialyzed versus PBS. The preparation thus obtained is the periplasmic fraction.

**Characterization of antibodies by ELISA**

Reactivity of the anti-HS antibodies with various molecules was evaluated in ELISA in two ways: a) by application of antibodies to wells of polystyrene plates coated with the test molecules, and b) by an inhibition assay in which the antibodies were incubated with the test molecule for 16 h at 22°C, followed by transfer to wells previously coated with HS (bovine kidney, (van Kuppevelt *et al.*, 1998)). Test molecules included HS from bovine kidney, aorta, lung, intestine, human aorta, pig intestine and whale lung, heparin, dermatan sulfate, chondroitin 4- and 6-sulfate, keratan sulfate, dextran sulfate, hyaluronate, K5 polysaccharide from *E. coli*, bovine serum albumin (BSA), marvel (the non-fat milk preparation using as a blocking reagent during panning) and DNA. To analyse which chemical groups in HS are involved in antibody-binding, a large number of modified HS preparations and synthetic HS-oligosaccharides were evaluated. They included HS from bovine kidney which was digested with heparinase I, II or III, or with HNO\(_2\) at pH 1.5, and chemically modified HS (bovine kidney) and heparins (porcine intestine). The latter included preparations that were completely desulfated and \( N \)-acetylated, preparations that were completely \( O \)-desulfated and \( N \)-sulfated, and preparations that were \( N \)-desulfated and \( N \)-acetylated. The antibodies were also analyzed using a panel of 12 defined synthetic HS oligosaccharides.

Bound antibodies were detected using anti c-Myc tag mouse monoclonal antibody 9E10 and alkaline phosphatase-conjugated rabbit anti-mouse IgG antibody. Enzyme activity was detected using p-nitrophenyl phosphate as the substrate and absorbance was read at 405 nm.
**Characterization of antibodies and localization of HS epitopes by immuno-histochemistry**

Various rat tissue specimens (Wistar, male, 3 months old) were snap-frozen in liquid isopentane cooled with liquid nitrogen, and stored at -70°C. Cryosections (5 μm) were rehydrated for 10 min in PBS, blocked with PBS containing 1% (w/v) BSA, and incubated with anti-HS antibodies for 90 min at 22°C. As a control, antibodies from non-related clones were used. These antibodies did not stain rat tissue sections. Antibodies were detected by incubation with mouse anti-c-Myc tag antibodies (9E10, culture supernatant), followed by alexa 488-conjugated goat anti-mouse IgG (1:200 in PBS containing 1% BSA), both for 90 min at 22°C. After each incubation, sections were washed in PBS (3 x 10 min). Additional controls were the omission of primary or conjugated antibody.

To evaluate the specificity of the antibodies, cryosections were pretreated with heparinase III (digests HS), and with chondroitinase ABC (digests dermatan and chondroitin sulfates). As control, sections were incubated in the reaction buffer without enzyme. After rinsing and blocking, the sections were incubated with antibodies and processed for immunofluorescence as described. The efficacy of heparinase III treatment was analyzed using antibodies (3G10) against HS “stubs” generated by heparinase III. The efficacy of chondroitinase ABC treatment was evaluated by incubation of sections with antibodies (2B6) against chondroitin sulfate “stubs” generated by chondroitinase ABC. Specificity of the anti-HS antibodies was further tested by overnight preincubation of the antibodies with various molecules, viz. HS (bovine kidney), heparin, dermatan sulfate, chondroitin 4- and 6-sulfate, keratan sulfate, dextran sulfate, hyaluronate and DNA as described (van Kuppevelt et al., 1998), followed by processing for immunofluorescence.

**Results**

**Selection of anti-heparan sulfate antibodies**

Ten antibodies were selected on basis of amino acid sequence of the complementarity determining region 3 and/or V<sub>H</sub>-gene (table 1; Tomlinson et al., 1992). Five antibodies were obtained against bovine kidney HS (antibodies HS3A8, HS3B7, HS4A5, HS4D4 and HS4E4), and three antibodies against human lung HS (antibodies EV3B2, EV3D1 and EV3C3). The antibodies against mouse and human skeletal muscle HS (antibodies AO4B08 and RB4EA12, respectively) have been described previously (Jenniskens et al., 2000).
### Table 1. Some characteristics of the anti-HS antibodies

<table>
<thead>
<tr>
<th>Antibody</th>
<th>DP- gene</th>
<th>VH - family</th>
<th>VH – CDR3 sequence</th>
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</thead>
<tbody>
<tr>
<td>HS3A8</td>
<td>DP – 38</td>
<td>VH – 3</td>
<td>GMRPRL</td>
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<td>VH – 1</td>
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<td>VH – 4</td>
<td>WVTPE</td>
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<td>VH – 3</td>
<td>GYRPRF</td>
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<td>VH – 3</td>
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<td>A04B08</td>
<td>DP – 47</td>
<td>VH – 3</td>
<td>SLRMNGWRAHQ</td>
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<td>RB4EA12</td>
<td>DP – 32</td>
<td>VH – 3</td>
<td>RRAYRDL</td>
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</tbody>
</table>

Given are the DP-gene number (according to Tomlinson et al., 1992), VH-germ line gene family, and amino acid sequence of the VH complementarity determining region 3 (CDR3).

### Table 2. Reactivity of anti-HS antibodies with various HS preparations (ELISA)

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<tr>
<th>Preparation</th>
<th>Antibody</th>
<th>HS3A8</th>
<th>HS3B7</th>
<th>HS4A5</th>
<th>HS4D4</th>
<th>HS4E4</th>
<th>EV3B2</th>
<th>EV3C3</th>
<th>EV3D1</th>
<th>A04B08</th>
<th>RB4EA12</th>
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<td>2 (0)</td>
<td>0 ± 0</td>
<td>4 ± 8</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
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<td>0 ± 0</td>
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<tr>
<td>3 (0.56)</td>
<td>5 ± 1</td>
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<td>0 ± 2</td>
<td>26 ± 15</td>
<td>20 ± 1</td>
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<td>15 ± 1</td>
<td>32 ± 1</td>
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<tr>
<td>4 (0.60)</td>
<td>8 ± 5</td>
<td>18 ± 4</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>232 ± 45</td>
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<td>10 ± 6</td>
<td>32 ± 1</td>
<td>56 ± 2</td>
<td>7 ± 10</td>
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<td>5 (0.84)</td>
<td>23 ± 2</td>
<td>44 ± 4</td>
<td>24 ± 3</td>
<td>3 ± 0</td>
<td>9 ± 10</td>
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<td>9 (&gt;1)</td>
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<td>10 (1.64)</td>
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<td>31 ± 1</td>
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</table>

The ratio of sulfate groups to disaccharide is indicated in parentheses. Values (in % of reactivity with HS from bovine kidney) represent mean ± SD (n = 3).

1, HS from bovine kidney; 2, K5 capsular polysaccharide (E. coli, similar to the HS precursor); 3, HS from bovine aorta; 4, HS from human aorta; 5, HS from bovine kidney (fraction A: elutes from an anion exchange column between 0.7 and 1.1 M NaCl); 6, HS from bovine lung; 7, HS from bovine intestine; 8, HS from bovine kidney (fraction B: elutes from an anion exchange column between 1.1 and 1.25 M NaCl); 9, HS from whale lung; 10, HS from porcine intestinal mucosa.

### Specificity of anti-heparan sulfate antibodies

Using ELISA, antibodies were shown to be specific for HS and heparin, and not to be reactive with other glycosaminoglycans like dermatan sulfate, chondroitin sulfates, hyaluronate and keratan sulfate, nor with other polyanionic molecules like dextran sulfate and DNA. Antibodies show a characteristic reactivity with HS preparations isolated from various sources like bovine kidney, aorta, lung and intestine, human aorta, and whale lung (table 2). None of the antibodies is reactive with the bacterial capsular polysaccharide K5 which is similar to the HS precursor polysaccharide. Therefore, all 10 antibodies recognise specific structural modifications.
Table 3. Reactivity of anti-HS antibodies with modified HS and heparin molecules (inhibition ELISA)

<table>
<thead>
<tr>
<th>GAG</th>
<th>Antibody</th>
<th>HS3A8</th>
<th>HS3B7</th>
<th>HS4A5</th>
<th>HS4D4</th>
<th>HS4E4</th>
<th>EV3B2</th>
<th>EV3C3</th>
<th>EV3D1</th>
<th>AO4B08</th>
<th>RB4EA12</th>
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Numbers (IC$_{50}$) represent the amount of test substance (starting with 100 µg/ml) which results in a 50% inhibition of the binding of the antibody to HS (n=3). See text for explanation.

- 1, HS (bovine kidney); 2, heparin (porcine intestinal mucosa); 3, completely desulfated/N-acetylated heparin; 4, completely desulfated/N-sulfated heparin; 5, N-desulfated/N-acetylated heparin; 6, completely desulfated/N-acetylated HS; 7, completely desulfated/N-sulfated HS; 8, N-desulfated/N-acetylated HS; 9, HNO$_3$ (pH 1.5)-treated HS; 10, Heparinase I-digested HS; 11, Heparinase II-digested HS; 12, Heparinase III-digested HS.

HNO$_3$ cleaves glycosidic linkages involving glucosamine with the amine group either N-sulfated or unsubstituted. Heparinase I cleaves the linkages GlcNS$_6$S-GlcUAaMe; heparinase II the linkages GlcNAcS$_6$S-GlcUA-GlcUAaMe; and heparinase III the linkages GlcNAc-GlcUAaMe and GlcNAcS$_6$S-GlcUAaMe. IC$_{50}$ values in µg/ml.

Table 4. Reactivity of anti-HS antibodies with synthetic HS oligosaccharides (inhibition ELISA)

<table>
<thead>
<tr>
<th>GAG</th>
<th>Antibody</th>
<th>HS3A8</th>
<th>HS3B7</th>
<th>HS4A5</th>
<th>HS4D4</th>
<th>HS4E4</th>
<th>EV3B2</th>
<th>EV3C3</th>
<th>EV3D1</th>
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</table>

Numbers (IC$_{50}$) represent the amount of oligosaccharide (starting with 100 µg/ml) which results in a 50% inhibition of the binding of the antibody to HS (n=2). See text for explanation.

- 1, GlcNAcS$_6$S-GlcUAaMe; 2, IdoUA-GlcNAc-S-GlcUAaMe; 3, GlcNS$_6$S-GlcUA-GlcNAc-S-GlcUAaMe; 4, GlcN-GlcUAaMe; 5, IdoUA2S-GlcNS$_6$S-GlcNS$_6$SMe; 6, GlcUA-GlcNS$_6$S-GlcUAaMe; 7, GlcNAcS$_6$S-GlcUA-GlcUAaMe; 8, GlcNS$_6$S-GlcUA-GlcNAc-S-GlcUAaMe; 9, GlcNS$_6$S-GlcUA-GlcNS$_6$SdiS-GlcUAaMe; 10, GlcNS$_6$S-GlcUA-GlcNS$_6$SdiS-GlcUAaMe; 11, GlcNS$_6$S-GlcUA-GlcNS$_6$SdiS-GlcUAaMe; 12, GlcNS$_6$S-GlcUA-GlcNS$_6$SdiS-GlcUAaMe; GlcNS$_6$S, N-sulfated glucosamine; GlcUA, glucuronic acid; IdoUA, iduronic acid; 6S, 6-O-sulfation; 2S, 2-O-sulfation. IC$_{50}$ values in µg/ml.

Analysis of HS epitopes recognized by the antibodies

Antibodies were tested for reactivity with modified HS/heparin preparations (table 3), and with various synthetic HS oligosaccharides (table 4), to identify which modifications in HS are involved in binding. Results show that all antibodies recognize different structures in HS. In
all cases, sulfate groups are essential. HS and heparin stripped from their O- and N-sulfate groups are not reactive with the antibodies. Treatment of HS with HNO₂ at pH 1.5 ( cleavage at N-sulfated glucosamine residues (GlcNS) and the rare N-unsubstituted glucosamine residues) abolishes antibody binding. Treatment of HS with heparinase I (cleaves at sulfated domain structures) results in loss of binding activity of 8 out of 10 antibodies. For a number of antibodies, structural modifications of HS important for binding could be identified ( tables 3 and 4). HS3A8 recognizes highly sulfated domains within HS, since it reacts only with highly sulfated oligosaccharides. The antibody strongly prefers 6-O-, N-sulfated glucosamine (GlcNS6S) and prefers sulfated iduronic acid (IdoUA2S) over non-sulfated iduronic acid (IdoUA). The preferred sequence (starting at the non-reducing end) is GlcNS6S-IdoUA2S-GlcN6S6. Antibodies HS3B7 and RB4EA12 recognize IdoUA-GlcN6S containing domains, 2-O sulfation of IdoUA impeding binding (compare compound 10 and 12, and 11 and 12, table 4). Differences in topological distribution (see below), however, indicate that each antibody recognizes a different epitope. Antibody HS4A5 recognizes various sequences and the preferred sequence can best be described as GlcNS±6S - IdoU±2S - GlcN±6S. N-sulfation alone is not sufficient for binding (table 3). This generic sequence may indicate the recognition of a wide variety of structures, but the restricted topological distribution (blood vessel-associated basement membranes only, see below) argues against this (Fig. 1). Antibodies HS4D4 and AO4B08 are reactive with domains containing GlcNS6S-IdoUA2S-GlcN6S5 sequences. Non-sulfated IdoUA impedes binding (compare compound 10 and 12, table 4). For AO4B08 an additional 3-O-sulfate group on GlcN6S has no influence (compare compound 9 and 10, table 4). HS4D4, however, requires an additional modification possibly 3-O sulfation of GlcN6S (compare reactivity of HS4D4 and AO4B08 for compound 5, table 4). N-sulfation seems of minor importance (table 3). HS epitopes recognized by the two antibodies have a different topological distribution (see below). Antibody HS4E4 is not reactive with any of the synthetic HS oligosaccharides tested. It is the only antibody that reacts with completely desulfated/N-sulfated heparin/HS (table 3), suggesting that N-sulfation is of major importance. It reacts strongly with human aorta HS (table 2), which is largely devoid of 6-O-sulfation (Safaiyan et al., 2000), and which may indicate that this modification is inhibitory. Taken together, these data suggest that HS4E4 recognizes GlcNS-IdoUA2S containing domains. EV3B2 recognizes domains containing GlcNS±6S - UA - GlcNS±6S, where UA represent GlcUA or IdoUA. 2-O-sulfation of IdoUA improves binding (compare compounds 10 and 12, table 4); 3-O-sulfation of GlcNS seems to be of no importance (compare compound 9 and 10, table 4). EV3D1 needs GlcUA in a certain chemical context, preferably flanked by a GlcNS (±6S), but not by a non-sulfated IdoUA (compare compounds 6 and 7, and compounds 10 and 12, table 4). The final antibody, EV3C3, recognizes
HS structures which could not be clearly identified. All antibodies, except EV3C3, EV3D1, and EV3B2, bind to perlecain, a HS-proteoglycan isolated from the ECM of the Engelbreth-Holm-Swarm mouse sarcoma (Hassell et al., 1980) (data not shown).

Table 5. Distribution of HS-epitopes defined by antibodies in rat kidney

<table>
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<th>Antibody</th>
<th>HS3A8</th>
<th>HS3B7</th>
<th>HS4A5</th>
<th>HS4D4</th>
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<th>EV3B2</th>
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<td>6</td>
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</table>

Cryosections were processed for immunofluorescence as described (van Kuppevelt et al., 1998). 1, Glomerulus; 2, Bowman's capsule; 3, Peritubular capillaries; 4, Cortical tubules; 5, Smooth muscle cells; 6, Large blood vessel endothelium. Staining: ++, strong; +, moderate; +/-, weak; -, absent. * Only a subset of tubules is stained.

Localization of HS epitopes using immunohistochemistry

Each antibody showed that the HS structure it recognizes, has a defined topology. In the kidney, the anti-HS antibodies primarily stain basement membranes (Fig. 1, table 5). Antibodies HS4A5, RB4EA12, and HS4E4/AO4B08 (both have a similar topology) recognize structures with a clear different topological distribution compared to structures recognised by the other antibodies. These antibodies show a more or less similar tissue distribution, be it with some difference in staining intensity. Some antibodies (HS3A8 and HS4D4) show a similar topological staining in renal tissue, but a differential distribution in testicular tissue (Fig. 1). Staining of all the antibodies was abolished by pretreatment of sections with heparinase III, but not with chondroitinase ABC. Preincubation of antibodies with HS and heparin precluded staining. Incubation with other glycosaminoglycans, with dextran sulfate, or with DNA has no effect.

Discussion

Next to proteins and nucleic acids, polysaccharides may form a third group of information-dense biopolymers. This especially holds for HS, a class of linear polysaccharides involved in many basic cellular phenomena. Theoretically, cells can generate a large potential of different monosaccharide sequences (domains) in HS (see below). To study
Figure 1. Immunostaining of rat renal (a-f) and testicular tissue (g,h) with anti-HS antibodies. Cryosections were incubated with antibody HS4E4 (a), HS4A5 (b), RB4EA12 (c), a non-related phage display-derived antibody (d), HS3A8 (e, g) and HS4D4 (f, h). Note the similar staining of HS3A8 and HS4D4 in renal tissue (e,f) and the differential staining of these antibodies in testicular tissue (g, h). *: glomerulus. Arrow in g and h: smooth muscle cells. Scale bar: 25 μm (d, g, h) or 50 μm (a, b, c, e, f).

If such a potential indeed exists in vivo, we selected 10 anti-HS antibodies by phage display technology. This technology has proven to be very useful for obtaining antibodies against HS, which are almost non-immunogenic (van Kuppevelt et al., 1998; Jenniskens et al., 2000). All 10 antibodies recognize an unique epitope structure, the position of sulfate groups being of major importance. Together with the 5 anti-HS antibodies already described (van Kuppevelt et al., 1998; David et al., 1992; Raats et al., 2000), and which are different with respect to the ones described here, it implies that at least 15 different HS epitopes are present in the kidney, the nature of the epitopes being highly complex. All epitope structures have a defined topological tissue distribution, indicating that in vivo a tightly controlled biosynthetic apparatus for HS exists. This may also be true for chondroitin sulfates, since a number of anti-chondroitin sulfate antibodies have been generated that show a specific distribution in tissues (Couchman et al., 1984; Sorrell et al., 1990).
The biosynthesis of HS allows for a large number of different sequences. The HS precursor polysaccharide chain is made up of repeating disaccharide units which consist of a glucuronic acid and a N-acetylated glucosamine (GlcNAc) residue. These disaccharide units, of which there can be up to a 150 in HS, can undergo a number of modifications. Some GlcNAc residues are N-deacetylated and subsequently N-sulfated, and this step serves as a trigger for further modifications such as epimerization of GlcUA to IdoUA, 2-O sulfation of GlcUA and IdoUA, and 6-O- and/or 3-O-sulfation of GlcN. Modification occurs in specific regions of the polysaccharide chain leading to highly modified domains (N- and O-sulfate rich) interspaced by less modified domains (Kjellén and Lindahl, 1991; Salmivirta et al., 1996). The number of disaccharides units and their possible modifications can (theoretically) give rise to a tremendous structural variation. An octasaccharide, for instance, already has over a million possible monosaccharide sequences. The importance of specific modifications in vivo have been demonstrated. Genetic studies in Drosophila, mice and men reveal the involvement of HS in cell differentiation and morphogenesis. In Drosophila, mutations in genes encoding enzymes involved in HS biosynthesis (sugarless, sulfateless and tout velu) lead to a loss of specific signalling pathways (FGF, Wingless and Hedgehog), resulting in severe alterations in phenotype. In mice, a mutation in the gene encoding 2-O-sulfotransferase, leads to multiple developmental abnormalities, whereas targeted gene disruption in the genes encoding glucosaminyi N-deacetylase/N-sulfotransferase-1 and -2 result in lethal respiratory distress and mast cell abnormalities respectively (Perrimon and Bernfield, 2000). In men, mutations in glycosyltransferases involved in HS biosynthesis (EXT1 and EXT2) lead to hereditary multiple exostoses (McCormick et al., 1998). These findings indicate that specific HS modifications play a crucial role in developmental processes. Our data indicate that indeed a large number of HS epitopes exists in vivo and that these do not occur randomly, but are tightly, topologically, regulated. This indicates the existence of a tightly controlled pattern of modifications occurring during biosynthesis of HS.

Aberrations in HS fine structure have been implied in various pathological conditions including nephropathies, cancer, and bacterial and viral infections (Shieh et al., 1992; van Putten and Paul, 1995; Chen et al., 1997; Klieff et al., 1998; Raats et al., 2000; Shukla et al., 2000). The antibodies described here, and which probably have a Kd of about 0.1 μM (van Kuppevelt et al., 1998), can be used in ELISA, immunohistochemistry and FACS analysis. They can probably also be used for immunoblotting, although we did not test this.

With the emerging HS sequencing technology (Turnbull et al, 1999; Venkataraman et al., 1999), and new methods to prepare synthetic HS oligosaccharides (Petitou et al., 1999), the antibody-defined HS structures presented here may be characterised at the monosaccharide level. The
epitope defined by anti-HS antibody 10E4 was recently partially characterized (Leteux et al., 2001). Well defined anti-HS antibodies will make it possible to pin-point defined structural modifications, and perhaps even monosaccharide sequences, to biological processes in health and disease.

References


Chapter 4

Spatiotemporal Distribution of Heparan Sulfate Epitopes During Myogenesis and Synaptogenesis: A Study in Developing Mouse Intercostal Muscle

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Formation of a basal lamina (BL) ensheathing developing skeletal muscle cells is one of the earliest events in mammalian skeletal muscle myogenesis. BL-resident heparan sulfate proteoglycans (HSPGs) have been implicated in various processes during myogenesis, including synaptic differentiation. However, attention has focused on the proteoglycan protein core, ignoring the glycosaminoglycan moiety mainly because of a lack of appropriate tools. Recently, we selected a panel of anti-heparan sulfate (HS) antibodies that are applied here to study the spatiotemporal distribution of specific HS epitopes during myogenesis. In mouse intercostal muscle at E14, formation of acetylcholine receptor clusters at synaptic sites coincides with HS deposition. Whereas some HS epitopes show a general appearance throughout the BL, one epitope preferably clusters at synaptic sites, but does so only from E16 onwards. Significant changes in the HS epitope-constitution of both synaptic and extrasynaptic BL were observed during elongation and maturation of primary myotubes, a process preceding secondary myotube development. As a whole, the data presented here strengthen previous observations on developmental regulation by BL components, and add to the putative roles of specific HS epitopes in myogenesis and synaptogenesis.

Introduction

The synaptic cleft of the neuromuscular junction (NMJ) is occupied by a layer of extracellular material, which escheats all vertebrate skeletal muscle fibers: the basal lamina (BL). Several BL components are distributed in distinct synaptic and extrasynaptic regions, whereas others are common (Hall and Sanes, 1993). For many years, it has been recognized that BL-resident proteins play a regulating role in the differentiation and maintenance of both pre- and postsynaptic membranes (Burden, 1998; Meier and Wallace, 1998). Thus far, only few reports on the composition of the BL during myogenesis and synaptogenesis mention proteoglycans.

Proteoglycans are proteins which have a glycosaminoglycan (GAG) chain covalently attached to a core protein. In the case of heparan sulfate proteoglycans (HSPGs), the GAG moiety consists of heparan sulfate (HS), an unbranched chain of 30 to 150 disaccharides. Each HS disaccharide unit, consisting of a glucosamine and an uronic acid residue, can be modified by N-deacetylation, N-sulfation, 2-O-, 3-O-, and/or 6-O-sulfation and C-5 epimerization. The concert of these modifications potentially makes HS a very information-dense biopolymer, to which several proteins may bind in a monosaccharide sequence-specific way.
(Salmivirta et al., 1996; Bernfield et al., 1999; Tumova et al., 2000; Turnbull et al., 2001; Gallagher, 2001). In developmental processes however, the functions of HS have remained mostly under appreciated until recently (Perrimon and Bernfield, 2000).

In recent years, there have been a number of reports on the roles of BL-resident HSPGs in myogenesis and synaptogenesis. These include the roles of agrin in acetylcholine receptor (AChR) clustering (Ruegg and Bixby, 1998; Lin et al., 2001), the involvement of syndecan-2 in neural synaptogenesis (Hsueh et al., 1998), the binding of growth factors in NMJs by perlecan (Peng et al., 1998), the focal immobilization of BL-resident proteins like acetylcholine esterase (AChE) by perlecan (Peng et al., 1999), the involvement of perlecan in the assembly of laminin in the BL (Henry et al., 2001), and the maturation and maintenance of the NMJ and its synaptic BL by the dystrophin-glycoprotein complex and associated HSPGs (Grady et al., 2000; Jacobson et al., 2001).

As early as 1984, it was shown that synaptic and extrasynaptic parts of the muscle BL are subject to dynamic changes during synaptogenesis (Chiu and Sanes, 1984). More specific, expression of two HS-related epitopes (David et al., 1992), perlecan (Larraf et al., 1997a), alternatively spliced isoforms of agrin (Hoch et al., 1993), the HSPG-families of the syndecans (Bernfield et al., 1993; Larraf et al., 1997b; Sogos et al., 1998) and glypicans (Brandan et al., 1996; Litwack et al., 1998), the proteoglycans decorin (Velleman et al., 1999), and SPOCK (Charbonnier et al., 2000; Cifuentes-Diaz et al., 2000) have been shown to be developmentally regulated. Olguin and Brandan recently reported the temporal and spatial co-expression of HSPGs syndecan-3 and decorin with myogenin, a transcription factor responsible for the induction of terminal skeletal muscle differentiation (Olguin and Brandan, 2001). Moreover, Nurcombe and coworkers found evidence for differential glycosylation of a HSPG core protein during murine neural cell differentiation (Nurcombe et al., 1993; Brickman et al., 1998). HS structure itself is also subject to regulation during development and aging in vivo (Feyzi et al., 1998; Guimond et al., 2001). Taken together, HSPGs and especially the sulfation patterns of the HS moiety are considered candidate regulators of developmental signaling (Lander, 1998; Selleck, 2000; Dhoot et al., 2001). However, much has to be learned about the distribution of specific HS epitopes during myogenesis in vivo and the roles of this class of glycosaminoglycans in developmental processes.

Recently, we reported on the selection of a panel of phage display-derived antibodies that recognize specific HS epitopes (Jenniskens et al., 2000). In the present study, we describe the spatiotemporal distribution of several HS epitopes during myogenesis in mouse intercostal muscle, and their distribution with regard to developing synapses. For the first time, specific HS epitopes of the cellular HS constitution are shown to be subject to a dynamic distribution during myogenesis and synaptogenesis in vivo.
Materials and Methods

Materials

C3H mice were obtained from the University of Nijmegen Central Animal Laboratory. All chemicals used were from Merck (Darmstadt, Germany), unless stated otherwise. Bovine serum albumin (fraction V), and NaN₃ were obtained from Sigma (St. Louis, MO). Target Unmasking Fluid was purchased from Boehringer Mannheim (Mannheim, Germany), anti-pan sodium channel rabbit polyclonal IgG (SP19) from Alomone labs (Jerusalem, Israel). Anti-c-Myc tag goat polyclonal IgG (A-14) was from Santa Cruz Biotechnology (Santa Cruz, CA). Alexa 488-conjugated donkey anti-goat IgG, goat anti-rabbit IgG, and Alexa 594-conjugated α-bungarotoxin were purchased from Molecular Probes (Eugene, OR). Mowiol (4-88) was obtained from Calbiochem (La Jolla, CA).

Anti-heparan sulfate antibodies

Preparation of anti-heparan sulfate antibodies (periplasmic fractions) AO4B05, AO4B08, RB4CD12, RB4EA12, and control antibody MPB01 was performed as described (Jenniskens et al., 2000). Antibody MPB01 was randomly selected from scFv library #1. This antibody is a member of VH family 3, has DP segment 38, bears the CDR3 sequence PKRTRN (single-letter amino acid code), and is not reactive with HS or heparin from any source tested, as analyzed by ELISA and IFA techniques.

Pre- and postnatal mouse tissue

C3H mice were bred under standard conditions and monitored for vaginal plugs twice a day. The first day of occurrence of a vaginal plug was taken as day 0 of gestation (E0). Pregnant mice were quarantinised and kept solitary until the litter had reached the indicated E-number (12, 13, 14, 15, 16, 17, 18, and 20 days in utero). Mice were anesthetized and killed by cervical dislocation, in accordance with the ethic regulations of the university. Embryos were isolated either with (E12 and 13) or without (E14-E20) the surrounding uteric tissue, rinsed in PBS, snap-frozen in liquid nitrogen-cooled isopentane, and stored at -80°C. Birth occurred on average at day E20 (P0). Mice of postnatal day 2 (P2) were anesthetized prior to freezing. For each developmental stage, at least three individual mice, derived from two separate litters, were studied. Adult intercostal muscles were difficult to isolate in a way appropriate for cryosectioning, because of the presence of osteofied ribs. We therefore used specimens of the m. soleus, isolated from the pregnant females that were killed, for studying adult NMJs.
**Immunohistochemistry**

5 to 10 µm thick cryosections of whole embryos, newborns or adult tissue specimens were cut, mounted on slides, dried thoroughly, and stored at -80°C until use. Cryosections were rehydrated in PBS for 10 min. After 20 min blocking in PBS containing 0.1% (w/v) BSA, cryosections were incubated with anti-heparan sulfate antibodies for 90 min. Bound antibodies were visualized using anti-c-Myc goat polyclonal antibody A-14 and Alexa 488-conjugated donkey anti-goat IgG (60 min each). AChR clusters were stained by Alexa 594-conjugated α-bungarotoxin, included in the final incubation. Primary antibodies against voltage gated ion channel were applied for 60 min, after which bound antibodies were visualized using Alexa 488-conjugated goat anti-rabbit IgG for 60 min. Following each incubation, cryosections were washed three times 10 min with PBS. Finally, cryosections were fixed in 100% methanol, dried, and embedded in Mowiol (10% (w/v) in 0.1 M Tris-HCl, pH 8.5/ 25% (v/v) glycerol/ 2.5% (w/v) NaN₃). As a control, primary, secondary or conjugated antibodies were omitted. Also, a control phage display-derived scFv antibody that does not react with HS was used (MPB01). To investigate the possibility of epitope masking by tissue-resident HS-binding proteins, cryosections were incubated in high-salt buffer (PBS/1 M NaCl) or in Target Unmasking Fluid prior to immunostaining. Since no apparent changes in staining patterns were observed, we assume that the procedure described here allows for the staining of all HS epitopes present in the tissue. All incubations were performed at ambient temperature (21°C). Photographs were taken, using a constant shutter time, on a Zeiss Axioskop immunofluorescence microscope (Göttingen, Germany). Confocal images were made on a Nikon Diaphot inverted microscope attached to a BioRad MRC1024 ES confocal laser scanning microscope (Hemel Hempstead, UK). Digital images were processed using Confocal Assistant 4.02 and Adobe Photoshop 6.0 software.

**Results**

**Histogenesis of mouse intercostal muscle**

Intercostal muscle was chosen for studying muscle development and synaptogenesis, since its muscle mass is relatively homogeneous, well innervated, and easy to monitor (Theiler, 1989; Hogan et al., 1994). To improve the comparability between intercostal muscles of different developmental stages, we focused our study on the *m. intercostalis externus* between ribs 5 and 6.

Intercostal muscles are divided in two main muscle-masses: *m. intercostalis internus* and *m. intercostalis externus* (Fig. 1). The *m. intercostalis internus* is located at the internal side and has a function in drawing
adjacent ribs together during forced expiration. The *m. intercostalis externus* is located at the external side and elevates adjacent ribs during inspiration. During late-embryogenesis, we observed an asynchronous development of these intercostal muscles. From cranial to caudal, the *m. intercostalis externus* showed a more pronounced differentiation, as compared to the *m. intercostalis internus*. Whereas the latter consisted of a relatively heterogeneous population of loosely dispersed small muscle fibers, the *m. intercostalis externus* showed a more organized homogeneous distribution of fibers with a larger diameter. Moreover, the *m. intercosta-
lis externus contained more nerve bundles and blood vessels in corridors of perimysial connective tissue. The observed differences got more pronounced during the course of embryogenesis.

**Localization of HS epitopes**

Information about the anti-HS antibodies and their corresponding epitopes is given in table 1. Localization of individual epitopes is depicted in figures 2-7 and is summarized in table 2.

**Table 1.** Complementarity determining region 3 (CDR3) sequences and preferred HS sequences of the anti-HS antibodies used in this study.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>CDR3 sequence</th>
<th>preferred HS binding-sequence</th>
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<tbody>
<tr>
<td>AO4B05</td>
<td>LKQQGIS</td>
<td>ND</td>
</tr>
<tr>
<td>AO4B08</td>
<td>SLRNGWRAHQ</td>
<td>GlcNS6S-IdoUA2S-GlcNS6S&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>RB4CB9</td>
<td>HAPLRNRTNT</td>
<td>b</td>
</tr>
<tr>
<td>RB4CD12</td>
<td>GMRRPRL</td>
<td>GlcNS6S-IdoUA2S-GlcNS6S&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>RB4EA12</td>
<td>RRYALDY</td>
<td>GlcNS6S-IdoUA-GlcNS6S</td>
</tr>
<tr>
<td>MPB01</td>
<td>PKRTRN</td>
<td>not reactive with HS</td>
</tr>
</tbody>
</table>

CDR3 sequences are shown in single-letter amino acid code. The preferred HS sequence was deduced as described in Dennissen et al., 2002. Antibodies RB4CB9 and RB4CD12 (previously selected against human skeletal muscle GAGs) are identical to HS4E4 and HS4D4 (selected against a kidney HS preparation), respectively.

<sup>a</sup> Antibodies AO4B08 and RB4CD12 recognize different HS epitopes, as deduced by their differential reactivity in ELISA assay and different staining patterns in muscle and kidney (Jenniskens et al., 2000; Dennissen et al, 2002).

<sup>b</sup> Preferred sequence is not known, N-sulfation is indispensable, and 6-O sulfation is likely to be inhibitory.

ND: not determined.

**Table 2.** Immunostaining of the antibodies used in this study during myogenesis.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>E12-13</th>
<th>E14</th>
<th>E16</th>
<th>E18</th>
<th>E20</th>
<th>P2</th>
<th>adult</th>
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<tr>
<td>AO4B05</td>
<td>+/-, gr</td>
<td>++, c</td>
<td>++, c</td>
<td>++, c</td>
<td>++, c</td>
<td>++, c</td>
<td>++, c</td>
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<tr>
<td>AO4B08</td>
<td>+/-, gr</td>
<td>++, gr</td>
<td>++, c</td>
<td>++, c</td>
<td>++, c</td>
<td>++, c</td>
<td>++, c</td>
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<tr>
<td>RB4CB9</td>
<td>+/-, gr</td>
<td>++, gr</td>
<td>++, c</td>
<td>++, c</td>
<td>++, c</td>
<td>++, c</td>
<td>++, c</td>
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<tr>
<td>RB4CD12</td>
<td>+/-, gr</td>
<td>+, gr</td>
<td>++, c</td>
<td>++, c</td>
<td>++, c</td>
<td>++, c</td>
<td>++, c</td>
</tr>
<tr>
<td>RB4EA12</td>
<td>-</td>
<td>-</td>
<td>+&lt;sup&gt;a&lt;/sup&gt;</td>
<td>+&lt;sup&gt;b&lt;/sup&gt;</td>
<td>+&lt;sup&gt;b&lt;/sup&gt;</td>
<td>+&lt;sup&gt;b&lt;/sup&gt;</td>
<td>+&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>MPB01&lt;sup&gt;*&lt;/sup&gt;</td>
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</table>

Transversal sections of intercostal muscle at E12, E13, E14, E16, E18, E20, and P2, as well as adult m. soleus were stained for various HS epitopes and for AChR clusters. Immunostaining patterns of the antibodies presented in this study are summarized with regard to muscle BL reactivity. Staining intensity: ++, strong; +, moderate; +/-, weak; -, absent (n=5).

gr: granular staining of the BL.
c: continuous staining of the BL.
<sup>a</sup> staining of small intracellular granules.
<sup>b</sup> predominant staining of the synaptic BL.
<sup>*</sup> control antibody.
12 and 13 days in utero

At early developmental stages, intercostal muscle masses were scarcely detectable as such, when using immunohistochemical methods to stain for HS. Muscle primordia, at these stages, consist of fibroblasts and mononucleated myoblasts, the latter fusing to form primary myotubes. Upon myoblast fusion, patches of extracellular matrix material are formed on the myotube surface. Ongoing deposition of extracellular
Figure 3. Staining of developing skeletal muscle with antibody AO4B08. Transversal sections of intercostal muscle at E14 (a), E16 (b), E18 (c), E20 (d), and P2 (e), stained for HS epitope AO4B08 (a1-e1) and AChRs (a2-e2). At E14, the AO4B08 HS-epitope is present in BL, in a granular distribution, in synaptic areas (a, arrows) as well as in extrasynaptic areas. From E16 onwards, the HS-epitope is homogenously distributed throughout the BL, irrespective of synapses (b-e, arrows). Scale bar, 25 µm.

Material results in the formation of a continuous BL in the following days. Except for RB4EA12, the antibodies used in this study showed a faint, granular staining of extracellular material in myogenic regions. Control antibody MPB01 showed no staining whatsoever in all developmental stages examined.
14 days in utero

At 14 days in utero, the intercostal muscle appeared as a relatively loosely dispersed muscle mass. Nuclei were relatively large and located in the center of the myotubes. Blood vessels and nerve bundles were located in connective tissue surrounding myogenic areas.
Figure 5. Staining of developing skeletal muscle with antibody RB4EA12. Transversal sections of intercostal muscle at E14 (a), E16 (b), E18 (c), E20 (d), and P2 (e), stained for HS epitope RB4EA12 (a1-e1) and AChRs (a2-e2). At 14 days in utero, HS-epitope RB4EA12 could be detected in small perinuclear granules, whereas it was not present in muscle BL at all (a). During myogenic differentiation, staining for this HS epitope was poor and granular in extrasynaptic BL, but abundant in the synaptic portion of the BL (b-e, arrows) and nerve BL. Scale bar, 25 μm.

BL formation had progressed to a nearly continuous sheet, which could be stained with anti-HS antibodies AO4B05 (Fig. 2a), AO4B08 (Fig. 3a), RB4CB9, and, to a lesser extent, RB4CD12 (Fig. 4a). The HS epitope recognized by AO4B05 was present throughout the newly formed BL. AO4B05 staining was slightly more intense at some sites of AChR aggregation. Antibodies AO4B08 and RB4CB9 showed a more granular staining of the BL. Antibody RB4EA12 (Fig. 5a) did not stain muscle BL,
Figure 6. Staining of developing skeletal muscle with an antibody against \( \text{vgNa}^+ \) channels. Transversal sections of intercostal muscle at E14 (a), E16 (b), E18 (c), E20 (d), and P2 (e), stained for \( \text{vgNa}^+ \) channel (a1-e1) and AChRs (a2-e2). \( \text{vgNa}^+ \) channels, which are located in the muscle plasma membrane, could be visualized as early as E14, the stage in which AChR clustering occurs (a, arrow). In all stages of muscle development, these ion channels were evenly distributed in the membrane, apart from a slight decrease in density in synapses (a-e, arrows). Scale bar, 25 \( \mu \text{m} \).

but was present in small intracellular granules.

Consistent with reports in literature, AChR clustering was first visible at E14. The voltage-gated sodium channel (\( \text{vgNa}^+ \) channel), another post synaptic ion channel involved in excitation-contraction coupling (e-c coupling), showed a uniform distribution over the myotube membrane, with a decrease in density at AChR clusters (Fig. 6a).
16 days in utero

The diameter of the myotubes had increased by E16. Also, muscle masses were more compact and clusters of myotubes were separated by septa of connective tissue containing nerve bundles and blood vessels. In general, nuclei were smaller and were located either centrally or eccentrically, forming a heterogeneous set of multi-nucleated myotubes. The m. intercostalis internus and m. intercostalis externus were distinguishable, which enabled us to focus on the latter from this developmental stage onwards.

Antibodies AO4B05 (Fig. 2b), AO4B08 (Fig. 3b), RB4CB9, and RB4CD12 (Fig. 4b) stained the entire muscle BL. AO4B08 and RB4CB9 predominantly stained the muscle BL, whereas RB4CD12 epitopes were also present in blood vessels and in nerves located in the perimysium. RB4EA12 (Fig. 5b) continued to stain perinuclear granules but stained also, faintly, at sites of AChR clustering. VgNa’channels (Fig. 6b) showed a modest, overall distribution over the myotube membrane at this stage.

18 days in utero

By 18 days in utero, myotube diameter had further increased. Myotubes had become more homogeneous, both in size and in shape, approaching the regular pattern of mature muscle. Myonuclei were predominantly located eccentrically, although central nuclei could be observed occasionally.

Antibodies AO4B05 (Fig. 2c), AO4B08 (Fig. 3c), RB4CB9, and RB4CD12 (Fig. 4c) stained the muscle BL in a way comparable to that seen in 16-day fetuses. RB4EA12 (Fig. 5c) failed to stain cytosolic granules as well as the extrasynaptic BL, but showed a distinct staining of the synaptic BL. The vgNa’channel (Fig. 6c) continued to be evenly distributed over the sarcolemma.

20 days in utero/ birth

At birth, the m. intercostalis externus appeared more homogeneous and densely packed when compared to earlier developmental stages. This uniform maturation state is in line with the distribution of AO4B05 (Fig. 2d), AO4B08 (Fig. 3d), RB4CB9, and RB4CD12 (Fig. 4d) epitopes that stained the entire muscle BL. RB4EA12 (Fig. 5d) stained the extrasynaptic BL only to a minor extent, whereas a strong signal was achieved for synaptic and nerve BL.

VgNa’channels (Fig. 6d) were homogeneously distributed over the myotube membrane.
**Figure 7.** Comparison of the distribution of HS epitopes AO4B08 and RB4EA12 at synaptic sites. Confocal images of sections through neuromuscular junctions of adult *m. soleus* stained for AO4B08 (a1), RB4EA12 (b1), control antibody MPB01 (c1) and AChRs (a2, b2, and c2). Merged images are shown in color (a3, b3, and c3). HS-epitope AO4B08 is present throughout the endomysial BL, including synaptic regions (a1 and a3). In contrast, HS-epitope RB4EA12 is hardly present in the extrasynaptic BL, but enriched in synapses, where it colocalizes tightly with AChR clusters (b1 and b3). Control antibody MPB01 does not stain muscle BL at all (c1 and c3). Scale bar, 50 μm.

**2 days postnatal**

During early postnatal development, no major changes were observed in the distribution of HS epitopes: AO4B05 (Fig. 2e), AO4B08 (Fig. 3e), and RB4CB9 stained the entire BL. The RB4CD12 epitope (Fig. 4e) was evenly distributed over the muscle BL, but was more abundant in neural and capillary BL. RB4EA12 (Fig. 5e) was predominant in synaptic and neural BL. VgNa⁺channels (Fig. 6e) were homogeneously distributed over the cell membrane.

**Adult**

Following fetal development, staining patterns of the HS epitopes studied, did not undergo major changes with regard to BL location. Confocal imaging of the NMJs of *m. soleus* enabled us to visualize differences in the location of individual HS epitopes with regard to AChR clusters. Most antibodies stained the entire BL, and were located separate from AChR clusters, as exemplified by AO4B08 (Fig. 7a1-c1). RB4EA12 (Fig. 7a2-c2), however, showed a tight colocalization with AChR clusters.
Discussion

Skeletal muscle tissue is derived from embryonic mesenchyme of the somites (Grinnel, 1995). During early mouse embryogenesis, organ primordia are established, followed by organogenesis from E10 until E14, and subsequent fetal growth and development (Hogan, 1994). In extensor digitorum longus muscle at embryonic day 12, motor nerves reach muscle primordia, which already contain some multinucleate primary myotubes surrounded by myoblasts. Primary myotubes are elongated by myoblast fusion during two days, after which myogenensis resumes with the generation of secondary myotubes. The second generation of myotubes is formed on the scaffold of primary myotubes between E14 and E16. This biphasic character of myotube development is thought to be caused by the necessity of primary myotubes to first reach a certain stage of maturation, including sarcolemmal changes, before secondary myotube development can be supported (Ontell and Kozeka, 1984; Jansen and Fladby, 1990). Extracellular material is deposited on the myotube surface, beginning on E14, to form a continuous BL by E19 (Chiu and Sanes, 1984).

One of the HS epitopes examined in this study (AO4B05) surrounds primary myotubes in a relatively continuous staining at the onset of BL formation, at E14. Other HS epitopes that are also present throughout the muscle BL (AO4B08, RB4CB9, and RB4CD12) show a less continuous, granular distribution. Thus, a specific HS epitope is present in a more or less continuous sheet surrounding primary myoblasts before BL completion, whereas other HS epitopes are deposited in the course of BL formation. Since BL formation during maturation of primary myotubes may be causal for the onset of generation of secondary myotubes, we suspect significant roles for individual HS epitopes in the formation and integrity of developmental stage-specific BL. Deposition and accumulation of certain HS epitopes, both qualitatively and quantitatively, may add to form a BL capable of directing developmental processes. Moreover, spatial differences in the density of specific HS epitope deposits may be causal for the induction of local adaptations, such as synaptic specializations.

AChR clustering at sites of neuromuscular contact first occurs approximately on day E14. At this time, the synaptic cleft already contains BL material (Nakajima et al, 1980), of which the HSPG agrin is involved in the clustering of AChR (Ruegg and Bixby, 1998). During fetal growth, a faint staining of AChRs in the extrasynaptic cell membrane is observed, as well as a stronger cytosolic staining of this protein in and around synaptic areas (see e.g. fig 2a2-e2). This elevated staining may be due to higher expression levels of AChR from synaptic nuclei. Newly formed AChRs are detected in the cytosol, most probably located within Golgi vesicles while being transported to the sarcolemma, and in the cell.
membrane before clustering at synaptic sites. Specializations of the synaptic regions take place during the days of myogenesis following E14. A few days postnatally, these alterations culminate in functional neuromuscular junctions containing a continuous sBL, a thickened postsynaptic membrane, and junctional folds. One of the prominent features of the developing NMJ, the accumulation of AChE, is detectable soon after AChR clustering; nearly all junctional areas are rich in AChE by E18 (Chiu and Sanes, 1984). This process is mediated by BL-resident dermatan sulfate proteoglycans and HSPGs (Brandan et al., 1985; von Bernhardi and Inestrosa, 1990), among which perlecan (Peng et al., 1999).

The most striking staining pattern was observed for HS epitope RB4EA12, which preferably clusters at synaptic sites by E16 and accumulates at these locations thru E20. AO4B05, AO4B08, and RB4CB9 are present in the sBL as early as E14, during the onset of AChR clustering. Such regulated appearance of HS epitopes simultaneous with the aggregation of synapse-specific proteins is indicative for a possible involvement of these HS epitopes in the processes involved. The late appearance of RB4EA12 and its persisted synaptic location in adult tissue, occur parallel to the clustering and retention of AChE in sBL. However, this HS epitope colocalizes tightly with AChR clusters that are located in the postsynaptic membrane. This may indicate that HS epitope RB4EA12 is possibly present in cell surface HSPGs like glypicans and syndecans, rather than in BL-resident HSPGs like agrin and perlecan. It should be noted however, that a single HS chain can be up to 100 nm in length and is therefore able to span the entire synaptic cleft regardless of the location of the protein core. Other HS epitopes show a distinct spatiotemporal distribution with regard to AChR clusters and VGNa+ channels, but stain the entire BL surrounding the muscle fibers. At present, we have no information as to a possibly restricted occurrence of unique HS epitopes on specific proteoglycans. Defining the HS epitope-profiles of individual core proteins forms a major challenge for future studies.

Polypeptide growth factors, like epidermal growth factor, fibroblast growth factor-2, insulin-like growth factors and transforming growth factor-β, play key roles in myogenesis (McCusker and Clemmons, 1994). Some of the growth factors are known to interact with HS or heparin. Fibroblast growth factors, for instance, regulate the growth and differentiation of embryonic primary myoblasts (Itoh et al., 1996; Flanagan-Steet et al., 2000) as well as secondary and adult myoblasts (Flanagan-Steet et al., 2000). Moreover, fibroblast growth factor-2 is involved in both presynaptic (Dai and Peng, 1995) and postsynaptic (Peng et al., 1991) differentiation. Therefore, we studied the spatiotemporal distribution of several growth factors (epidermal growth factor, fibroblast growth factor-2, insulin-like growth factor, and transforming growth factor-β), with regard to the HS epitopes studied here. We were unable to detect correlations between the distribution of either of these growth factors.
factors with regard to the distribution patterns of the HS epitopes (data not shown). In future studies, it will be interesting to determine any potential interactions between HS-binding growth factors and the HS epitopes defined by the antibodies studied here. Such studies require a more biochemical approach and would significantly contribute to our understanding of the mechanisms underlaying growth factor mediated processes.

In recent years, it has become widely accepted that especially the sulfation pattern of HS domains regulates specific interactions with HS-binding proteins (Bernfield et al., 1999; Tumova et al., 2000). Binding of fibroblast growth factor-2 requires 2-O-sulfation of iduronic acid residues, whereas additional 6-O-sulfation of the glucosamine residue is required for the binding of fibroblast growth factor-1 (Kreuger et al., 2001). Previously, carbohydrates (e.g. N-acetylated galactosamine) were shown to be primarily located in the sBL, and differentially located within the synapse, on basis of lectin and antibody-binding profiles (Sanes and Cheney, 1982; Iglesias et al., 1992; Martin et al., 1999). By regulating the structure of HS and the spatiotemporal distribution of specific HS epitopes, the muscle and the innervating nerve may create a specific microenvironment, which attracts and sequesters HS binding proteins necessary for the correct development of the NMJ.

Synaptic development is a lifelong process rather than an embryonic event. NMJs persist for a lifetime, but undergo structural and functional changes due to a dynamic equilibrium that permits remodeling in response to alterations in activity (reviewed by Sanes and Lichtman, 1999). Following fetal development, HS is also shown to be subject to age-related changes (Feyzi et al., 1998).

In summary, considering the tight and dynamic regulation of HSPG expression during myogenesis, we looked in detail to the occurrence of specific HS epitopes. Our results indicate a highly regulated spatiotemporal expression of various HS epitopes during muscle development. The results presented in this study argue for roles of individual HS epitopes in developmental processes underlying myogenesis and synaptogenesis.

References


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Chapter 5

Disturbed Calcium Kinetics in N-Deacetylase/N-Sulfotransferase-1 Defective Myotubes

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Submitted.
The biosynthesis of heparan sulfate (HS), present on the cell surface and in the basal lamina surrounding cells, is a multi-step process of which each step is mediated by a specific enzyme. The initial modification of the precursor polysaccharide, N-deacetylation followed by N-sulfation of selected N-acetyl-D-glucosamine residues, is catalyzed by the enzyme glucosaminyl N-deacetylase/ N-sulfotransferase (NDST). This event is a key step that regulates the overall sulfate content of the polysaccharide. Here, we report on the effects of NDST deficiency on calcium kinetics in myotubes from NDST-1 and NDST-2-defective mice, indicating a novel role for HS in skeletal muscle physiology.

Immunostaining for specific HS epitopes shows major changes in HS composition in cultured myotubes and in skeletal muscle tissue derived from NDST-1-/- mice. No changes were observed for NDST-2-/- and heterozygous mice. Using high-speed UV confocal laser scanning microscopy, aberrant calcium kinetics were observed in NDST-1-/- myotubes, but not in NDST-2-/- or heterozygous myotubes. Electrically-induced calcium spikes had significantly lower amplitudes, and a reduced removal rate of cytosolic calcium, indicating the importance of HS in muscle calcium kinetics.

Introduction

Heparan sulfate proteoglycans present on the cell surface and in the basal lamina (BL) surrounding cells are involved in various biological processes. The HS chains, which are covalently attached to a protein core, exert their biological functioning through the selective binding of proteins. These interactions depend on HS domains with specific patterns of sulfation (Kjellén and Lindahl, 1991; Salmivirta et al., 1996; Lindahl et al., 1998; Bernfield et al., 1999). The complex processes involved in the structural modification of the HS chain that lead to the generation of such domains are beginning to be unraveled. The proposed pathway of HS biosynthesis involves a cascade of enzymes, each responsible for a single modification step (recently reviewed in: Habuchi, 2000; Selleck, 2000; Sugahara and Kitagawa, 2000; Gallagher, 2001).

After formation of a HS precursor polysaccharide, consisting of repeating D-glucuronic acid and N-acetyl-D-glucosamine residues, the bifunctional enzyme glucosaminyl N-deacetylase/ N-sulfotransferase (NDST) catalyzes the first modifications of the precursor polysaccharide, where the acetyl group of selected N-acetyl-D-glucosamine residues is replaced with a sulfate group. This reaction is a prerequisite for all other
modifications such as C-5 epimerization, 2-O-sulfation, 3-O-sulfation, and 6-O-sulfation, which only occur in the vicinity of N-sulfate groups (Lindahl et al., 1998).

Four isoforms of NDST have been identified (Hashimoto et al., 1992; Eriksson et al., 1994; Orellana and Hirschberg, 1994; Aikawa and Esko, 1999). NDST-1 and NDST-2 are widely distributed (Humphries et al., 1997; Humphries et al., 1998; Kusche-Gullberg et al., 1998; Aikawa et al., 2001), while NDST-3 and NDST-4 have a more restricted expression. Disruption of the genes encoding NDST-1 (Fan et al., 2000; Ringvall et al., 2000) and NDST-2 (Forsberg et al., 1999; Humphries et al., 1999) gave insights in the roles of these enzymes in the biosynthesis and physiological roles of HS and heparin, a highly sulfated form of HS. NDST-1-deficient mice are capable of synthesizing N-sulfated HS, but the sulfate content is significantly lower than that of HS from wild type mice, indicating the importance of this enzyme in HS biosynthesis (Ringvall et al., 2000). So far, no obvious defects in HS have been found in NDST-2-deficient mice, which instead are unable to synthesize fully sulfated heparin (Humphries et al., 1999; Forsberg et al., 1999). However, the increased lethality of NDST-1 and NDST-2 double knock-out mice, compared to NDST-1-deficient mice, suggests that NDST-2 also plays a role in HS biosynthesis (Forsberg and Kjellén, 2001).

Different roles for HS or heparin in calcium kinetics have been suggested, such as the buffering of calcium (Bezprozvanny et al., 1993), influencing growth factor signaling (Patel et al., 1998), or regulating the activity of ion channels such as the dihydropyridine receptor (Knaus et al., 1990; Lacrinova et al., 1993; Martinez et al., 1996) and the ryanodine receptor (Bezprozvanny et al., 1993; Ritov et al., 1985). The availability of mice deficient in NDST-1 and NDST-2 provides a means to further investigate the role of HS in skeletal muscle calcium kinetics in a physiological setting.

In the present study, we report on the effects of the HS deficiency on electrically-induced calcium spikes. Immunostaining for specific HS epitopes is reduced in skeletal muscle and in cultured myotubes of NDST-1-/-, but not in other genotypes. NDST-1-/- myotubes show a significant decrease in the amplitude of calcium spikes and a slower removal of cytosolic calcium. These results strongly indicate the involvement of HS in skeletal muscle calcium kinetics.

**Materials and Methods**

**Materials**

All chemicals used were from Merck (Darmstadt, Germany), unless stated otherwise. Bacterial media (2xTY and LB) and cell culture media
were from Life Technologies (Paisley, Scotland) and tissue culture plastics were from Greiner (Frickenhausen, Germany). Bovine serum albumin (fraction V) and NaN₃ were obtained from Sigma (St. Louis, MO). Anti-c-Myc tag mouse monoclonal IgG (clone 9E10) was from Boehringer Mannheim (Mannheim, Germany) and anti-c-Myc tag goat polyclonal IgG (A-14) was from Santa Cruz Biotechnology (Santa Cruz, CA). Alexa 488-conjugated donkey anti-goat IgG and goat anti-mouse IgG, Alexa 594-conjugated α-bungarotoxin, Indo-1/AM, and Pluronic F127 were purchased from Molecular Probes (Eugene, OR) and Mowiol (4-88) was from Calbiochem (La Jolla, CA). All experiments were performed at ambient temperature (21°C), unless stated otherwise.

**Anti-heparan sulfate antibodies**

Preparation of phage display-derived c-Myc-tagged anti-HS antibodies AO4B05, AO4B08, AO4F12, RB4CB9, RB4CD12, RB4EA12, and MPB01 was performed as described (Jenniskens et al., 2000). Control antibody MPB01 was randomly picked from phage display ‘scFv library #1’ (Nissim et al., 1994). This antibody does not recognize HS, or heparin, and is not reactive with any of the cells or tissues tested (muscle, brain, kidney, lung) as judged by ELISA and immunohistochemistry.

**Mouse skeletal muscle tissue**

C57BL/6 Mice heterozygous for NDST-1 and NDST-2 were intercrossed to generate offspring of all genotypes as described (Forsberg et al., 1999; Ringvall et al., 2000). The first day of occurrence of a vaginal plug was taken as day 0 of gestation (E0). E18.5 (18.5 days in utero) embryos were isolated by caesarian delivery and killed by decapitation, in accordance with the ethic regulations of the University. Embryos were rinsed in PBS, snap-frozen in liquid nitrogen-cooled isopentane, and stored at -80°C.

**Immunohistochemistry**

5 to 10 μm thick cryosections of whole embryos were cut, mounted on slides, dried thoroughly, and stored at -80°C until use. Cell cultures were washed three times with PBS, dried overnight, and stored at -80°C until use. Cryosections and cell cultures were stained with anti-heparan sulfate antibodies and α-bungarotoxin as described (Jenniskens et al., 2000). Photographs were taken, using a constant shutter time, on a Zeiss Axioskop immunofluorescence microscope (Göttingen, Germany). For each genotype, skeletal muscle specimens of individual embryos of two separate litters were studied.
Primary myoblast culture

To obtain primary myoblast cultures, E18.5 embryos were isolated by caesarian delivery, killed by decapitation, and dissected (disposed of skin and other organs). Skeletal muscle tissue was isolated from the remaining carcasses, triturated with needles and incubated in an enzyme solution (300 U collagenase/ml, 0.15% trypsin, and 0.08% BSA in PBS, pH 7.3) at 37°C for 15 min. To the cell suspension, containing satellite cells (precursors of myoblasts), an equal volume of neutralization solution (DMEM/ 50% fetal bovine serum (FBS)) was added. The dissociation procedure was repeated three times. Subsequently, cell suspensions were sieved through a 40 μm cell strainer and cells were collected by centrifugation (10 min, 50xg). Cell pellets were resuspended and enriched for myoblasts by pre-plating twice for 30 min, after which cells were seeded at 5x10⁴ cells per well in a 6 wells format. Primary myoblast cultures were maintained as proliferating cells for up to 6 passages in DMEM supplemented with 10% brain extract and 1% Ultroser G (Portier et al. 1999). Cells were plated at 2x10⁶ (6 wells format) or 5x10⁴ (24 wells format) cells per well and grown overnight in proliferation medium to reach 50-60% confluency the next day. At confluency, culture medium was replaced by differentiation medium (DMEM/ 10% brain extract/ 0.4% Ultroser G). Differentiation medium was refreshed every second day. For immunocytochemistry, H₂SO₄-etched 13 mm diameter coverslips were used in 24 well-format. For measurements of cytosolic calcium, cells were plated on H₂SO₄-etched 24 mm diameter glass coverslips in 6 well-format. Cultures were grown and differentiated at 37°C in a humidified 5% CO₂/ 95% air atmosphere. For each genotype, primary myoblast cultures derived from at least two individual embryos of two (NDST-2) or three (wild type and NDST-1) separate litters were studied.

Ratiometric measurements of intracellular calcium with Indo-1

Coverslips with confluent monolayers of primary myoblast cultures, differentiated for three days, were washed twice with physiological salt solution (PSS; 125 mM NaCl, 10 mM NaHCO₃, 1 mM NaH₂PO₄, 5 mM KCl, 2 mM MgSO₄, 1.8 mM CaCl₂, 10 mM HEPES and 10 mM glucose, pH 7.4) and loaded with Indo-1 using an Indo-1/AM solution (5 μM Indo-1/AM and 1 μM Pluronic F127 in PSS) for 45 min. Cell loading was performed at 37°C in a humidified 5% CO₂/ 95% air atmosphere. Cells were washed twice with PSS, placed in a Leiden chamber (Ince et al., 1985), and incubated in PSS for another 15 min. The Leiden chamber was placed on a Nikon diaphot inverted microscope attached to an OZ confocal laser scanning microscope (Noran instruments, Madison, WI). Electrical stimulation of myotubes was performed at 15 to 20 V with 10 ms duration and 0.025 ms delay. Repetitive stimuli were generated at a frequency of 1 Hz. Stimulation was performed using a pair of parallel
oriented platinum electrodes (5 mm interspaced, placed beside the monolayer), connected to a Grass SD9 electric pulse generator. Calcium free medium was obtained by the use of Ca$^{2+}$-free PSS, supplemented with 1 mM EGTA. For ratiometric Ca$^{2+}$ recordings, Indo-1 was excited at 351 nm using a hardware-modified high power argon-ion laser (Coherent enterprise, Santa Clara, CA). After being separated by a 455 DCLP dichroic mirror, Indo-1 fluorescence intensity was collected at 405±45 and 485±45 nm using photo multipliers. All measurements were performed at room temperature, using a Nikon x40 water-immersion objective, NA 1.2 with high UV transmission. The estimated laser intensity at the end of the objective lens was 28 μW. Confocality (optical section thickness: 1 to 2 μm) was achieved by using a slit width of 100 μm (Koopman et al., 2001). User-based hardware set-up and data acquisition was controlled by Intervision software (Version 1.5, Noran Instruments), running under IRIX 6.2 on an Indy workstation (Silicon Graphics Inc., Mountain view, CA) equipped with 128 MB of RAM. To improve signal-to-noise ratio, single full-frame images (512x480 pixels) were collected at 30 Hz by averaging of 32 images for each Indo-1 emission wavelength. Pairs of fluorescence intensities were saved in SGI-movie (mv) format, ready for off-line analysis using the Intervision 2D software package (Noran Instruments). Regions of interest (ROI) were drawn and numerical values were stored in ASCII-format. Additional data-analysis was carried out by using Origin 6.0 (Microcal, Northampton, MA), supplemental image analysis and visualization were performed with Image Pro-plus 4.1 (Media Cybernetics, Silver Spring, MD).

Results

**Occurrence of HS epitopes in situ in skeletal muscle**

We have previously selected antibodies that recognize specific HS epitopes using phage display (van Kuppevelt et al., 1998; Jenniskens et al., 2000). Some of these antibodies were used in this study to investigate the occurrence of various HS epitopes (AO4B05, AO4B08, AO4F12, RB4CB9, RB4CD12, and RB4EA12) in intercostal muscle of mouse (E18.5) embryos bearing a targeted disruption of the NDST-1 or the NDST-2 loci (Forsberg et al., 1999; Ringvall et al., 2000). All epitopes studied were present in the BL surrounding muscle fibers and/or nerves of wild type embryos (Fig. 1a, e) and in embryo's heterozygous for NDST-1 and NDST-2 (data not shown). Staining for HS epitopes in NDST-1-/- muscle BL was significantly reduced, except for the neuromuscular region (Fig. 1c). In contrast, NDST-2-/- muscle BL stained normally (Fig. 1g). The dia-
Figure 1. Reduction of HS epitopes in NDST-1/− skeletal muscle. Immunofluorescence staining for HS-epitope AO4B05 (a, c, e, and g) and acetylcholine receptor clusters (b, d, f, and h) in intercostal muscle of E18.5 embryos of wild type (a, b, e, and f), NDST-1/− (c and d), and NDST-2/− (g and h). Overall staining in NDST-1/− is significantly reduced, as compared to wild type and NDST-2/− staining, except for the BL in the NMJs and their direct vicinity (n=5). Scale bar, 50 μm.

meter of myofibers was slightly less uniform in NDST-1/− muscle compared to other genotypes, whereas the occurrence and size of neuromuscular junctions (NMJs) appeared normal.

**Culturing of primary myoblasts**

Primary myoblast cultures were generated from muscle tissue of E18.5 embryos. At three days of differentiation, multi-nucleated myotubes were present in all cultures. From day three of differentiation onwards, spontaneous contractions were regularly observed in wild type and heterozygous myotubes, but only occasionally in knock out myotubes. In wild type and heterozygous cultures, spontaneous contracting myotubes caused monolayers to peel off from the culture plastics, an effect which was not observed in NDST-1/− and NDST-2/− cultures.

**Occurrence of HS epitopes in vitro**

To analyze the occurrence of HS epitopes in cultured myotubes, three day-differentiated cultures were stained immunologically. All antibodies stained the BL of wild type (Fig. 2a, e) and heterozygous (data not shown) myotubes. Normal BL staining was present in NDST-2/− cultures (Fig. 2g). However, NDST-1/− cultures showed hardly any HS staining, even
Figure 2. Reduction of HS epitopes in NDST-1⁻/⁻ primary muscle cell culture. Immunofluorescence staining for HS-epitope AO4B05 (a, c, e, and g) and acetylcholine receptor clusters (b, d, f, and h) in wild type (a, b, e, and f), NDST-1⁻/⁻ (c and d), and NDST-2⁻/⁻ (g and h) primary muscle culture after three days of differentiation. HS staining is absent in NDST-1⁻/⁻ derived myotubes and mononuclear cells. In contrast, NDST-2⁻/⁻ cultures are not affected. Myotubes in all cultures show spontaneous acetylcholine receptors clusters (arrows; n=5). Scale bar, 50 μm.

at sites of spontaneous acetylcholine receptor clustering (Fig. 2c), indicating the inability of NDST-1 deficient myotubes to synthesize the HS epitopes recognized by the antibodies. The clustering of acetylcholine receptors appeared normal in all genotypes, as judged by α-bungarotoxin staining (Fig. 2, arrows).

**Electrically induced calcium spikes in NDST-1⁻/⁻ affected myotubes**

Ratiometric measurements of the [Ca²⁺]ᵢ were performed in myotubes of all genotypes, using a high-speed UV confocal laser scanning microscope and electrical stimulation. Average amplitudes of electrically induced calcium spikes decreased gradually in the following order: wild type > NDST-2⁺/− > NDST-2⁻/− > NDST-1⁺/− > NDST-1⁻/−, but only NDST-1⁻/− differed significantly from the wild type (Table 1, Fig. 3). All calcium spikes were independent of the presence of calcium in the extracellular environment, indicating that the released calcium originated from intracellular stores. Upon electrical stimulation, [Ca²⁺]ᵢ reached its maximum within 99 ms (three data points), after which it rapidly declined to a basal level (Fig. 3), with mono-exponential kinetics ($Y_t = Y_0 + Ae^{-t/\mu}$ ($Y_t$ = ratio as a function of time (reflecting the [Ca²⁺]ᵢ); $Y_0$ = ratio at t=0 (basal ratio, reflecting the basal cytosolic [Ca²⁺]); $A$ = scaling factor; $t$ = time; $\mu$ =...
Table 1. Kinetics of electrically induced calcium spikes in NDST-1/-2 affected myotubes.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>n*</th>
<th>Amplitude (ratio)</th>
<th>Decay constant μ (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>12</td>
<td>1.882 ± 0.255</td>
<td>0.264 ± 0.098</td>
</tr>
<tr>
<td>NDST-2+/-</td>
<td>10</td>
<td>1.872 ± 0.335</td>
<td>0.302 ± 0.142</td>
</tr>
<tr>
<td>NDST-2-/-</td>
<td>12</td>
<td>1.755 ± 0.242</td>
<td>0.225 ± 0.096</td>
</tr>
<tr>
<td>NDST-1+/-</td>
<td>13</td>
<td>1.678 ± 0.092</td>
<td>0.319 ± 0.103</td>
</tr>
<tr>
<td>NDST-1-/-</td>
<td>8</td>
<td>1.594 ± 0.108 **</td>
<td>0.426 ± 0.132 **</td>
</tr>
</tbody>
</table>

Ratiometric measurements of [Ca\(^{2+}\)] were performed in myotubes of all genotypes, using a high-speed UV confocal laser scanning microscope. Amplitudes and decay constants of electrically-induced calcium spikes are given as average values of the number of myotubes analyzed ± SD.

* Analysis was performed on data obtained from myotubes from separate cultures that were derived from individual embryos.
** p<0.01

Discussion

Mice deficient in NDST-1 or NDST-2 were used to study the effect of disturbances in HS biosynthesis on excitation-induced calcium spiking. Immunostaining of intercostal muscle showed a reduced staining for all HS epitopes studied in the BL of NDST-1-/- mice, except for neuromuscular regions. In NDST-2-/- and heterozygous mice staining was normal. The loss of HS epitopes in NDST-1-deficient muscle indicates a major role for NDST-1 in HS biosynthesis. As previously suggested, NDST-2 appears to be essential for the synthesis of heparin in mast cells.
but may be only marginally involved in HS biosynthesis (Forsberg et al., 1999; Ringvall et al., 2000). All four NDST-isoform transcripts are present in skeletal muscle, NDST-1 and NDST-2 mRNA being more abundant than NDST-3 and NDST-4 transcripts (Aikawa et al., 2001). Thus, other NDST isoforms may have (partially) compensated for the loss of NDST-1, resulting in the low N-sulfation levels of HS detected in NDST-1/− embryos (Ringvall et al., 2000). Although staining for HS epitopes was considerably reduced in skeletal muscle from NDST-1/− mice, the neuromuscular region appeared unaffected. Other NDST isoforms may be active in this highly specialized area, thus compensating for the NDST-1 deficiency. No obvious effects on the presence of HS epitopes were seen in mice heterozygous for NDST-1, indicating a compensatory effect of the single functional allele present.

In primary myoblast cultures, similar effects were seen with regard to the presence of HS epitopes. Staining for HS epitopes in NDST-1/− cultures was strongly reduced, whereas in NDST-2/− cultures or in cultures derived from heterozygous mice, staining was normal. The occurrence and size of acetylcholine receptor clusters appeared similar in all genotypes both in vivo and in vitro, indicating that the loss of either enzyme does not interfere with the proper clustering of this receptor.

Calcium kinetics was affected to various degrees in myotubes of individual cultures. The rate of [Ca2+] removal correlated with the amplitudes of calcium spikes, both of which were significantly decreased for NDST-1/− myotubes. The lower amplitude in NDST-1/− myotubes is indicative of a lowered calcium flux over the sarcoplasmic reticulum.
membrane. Heparin, a highly sulfated HS, binds with high affinity to the extracellular domain of the voltage-dependent L-type Ca\(^{2+}\) channel, the dihydropyridine receptor, of skeletal muscle cells (Knaus et al., 1990). Upon stimulation, this receptor induces a conformational change in the calcium release channel of the sarcoplasmic reticulum (the ryanodine receptor), resulting in a calcium flow from the sarcoplasmic reticulum to the cytosol. HS may be involved in the correct functioning of the dihydropyridine receptor and alterations in HS may reduce the calcium flow to the cytosol. Alternatively, the effect of HS may be indirect, e.g. by modulating growth factor signaling. The effect of decorin, a dermatan sulfate proteoglycan, on calcium kinetics is mediated through the EGF receptor (Patel et al., 1998). Also, there may be a lowered calcium concentration in the sarcoplasmic reticulum (and thus a lowered calcium flux) as a result of the NDST-1\(-/-\) deficiency. Also, the [Ca\(^{2+}\)]\(_{\text{cyt}}\) removal rate is affected in NDST-1\(-/-\) myotubes. Since this process is mainly effectuated by sarcoplasmic reticulum calcium ATPases, an effect of HS deficiency on the overall calcium-pumping rate of this ion pump may be envisioned. The occurrence of skeletal muscle differentiation characteristics such as fusion to multinucleated myotubes, spontaneous acetylcholine receptor clustering, and excitability indicate that myotubes of all genotypes were differentiated to a similar degree. Therefore, the effects observed are not likely the result of disturbed differentiation.

NDST-1-deficient cultures occasionally failed to generate calcium spikes upon electrical stimulation. Such aberrant calcium kinetics were not observed in any other genotype, arguing for these effects to be a result of the loss of this enzyme. NDST-1\(-/-\) mice die shortly after birth due to respiratory failure. This effect has been attributed to immature type II pneumocytes, resulting in shortage of lung surfactant (Fan et al., 2000). However, the loss of muscle contraction, likely to be the result of disturbed calcium kinetics, may also contribute to the lack of pulmonary function.

In this study, we report the effects of the deficiency of NDST-1 or NDST-2 on electrically-induced calcium spiking in skeletal muscle. The results presented here strongly indicate the involvement of HS in skeletal muscle calcium kinetics.

References


Chapter 6

Phenotypic Knock Out of Heparan Sulfates in Myotubes Impairs Excitation-Induced Calcium Spiking

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Submitted.
Little is known about the physiological functions of heparan sulfates (HS), present in the basal lamina surrounding skeletal muscle fibers. Here, we present a new system in which HS is phenotypically knocked out by endogenous expression of epitope-specific anti-HS antibodies. Single chain antibodies, containing an immunoglobulin leader peptide, were produced using various expression systems. Antibodies were detected in the Golgi apparatus, the site of HS biosynthesis. Likewise, the HS-degrading enzyme heparanase was expressed. Endogenous expression of antibodies or heparanase in myoblasts resulted in HS-defective myotubes. Excitability and calcium kinetics of HS-defective myotubes were severely compromised, as determined by analysis of electrically induced calcium spikes using video-speed UV confocal laser scanning microscopy. Phenotypically knocking out of individual HS epitopes resulted in specific effects on excitability and calcium kinetics. These data indicate important roles for HS in skeletal muscle calcium kinetics.

**Introduction**

Heparan sulfates (HS) are members of the glycosaminoglycan family, consisting of repeating disaccharide unit backbones onto which modification patterns are superimposed. The addition of sulfate groups leads to the generation of specific motifs that make HS a highly versatile, protein-binding, cell regulator (Spillmann and Lindahl, 1994; Bernfield et al., 1999; Turnbull et al., 2001; Gallagher, 2001).

The basal lamina (BL) and the cell membrane of skeletal muscle cells contain various heparan sulfate proteoglycans (HSPGs), which have been implicated in developmental and regulatory processes. Regulatory roles include the clustering of acetylcholine receptors (Wallace, 1990; Ferns et al., 1993; Campanelli et al., 1994; Hoch et al., 1994), the BL-localization of certain proteins (Brandan et al., 1985; Patton et al., 1997), and the maintenance of BL integrity (Costell et al., 1999). Unc-52, the *Caenorhabditis elegans* orthologue of perlecan, has important roles in muscle integrity (Rogalski et al., 1993). The BL-HSPG perlecan is mutated in Schwartz-Jampel syndrome, a human myotonic disorder, implicating a function of HSPGs in muscle excitability in men (Nicole et al., 2000).

Due to their location in the muscle BL and on the cell membrane, HSPGs may fulfill regulatory functions in skeletal muscle excitation-contraction (e-c) coupling. This may be accomplished by binding of calcium, by a direct interaction with ion channels, or by binding to growth factors and their receptors. Extracellular heparin, a highly sulfated form

PHENOTYPIC HEPARAN SULFATE KNOCK OUT MYOTUBES
of HS, interacts with the dihydropyridine receptor (DHPR), an L-type calcium channel, and profoundly affects its kinetic properties (Knaus et al., 1990; Lacinova et al., 1993; Martinez et al., 1996). The ryanodine receptor (RyR) is activated by heparin in vitro (Ritov et al., 1985; Bezprozvanny et al., 1993), whereas the structurally similar inositol trisphosphate receptor is inhibited (Gosh et al., 1988; Kobayashi et al., 1988). Decorin, a small leucine-rich (dermatan sulfate) proteoglycan, mediates increases in cytosolic calcium through the epidermal growth factor receptor (Patel et al., 1998). Intracellular application of heparin blocks e-c coupling in an activation-dependent manner in toad, but not in rat muscle fibers (Lamb et al., 1994).

Little is known about the precise functions of HS epitopes in muscle physiology. For molecules like HS, which are not genetically encoded, it has been difficult to generate dominant negative in vitro or in vivo research systems (Lander and Selleck, 2000, Iozzo, 2001). Recently, we selected single chain variable fragment (scFv) antibodies directed against specific HS epitopes, and showed HS epitopes to be differentially distributed in muscle and nerve BL (van Kuppevelt et al., 1998; Jenniskens et al., 2000).

In this study, we have used the DNA encoding these antibodies to produce intracellular antibodies (intrabodies (Biocca and Cattaneo, 1995; Marasco, 1997)) to phenotypically knock out specific HS epitopes in myoblasts. A cDNA encoding the HS-degrading enzyme heparanase (Hulett et al., 1999; Vlodavsky et al., 1999) was used likewise, thus generating HS-defective myoblasts. Using video-speed UV confocal laser scanning microscopy to measure electrically induced changes in cytosolic calcium concentration ([Ca^{2+}]_t), we show that excitability and calcium kinetics are severely impaired in HS-defective myotubes that differentiated from transfected myoblasts. To our knowledge, this is the first time a physiological role for HS epitopes in excitation-induced calcium spiking is demonstrated.

Materials and Methods

Materials

Mice (C3H, male, 70 d) and rats (Wistar, 10 d) were obtained from the University of Nijmegen Central Animal Laboratory. C_{2,12} (CRL1772) skeletal muscle cell line was purchased from American Type Culture Collection (Rockville, MD). Glycosaminoglycan-deficient myoblast (S_{2,2} cell line was a generous gift of Dr. Z. Hall (Dept. of Physiology, University of California, San Francisco, CA). Anti-syndecan antibody (2E9; syndecan-1 and -3) was a kind gift of Dr. G. David (Center for Human
Genetics, University of Leuven, Leuven, Belgium). Anti-perlecan antibody (EY-90) was a kind gift of Dr. D. Noonan (Dept. of Chemical Carcinogenesis, ICT, Genova, Italy).

All chemicals used were from Merck (Darmstadt, Germany), unless stated otherwise. Bacterial media (2xTY and LB), cell culture media, OptiMEM, LipofectAMIN/ PLUS reagents, and Geneticin G418 were from Life Technologies (Paisley, Scotland); tissue culture plastics from Greiner (Frickenhausen, Germany). Bovine serum albumin (fraction V), Cy3-labelled P5D4, and NaN₃ were obtained from Sigma (St. Louis, MO); Anti-c-Myc tag monoclonal IgG (clone 9E10) from Boehringer Mannheim (Mannheim, Germany); anti-c-Myc tag goat polyclonal IgG (A-14) from Santa Cruz Biotechnology (Santa Cruz, CA); Alexa 488-conjugated donkey anti-goat IgG, donkey anti-sheep IgG, and goat anti-mouse IgG, Alexa 594-conjugated goat anti-mouse IgG, Alexa 594-conjugated α-bungarotoxin, NBD C₆-Ceramide (N-1154), Indo-1/AM, and Pluronic F127 from Molecular Probes (Eugene, OR); Mowiol (4-88) from Calbiochem (La Jolla, CA). Polymerase chain reaction (PCR) chemicals, Taq polymerase (DNA polymerase from Thermus aquaticus), and Pfu-Taq polymerase were obtained from Promega (Madison, WI); PCR primers and synthetic oligonucleotides from Biolegio (Malden, The Netherlands); Ecdysone-inducible eukaryotic expression system, high level eukaryotic expression vector pcDNA3.1, Zeocin, Muresterone A, and Ponasterone A from Invitrogen (Groningen, The Netherlands); bicistronic vector pIRES2-EGFP from Clontech (Palo Alto, CA); Restriction enzymes and buffers from New England Biolabs (Beverly, MA); Unique site elimination kit from Amersham Pharmacia (Roosendaal, The Netherlands); ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit from PE Applied Biosystems (Norwalk, CT).

All experiments were performed at ambient temperature (21°C), unless stated otherwise.

**Generation of anti-HS antibody expression constructs**

For the inducible expression of scFv antibody fragments, an Ecdysone-inducible eukaryotic expression system, consisting of vectors pVgRXR and pIND, was used (No et al., 1996; Fig. 1). To allow Ncol-NotI cloning of antibody-coding fragments, two Ncol restriction sites in the pIND vector (at nucleotides 1544 and 2279) were eliminated by site directed mutagenesis using the unique site elimination kit with oligonucleotides pINDmutNco-1544: 5'-CTCCGCCCACATGC*CTGACTAATTTTTTATATATGC-3' and pINDmutNco-2279: 5'-GAGGATCTCGTCGTGACA*CATGGCGATGCCTGCTTGC-3', following the manufacturer’s protocol, to generate pINDmutNcoI. An immunoglobulin leader sequence (Ig-lead) cassette, compiled of two complementary oligonucleotides Ig-lead-codogene (sense): 5'-AGCTTATGGG-
ATGGAGCTGTATCATCCTCTTCTTGGTAGCAACAGCTACAG-
GTGTCCACTCCATGGGATCCGAT-3' and Ig-lead-noncodogene (antisense): 5'-ATCGGATCCCATGGAGTGGACACCTGTAGCTG-
TTGCTACCAAGAAGAGGATGATACAGCTCCATCCCATA-3', was inserted into the multiple cloning site of HindIII/ EcoRV-digested pINDmutNcoI to form pIND/Ig-lead. These oligonucleotides annealed to form an Ig-lead cassette bordered on the 5' end by a HindIII, and on the 3’ end by an EcoRV restriction site. In this cassette, the first three nucleotides following the HindIII restriction recognition site form the initiation codon (ATG) used for translation. As a marker for expressed proteins, a VSV tag was cloned in frame with the Ig-lead sequence. Complementary oligonucleotides VSV-codogene (sense): 5'-ATCGCGGCGCATATACAGACATAGAGATGAACCGACTTGGAAAGTGAGC-3' and VSV-noncodogene (antisense): 5'-TCGAGCTCACTTTCCAAGTCGGTTCATCTCTATGTCTGTATATGCGGCCGAT-3' were annealed and inserted into pIND/Ig-lead as an EcoRV-XhoI cassette, generating pIND/Ig-lead/VSV. The VSV cassette contains an opal stop codon (TGA) directly following the VSV tag coding sequence, to terminate protein synthesis. Ig and VSV cassettes were constructed such that NcoI-NotI scFv cassettes could be cloned into pIND/Ig-lead/VSV in frame, with only a stretch of three alanine codons inserted between the scFv fragment and the VSV tag. ScFv coding regions were digested from original pHEN clones (Jenniskens et al., 2000) as NcoI-NotI fragments, and inserted in between the Ig and VSV sequences, to generate pIND/Ig-lead/scFv/VSV clones. In this study, the following anti-HS scFv antibodies were used (Table 1): A04B05, A04B08, AO4F12, RB4CB9, RB4CD12, and RB4EA12. As a control, a scFv antibody (MPB01), which does not recognize HS, nor heparin, nor any cellular structure was used (see below). Next to construction in the pIND vector, Ig-lead/scFv/VSV cassettes were cloned into pcDNA3.1 and pIRES2-EGFP as NheI-XhoI fragments (containing the full-length expression cassette, including start and stop codons) to generate high expression and bicistronic expression systems, respectively.

**Generation of human heparanase expression construct**

Human heparanase cDNA (AF/44325) was amplified from the original clone (Vlodavski et al., 1999), using Pfu-Taq polymerase and primers Hep-
s3 (sense): 5’-CCCAGGTGAGCCCACTGGGCTGCGCTCAGAAGCC-3' and Hep-a1 (antisense): 5’-AATATCTTGCGGCCCAGCATGGTATATATATTTCAGATGCA-3’. Via their non-complementary regions, these oligonucleotides introduced restriction sites at both sides of the heparanase-coding region: at the 5’ end, an NcoI site was introduced and at the 3’ end, a NotI site was generated. The PCR product was digested with NcoI and NotI, and ligated in similarly digested pIND/Ig-
lead/VSV, generating pIND/Ig-lead/Hep/VSV. In the construct thus obtained, the heparanase-coding region directly followed the Ig sequence, and amino acid #2 of the enzyme was mutated from a leucine (CTG) to a valine (GTG). The two in frame stop codons (opal (TGA) and ochre (TAA)) directly following the coding region are conserved.

**Cell culture, transfection, and induction**

C₂C₁₂ cells were maintained as proliferating myoblasts in DMEM supplemented with 10% fetal bovine serum (FBS). CHO and COS-7 cells were cultured in M505 medium (50% DMEM, 50% Ham’s F12 nutrient medium). Cells were plated at 2x10⁵ (6 wells format) or 5x10⁴ (24 wells format) cells per well and grown overnight in proliferation medium (DMEM/ 10% FBS), to reach 50-60% confluency the next day. For measurements of intracellular calcium, cells were plated on H₂SO₄-etched 24 mm diameter glass coverslips in 6 well-format. For immunocytochemistry, 13 mm diameter coverslips were used in 24 well-format. For transfection, two solutions were prepared (volumes are given per well in 24 well format; for 6 well format, all volumes are to be multiplied by 4): (solution 1) 110 µl OptiMEM, 1.5 µl PLUS reagent, and 600 ng plasmid DNA (A₂₆₀/A₂₈₀ >1.8), and (solution 2) 110 µl OptiMEM and 2 µl LipofectAMIN. After 15 min, solutions 1 and 2 were mixed, and incubated for another 15 min. Meanwhile, half-confluent cultures were washed three times with OptiMEM. 300 µl OptiMEM was left on the culture, and the transfection solution was added. After 4 h incubation, the transfection medium was substituted with DMEM/ 10% FBS, and cells were allowed to recover overnight. For the Ecdysone-inducible eukaryotic expression system, a mix of pIND/Ig-lead/scFv/VSV, pIND/EGFP, and pVgRXR plasmids (in the ratio 6:2:2) was used. Induction was achieved the next day by applying Muresterone A or Ponasterone A, at a final concentration of 5 or 10 mM respectively, to the culture medium. PIRE2 and pcDNA 3.1 constructs were transfected separately. At confluency, one day post transfection, C₂C₁₂ culture medium was replaced by differentiation medium UBE (DMEM/ 10% brain extract/ 0.4% Ultroser G (Portier et al., 1999)), supplemented with Muresterone A or Ponasterone A if necessary. Cultures were grown, transected, and differentiated at 37°C in a humidified 5% CO₂/ 95% air atmosphere. Differentiation medium was refreshed every second day.

Following validation of the expression system in transiently transfected cells, stably transfected C₂C₁₂ cell lines were prepared using the pcDNA3.1-based expression system. Although antibody-encoding mRNA could be detected in stably transfected cells by RT-PCR, no antibodies could be detected neither with immunocytological nor with immunoblotting techniques. We therefore did not pursue this.
**Ratiometric measurements of intracellular calcium with Indo-1**

Coverslips with confluent monolayers of C2C12 myotubes, differentiated for three days, were washed twice with physiological salt solution (PSS; 125 mM NaCl, 10 mM NaHCO3, 1 mM NaH2PO4, 5 mM KCl, 2 mM MgSO4, 1.8 mM CaCl2, 10 mM HEPES and 10 mM glucose, pH 7.4) and loaded with Indo-1 using an Indo-1/AM solution (5 μM Indo-1/AM and 1 μM Pluronic F127 in PSS) for 45 min. Cell loading was performed at 37°C in a humidified 5% CO2/95% air atmosphere. Cells were washed twice with PSS, placed in a Leiden chamber (Ince et al., 1985), and incubated in PSS for another 15 min. The Leiden chamber was placed on a Nikon diaphot inverted microscope attached to an OZ confocal laser scanning microscope (Nikon instruments, Madison, WI). Electrical stimulation of myotubes was performed at 15 to 20 V with 10 ms duration and 0.025 ms delay. Repetitive stimuli were generated at a frequency of 1 Hz. Stimulation was performed using a pair of parallel oriented platinum electrodes (5 mm interspaced, placed beside the monolayer), connected to a Grass SD9 electric pulse generator. Calcium free medium was obtained by the use of Ca2+-free PSS, supplemented with 1 mM EGTA. Identification of EGFP-positive cells was performed by collecting fluorescence above 500 nm (500 LP filter), after excitation at 488 nm with an argon-ion laser (Omnichrome, Chino, CA). For ratiometric Ca2+ recordings, Indo-1 was excited at 351 nm using a hardware-modified high power argon-ion laser (Coherent enterprise, Santa Clara, CA). After being separated by a 455 DCLP dichroic mirror, Indo-1 fluorescence intensity was collected at 405±45 and 485±45 nm using photo multipliers. All measurements were performed at room temperature, using a Nikon x40, water-immersion objective, NA 1.2 with high UV transmission. The estimated laser intensity at the end of the objective lens was 28 μW. Confocality (optical section thickness: 1 to 2 μm) was achieved by using a slit width of 100 μm (Koopman et al., 2001). User-based hardware setup and data acquisition was controlled by Intervision software (Version 1.5, Noran Instruments), running under IRIX 6.2 on an Indy workstation (Silicon Graphics Inc., Mountain view, CA) equipped with 128 Mb of RAM. To improve signal-to-noise ratio, single full-frame images (512x480 pixels) were collected at 30 Hz by averaging of 32 images for each Indo-1 emission wavelength. Pairs of fluorescence intensities were saved in SGI-movie (mv) format, ready for off-line analysis using the Intervision 2D software package (Noran Instruments). Regions of interest (ROI) were drawn and numerical values were stored in ASCII-format. Additional data-analysis was carried out by using Origin 6.0 (Microcal, Northampton, MA), supplemental image analysis and visualization were performed with Image Pro-plus 4.1 (Media Cybernetics, Silver Spring, MD). To ensure the correct functioning of the stimulatory electric field, only measurements containing at least one excitable myotube were analyzed.
**Anti-heparan sulfate antibodies**

Preparation of c-Myc-tagged anti-HS antibodies (periplasmic fractions) AO4B05, AO4B08, AO4F12, RB4CB9, RB4CD12, RB4EA12, and control antibody MPB01 was performed as described previously (Jenniskens et al., 2000). Antibody MPB01 was randomly picked from scFv library #1 (Nissim et al., 1994). This antibody does not recognize HS, nor heparin, and is not reactive with any of the cells or tissues tested (muscle, brain, kidney, lung) by ELISA and IFA techniques. This control antibody was used in all three expression systems.

**Immunohistochemistry**

Cell cultures were washed three times with PBS, dried overnight, and stored at -80°C until use. Cell cultures were rehydrated in PBS for 10 min. After 20 min blocking in PBS containing 0.1% (w/v) BSA, fixed cultures were incubated with c-Myc-tagged anti-HS antibodies overnight. Bound c-Myc-tagged and endogenously produced VSV-tagged anti-HS antibodies were visualized using anti-c-Myc goat polyclonal A-14 and anti-VSV monoclonal P5D4 antibodies for 90 min, followed by Alexa 594-conjugated donkey anti-goat IgG and Alexa 488-conjugated goat anti-mouse IgG (60 min each). Perlecan and syndecan were visualized together with VSV-tagged intrabodies in a similar way, using Cy3-labelled P5D4 for VSV staining, antibody EY-90 for perlecan, and syndecan-1 and -3 antibody 2E9. Following each incubation, cultures

<table>
<thead>
<tr>
<th>Antibody</th>
<th>CDR3 sequence</th>
<th>Preferred HS sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>AO4B05</td>
<td>LKQQGIS</td>
<td>ND^</td>
</tr>
<tr>
<td>AO4B08</td>
<td>SLRMNGWRAHQ</td>
<td>GlcNS6S-IdoUA2S-IdoUA2S-GlcNS6S</td>
</tr>
<tr>
<td>AO4F12</td>
<td>AMTQKKPRKSL</td>
<td>ND^</td>
</tr>
<tr>
<td>RB4CB9</td>
<td>HAPLRNTRNT</td>
<td>1</td>
</tr>
<tr>
<td>RB4CD12</td>
<td>GMRPRL</td>
<td>GlcNS6S-IdoUA2S-GlcNS6S</td>
</tr>
<tr>
<td>RB4EA12</td>
<td>RRYALDY</td>
<td>GlcNS6S-IdoUA-GlcNS6S</td>
</tr>
<tr>
<td>MPB01</td>
<td>PKTRRN</td>
<td>not reactive with HS</td>
</tr>
</tbody>
</table>

CDR3 sequences are shown in single-letter amino acid code. The preferred HS sequence was deduced as described in Dennissen et al., 2002. Antibodies RB4CB9 and RB4CD12 (previously selected against human skeletal muscle GAGs) are identical to HS4E4 and HS3A8 (selected against a kidney HS preparation), respectively.

^ Antibodies AO4B05 and AO4F12 recognize different HS epitopes, as deduced by their differential reactivity in ELISA assay and different staining patterns in Torpedo marmorata electric organ (Jenniskens et al., 2000)

^ Preferred sequence is not known, N-sulfation is indispensable, 6-O-sulfation is likely to be inhibitory

^ Antibodies AO4B08 and RB4CD12 recognize different HS epitopes, as deduced by their differential reactivity in ELISA assay and different staining patterns in muscle and kidney (Jenniskens et al., 2000; Dennissen et al., 2002)

ND: not determined.
Figure 1. Construction of the vectors for antibody expression. Initially, the pIND expression vector was adapted by elimination of two NcoI sites of the original pIND vector (at nucleotides 1544 and 2279). Next, an immunoglobulin leader cassette (Ig-lead (single-letter amino acid code); MGWSCIILFLVATATGVHS, bold) and a VSV cassette (VSV tag (single-letter amino acid code); YTDIEMNRLGK, bold) were inserted in the multiple cloning site as HindIII-EcoRV and EcoRV-XhoI fragments respectively (DNA sequence is depicted in lower case), generating pIND/Ig-lead/VSV. Ig-lead and VSV cassettes were constructed such that scFv cassettes, NcoI-NotI digested from original pHEN clones (Jenniskens et al., 2000), can be inserted in frame into pIND/Ig-lead/VSV (NcoI and NotI recognition sequences are in bold and underlined). The first codon following the HindIII recognition site (following an in frame opal codon (tga) at nucleotides 432-434) is the translation initiation codon (atg; bold, italics) and the opal codon (tga; bold, italics) downstream of the VSV-tag sequence functions as stop codon. The Ig-lead/scFv/VSV expression cassettes were cloned into pIRES2/EGFP and pcDNA3.1 vectors as NheI-XhoI fragments.

were washed three times 10 min with PBS. Finally, cultures were fixed in 100% methanol, dried, and embedded in Mowiol (10% (w/v) in 0.1 M Tris-HCl, pH 8.5/ 25% (v/v) glycerol/ 2.5% (w/v) NaN₃). As a control, primary, secondary or conjugated antibodies were omitted. All incubations were performed at ambient temperature (21°C). Photographs were taken, using a constant shutter time, on a Zeiss Axioskop immunofluorescence microscope (Göttingen, Germany). Confocal images were made on a Nikon Diaphot inverted microscope attached to a BioRad MRC1024 ES confocal laserscanning microscope (Hemel Hempstead, UK). Digital images were processed using Confocal Assistant 4.02 and Adobe Photoshop 6.0 software.
Results

**Anti-HS intrabody and heparanase expression systems**

Phage display-derived antibodies have the advantage of the direct availability of the genetic material coding for the antibody in a single chain variable fragment (scFv) conformation. In this study we used several anti-HS antibodies from scFv library #1, selected against skeletal muscle-HS (Table 1; Jenniskens et al., 2000). From the original pHEN plasmid, the scFv antibody-coding region was excised, provided with an immunoglobulin leader sequence (Ig-lead), and cloned into three different eukaryotic expression vectors. Plasmid pcDNA3.1 was used for high-level expression, pIRES2-EGFP was used for bicistronic expression together with cytosolic enhanced green fluorescent protein (EGFP), and pIND was used for Ecdysone-inducible expression (see Methods, Fig. 1, and Table 1). All three expression systems were used in this study, and the results obtained were similar.

The immunoglobulin leader peptide was used to guide the anti-HS antibodies to the secretory pathway through the lumen of the Golgi apparatus. This approach was based on the assumption that antibodies may bind their HS epitopes while these are being synthesized in the lumen of this organelle. For all three expression systems, antibodies were found in cytosolic granules as demonstrated in CHO, COS-7, and C₂C₁₂ (myoblast) cells. In these cells a polarized, peri-nuclear expression was observed, which colocalized with NBD C₆-ceramide staining, a selective dye for the Golgi network (Fig. 2).

![Figure 2](image_url)

**Figure 2.** Golgi location of anti-HS antibodies. COS-7 cells were transfected with the ecdyson-inducible expression vector encoding anti-HS antibody AO4B05. Antibodies could be detected by immunologic detection of their VSV-tag (a) and were shown to colocalize with the Golgi network as visualized by NBD C₆-ceramide staining (b). Similar results were obtained for all antibodies. Arrows point at cell margin; N, nucleus. Scale bar, 10 μm.
Figure 3. Proliferation and differentiation of C2C12 myoblasts expressing control antibody MPB01 and cytosolic EGFP. C2C12 myoblasts were transfected with ecdyson-inducible expression vectors, encoding control antibody MPB01 and cytosolic EGFP, at 50-60% confluency. Transfected myoblasts display EGFP expression (arrows) as early as two hours post transfection (a). At confluence, myoblasts align and stretch (b). After two days of differentiation, myoblasts have fused to form myotubes (c, arrows). After five days of differentiation, myotubes dominate the culture (d, arrows), oriented in the parallel pattern typical for highly differentiated cultures. Expression of control antibody MPB01 and concomitant cytosolic expression of EGFP have no adverse effects on in vitro myogenesis. Scale bar, 100 μm.

Transfection efficiency in C2C12 myoblasts was 15-20%. Transfected myoblasts fused with neighboring wild type myoblasts to form morphologically normal myotubes (studied for all anti-HS antibodies; Fig. 3). Transfected myotubes showed EGFP distributed throughout the cytosol, whereas endogenously expressed VSV-tagged anti-HS antibodies were contained in Golgi vesicles, similar as in myoblasts. Both transfected and non-transfected myotubes contained 5 to 10 nuclei and displayed clusters of acetylcholine receptors (as visualized by α-bungarotoxin staining, data not shown). Also, in both transfected and in non-transfected myotubes, electrical excitability was first observed at day three of differentiation. These data indicate that differentiation of transfected (hybrid) myotubes resembles that of wild type myotubes.

Expression of anti-HS intrabodies or heparanase results in phenotypic HS-knock out myotubes

Endogenous expression of each anti-HS antibody blocks the specific epitope recognized by this antibody. This could be demonstrated by visualizing the endogenously expressed, VSV-tagged anti-HS intrabodies, which were located within Golgi vesicles, in transiently transfected C2C12 myoblasts (Fig. 4a1-a6). Simultaneously, using a c-Myc-
Figure 4. Expression of anti-HS intrabodies blocks the expression of the involved HS epitope at the cell surface. C2C12 myoblasts were transfected with ecdysone-inducible expression vectors encoding VSV-tagged anti-HS antibody AO4B05 (a1-3) or AO4F12 (a4-6). Antibodies were detected in cytosolic granules, most likely complexed with the HS epitope to which they bind. Counter-staining for BL-HS using c-Myc-tagged AO4B05 (b1-3) and AO4F12 (b4-6) reveals the presence of these HS epitopes at the surface of non-transfected cells, but not at the surface of transfected cells. The merge of images a and b (c1-6) shows in green the VSV-tagged intrabodies and in red the extracellular HS epitopes. Similar results were obtained for all antibodies. Scale bar, 25 μm.

tagged antibody against the same HS epitope, this epitope could not be detected in the extracellular matrix surrounding these cells (Fig. 4b1-b6). Using antibody-expressing C2C12 myoblasts, it was shown that phenotypic knocking out of one HS epitope does not necessarily impede the functional synthesis of another epitope, thus providing evidence for the presence of unique epitopes on separate HS molecules (Fig. 5a-d). This effect was studied and observed for HS epitopes AO4B05, AO4F12, and RB4CD12.

In the direct vicinity of transfected myotubes, the staining intensity of extracellular HS with c-Myc-tagged antibodies against the same HS epitope was less intense. Also, anti-VSV staining for intrabodies often
Figure 5. Phenotypically knocking out one HS epitope does not impede the cell surface expression of another epitope. C\textsubscript{2}C\textsubscript{12} myoblasts were transfected with ecdyson-inducible expression vectors encoding anti-HS antibody AO4B05 and cytosolic EGFP (a-d). Transfected cultures were FACS sorted to obtain homogeneous populations of AO4B05-expressing and of control myoblasts, using EGFP expression as a selection marker for transfected cells. Control myoblasts (a and c) differentiated into myotubes with BL staining for HS epitopes AO4B05 (a) and AO4F12 (c). In contrast, homogenous cultures of myotubes endogenously expressing antibody AO4B05 (b and d) showed no BL staining for the AO4B05 epitope, the antibody-HS complex being localized in the peri-nuclear Golgi-region (b). In contrast, HS epitope AO4F12 could readily be detected in the BL (d). Similar results were obtained for any combination of antibodies AO4B05, AO4F12, and RB4CD12. Scale bar, 50 \mu m.

resulted in labeling of BL-resident HS epitopes of surrounding myotubes, indicating that a part of these antibodies are secreted and are reactive with HS epitopes in the extracellular environment (see e.g. Fig. 4c3). The occurrence of HSPG core proteins on the surface and in the BL of transfected myotubes was examined for syndecan and perlecan, respectively (fig. 6). Elimination of HS epitopes AO4B05, AO4F12, or RB4CD12 had no effect on the occurrence and distribution of the core proteins.

Similar to the expression of anti-HS antibodies, human heparanase could be functionally expressed in C\textsubscript{2}C\textsubscript{12} cultures. By ELISA assay, a modest heparanase activity was detected in the culture supernatant of transfected cultures, indicating the secretion of functional heparanase by myoblasts. Moreover, heparanase expressing myoblasts stained poorly for any of the HS epitopes used in this study (results not shown). Hybrid myotubes were formed, with morphological characteristics similar to wild type and anti-HS antibody-transfected myotubes.
Table 2. The effect of endogenous expression of anti-HS intrabodies and heparanase on the excitability of 3 day-differentiated C2C12 myotubes.

<table>
<thead>
<tr>
<th>Culture</th>
<th>n</th>
<th>Excitability (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C2C12 Mocka</td>
<td>14</td>
<td>36</td>
</tr>
<tr>
<td>C2C12 MPB01b</td>
<td>21</td>
<td>29</td>
</tr>
<tr>
<td>C2C12 AO4B05</td>
<td>14</td>
<td>21</td>
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<tr>
<td>C2C12 AO4B08</td>
<td>19</td>
<td>11</td>
</tr>
<tr>
<td>C2C12 AO4F12</td>
<td>13</td>
<td>8</td>
</tr>
<tr>
<td>C2C12 RB4CB9</td>
<td>15</td>
<td>0*</td>
</tr>
<tr>
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<td>5*</td>
</tr>
<tr>
<td>C2C12 RB4EA12</td>
<td>23</td>
<td>17</td>
</tr>
<tr>
<td>C2C12 H.Hep</td>
<td>50</td>
<td>6*</td>
</tr>
</tbody>
</table>

Cytosolic calcium spikes of individual EGFP-positive C2C12 myotubes were monitored upon electric stimulation. The percentage of myotubes that responded to stimuli by generating calcium spikes (i.e. excitable) was determined. n represents the number of myotubes analyzed.

a Transfected with either pIRES2-EGFP, or with pVgRXR, pIND/EGFP, and pIND/Ig-lead/VSV (both lacking anti-HS antibodies or heparanase)

b Transfected with either pIRES2/MPB01-EGFP, or with pVgRXR, pIND/EGFP, and pIND/Ig-lead/MPB01/VSV

* P value < 0.05 (against C2C12 MPB01).

Excitation-induced calcium spiking is disrupted in HS-knock out myotubes

To examine the effect of the changes in cellular HS composition on calcium kinetics, ratiometric measurements of [Ca2+]i were performed in EGFP-positive myotubes of transiently transfected cultures after 3 days of differentiation. In these myotubes, the expression of EGFP is linked directly to antibody expression levels. We did not observe differences in calcium kinetics in myotubes with high EGFP expression levels (determined optically), as compared to myotubes with low or moderate EGFP expression. Indo-1 ratio signals were recorded within a region of interest (ROI) of individual myotubes using a high-speed UV confocal laser scanning microscope.

After 3 days of differentiation, 36% of the mock-transfected EGFP-positive myotubes responded to electrical stimuli by generating calcium spikes (Table 2). Upon co-expression of control antibody MPB01, the percentage excitable EGFP-positive myotubes was 29%. The kinetics of the calcium spikes of both control myotubes (Fig. 7a, trace 1) were identical to wild type calcium spikes, and no spontaneous calcium spikes were recorded. [Ca2+]i reached its maximum within 99 ms (three data points), after which it rapidly declined to basal level.

Expression of anti-HS antibodies or heparanase resulted in a marked decrease in the excitability of myotubes (Table 2). Moreover, all excitable EGFP-positive myotubes responded aberrantly to excitatory stimuli, in contrast to mock and MPB01-transfected myotubes, which in all recordings showed wild type responses. Anomalies included: (1) frequent skipping of calcium spikes upon electrical stimulation, (2) considerably slow decay resulting in an elevated plateau at high
Figure 6. Expression and distribution of the core proteins of HSPGs syndecan and perlecan is not affected in HS-knock out myotubes. Wild type C2C12 myoblasts (a) were transfected with a bicistronic expression vector encoding anti-HS antibody AO4F12 (b-d) and cytosolic EGFP. Subsequently, cultures were stained for syndecan (a1, c1) or perlecan (a2, c2). Transfected myotubes were identified by staining for the VSV-tag, present in the anti-HS antibodies (b). Expression of anti-HS antibodies does not affect the expression and distribution of either HSPG core protein (c). The merge (d) of images b and c shows in red the VSV-tagged intrabodies and in green the extracellular core proteins. Similar results were obtained for antibodies AO4B05 and RB4CD12. Scale bar, 50 μm (a); 20 μm (b-d).

frequency stimulation, (3) differences in amplitudes, (4) spontaneous generation of calcium spikes, (5) continuous firing, and (6) fluctuations in basal [Ca$^{2+}$], (Fig. 7b). Skipping of calcium spikes was observed most often in myotubes expressing heparanase and anti-HS antibodies AO4B05 and RB4EA12. Differences in amplitudes of calcium spikes were more frequently observed in AO4B08 and RB4EA12 expressing myotubes. Spontaneous generation of calcium spikes, in irregular or regular patterns, occurred most often in myotubes expressing AO4B05 and heparanase. Periodical fluctuations in the basal [Ca$^{2+}$] occurred especially upon expression of heparanase and anti-HS antibodies AO4B08 and RB4CD12. All calcium spikes were independent of the presence of calcium in the extracellular environment, indicating that the released calcium originated from intracellular stores (data not shown).
Figure 7. Ratiometric measurements of cytosolic calcium in transfected C2C12 myotubes. C2C12 myoblasts were transfected with a bicistronic expression vector, encoding control antibody MPB01 and cytosolic EGFP (a), or with an expression vector encoding an anti-HS antibody or heparanase combined with cytosolic EGFP (b). Upon confluency, C2C12 myotubes were differentiated for 3 days. Regions of interest (ROIs) were drawn on EGFP images (488 nm) of the myotubes (exemplified by the ROIs in a and b). Changes in intracellular calcium were measured in ROIs, corrected for background, and plotted against time as ratio values (405 nm/485 nm); bars below ratio traces indicate time points of electrical stimulation. Excitable myotubes transfected with control-antibody MPB01 show wild type calcium spikes upon stimulation (a; trace 1). Myotubes transfected with anti-HS antibodies or with heparanase gave on no occasion wild type calcium spikes, but displayed six types of anomalies (b; traces 1-6): (1) frequent skipping of calcium spikes upon electrical stimulation, (2) considerably slow decay resulting in an elevated plateau at high frequency stimulation, (3) differences in amplitudes, (4) spontaneous generation of calcium spikes, (5) continuous firing, and (6) fluctuations in basal $[\text{Ca}^{2+}]$. The table in the right panel depicts the occurrence of the anomalies for each expressed protein. Individual 405 nm and 485 nm signals are depicted for the underlined part of each trace. The physiological relevance of the ratio traces is demonstrated by the rise of the 405 nm signal (calcium-bound Indo-1), simultaneous with the decline of the 485 nm signal (calcium-unbound Indo-1). Similar results were obtained using either the ecdysone-inducible or the bicistronic expression system.
Excitation-induced calcium spiking is disrupted in mutant GAG-deficient myotubes

To verify the relationship between HS depletion and its effects on excitation-induced calcium spiking, we measured the calcium kinetics of myotubes of the C2C12-derived myoblast cell line S27 (Gordon and Hall, 1989; Bowen et al., 1996). S27 myoblasts differentiate normally, but are defective in GAG biosynthesis (shorter, undersulfated GAG chains are formed). The expression of HSPG core proteins (as judged on basis of the perlecan core protein) is normal. Staining for HS epitopes, applying the same antibodies as used in this study, is negative (Jenniskens et al., 2000). Only few (approximately 2%) S27 myotubes were excitable, and those that could be triggered generated modest calcium spikes (Fig. 8). Excitable S27 myotubes had a very low signal to noise ratio, in contrast to wild type myotubes that displayed sharp calcium spikes.

**Figure 8.** Ratiometric measurements of cytosolic calcium in wild type and in GAG-deficient myotubes. Wild type (a; C2C12) and GAG-deficient (b; S27) myotubes were differentiated for 3 days. Regions of interest (ROIs) were drawn on Indo-1 images (485 nm) of the myotubes (exemplified by the ROIs in a and b). Changes in intracellular calcium were measured in ROIs, corrected for background, and plotted against time as ratio values (405 nm/485 nm); bars below ratio traces indicate time points of electrical stimulation. Upon stimulation, excitable wild type myotubes showed sharp calcium spikes (a; trace). Only few S27 myotubes were excitable, and these showed minor calcium fluxes with a very low signal to noise ratio (b; trace). Individual 405 nm and 485 nm signals are depicted for the underlined part of each trace. The physiological relevance of the ratio traces is demonstrated by the rise of the 405 nm signal (calcium-bound Indo-1), simultaneous with the decline of the 485 nm signal (calcium-unbound Indo-1). The table depicts the number of excitable myotubes as a percentage of the total number measured; C2C12, n=1875; S27, n=140.

<table>
<thead>
<tr>
<th>Culture</th>
<th>n</th>
<th>Excitability (%)</th>
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</thead>
<tbody>
<tr>
<td>C2C12 (a)</td>
<td>1875</td>
<td>40</td>
</tr>
<tr>
<td>S27 (b)</td>
<td>140</td>
<td>2</td>
</tr>
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Discussion

With the generation of a panel of antibodies that recognize specific, discretely located HS epitopes in skeletal muscle (Jenniskens et al., 2000), we recently developed tools appropriate for in vitro studying of the physiological roles of HS epitopes. In this paper, we report on the intracellular expression of these antibodies and of the HS-degrading enzyme heparanase, resulting in phenotypic knock out of HS in the C2C12 myoblast cell line. We show that the calcium kinetics in HS-defective myotubes is impaired, indicating that HS epitopes have a role in the proper functioning of excitation-induced calcium spiking in skeletal muscle.

Expression of anti-HS intrabodies and heparanase in the C\textsubscript{2}C\textsubscript{12} myoblast cell line was accomplished by either of three expression systems: (1) pcDNA3.1 for high-level expression, (2) pIRE\textsubscript{2}-EGFP for bicistronic expression together with cytosolic EGFP in transient transfectants, and (3) pIND of the Ecdysone-inducible eukaryotic expression system for inducible expression together with cytosolic EGFP in transient transfectants. All three expression systems gave essentially the same results. Using an inducible expression system, we anticipated on a possible future implementation of the system in a transgenic mouse model, thus enabling the regulation of expression of the antibodies \textit{in vivo} in time. Whereas stably transfected myoblasts were unable to produce detectable quantities of antibodies using either expression system, transiently transfected myoblasts expressed antibodies in amounts sufficient for immunologic detection and for studying their physiological effects. We therefore did not pursue the generation of stably transfected cell lines, but studied the effects of the expression of anti-HS antibodies in transiently transfected cells. Differences in expression levels are probably caused by the presence of multiple copies of the expression constructs in transient transfectants as compared to stably transfected myoblasts, or by the lack of transcriptional activity due to the place of insertion in the genome.

Targeting of the intrabodies to the secretory pathway was realized by using an immunoglobulin leader peptide. Anti-HS intrabodies bind to HS epitopes in the Golgi lumen, and the antibody-HS complexes formed may either be secreted into the extracellular environment or recognized and degraded by the cellular quality control system. Unbound antibodies are secreted and bind to HS epitopes on cells surrounding transfectants. Endogenously expressed heparanase possibly acts in a similar fashion, digesting HS within the Golgi lumen and being secreted to the extracellular space in an active conformation. The effect of the expression of anti-HS intrabodies or heparanase is the inactivation of newly formed HS, resulting in phenotypic HS-knock out myoblasts.

Blocking one HS-epitope with a specific antibody may result in the removal of the whole HS chain, possibly harboring other HS-epitopes.
However, our results show that knocking out one HS epitope does not necessarily interfere with the synthesis of another epitope, arguing for the occurrence of individual epitopes on physically separated HS molecules. This phenomenon, however, was not investigated further. Phenotypic knock out of HS epitopes did not interfere with the occurrence and distribution of cell surface and BL-resident HSPG core proteins. HS-knock out myotubes stained normally for syndecan and perlecan, respectively, and did not have either core protein retained within the Golgi apparatus. This situation is analogous to effects seen in xyloside-treated cells (Rapraeger et al., 1996), in which proteoglycan core proteins have shorter or no GAG chains and are transported and located similar to intact proteoglycans. Blocking or digestion of (a specific) HS epitope(s) within the Golgi apparatus apparently does not necessarily interfere with the proper routing of the HSPG core protein. In this context, it will be interesting to investigate whether the HS epitopes are specific for individual HS chains and for certain HSPG core proteins.

Cytosolic co-expression of the bioluminescent protein EGFP was used to identify transfected myotubes. Since myotubes are formed by the fusion of neighboring myoblasts, heterogeneous syncytia originate from both transfected and non-transfected myoblasts. The dilution of EGFP-, antibody-, and heparanase-coding vectors due to myoblast fusion and the degradation over time by nucleases may result in less effective expression levels of these proteins. Moreover, the synthesis of HSPGs from various nuclei within a myotube may compensate for the phenotypic HS-knock out effectuated by the expression of antibodies or enzyme. Three days of differentiation was determined as an optimum between the myotube maturation grade and the dilution and degradation of the transfected plasmids. At this stage, excitable myotubes occurred for the first time (simultaneously) in both wild type and in transfected myoblast cultures, indicating a similar level of differentiation. EGFP-negative myotubes may prove to be falsely negative since their HS epitopes may be blocked by antibodies or digested by heparanase secreted by neighboring (transfected) myotubes. Hence, mock transfected and control antibody-transfected EGFP-positive myotubes were used as a control, rather than EGFP-negative myotubes neighboring transfected myotubes.

The effect of the depletion of HS-epitopes was evaluated by ratiometric measurements of the \([Ca^{2+}]_c\) on transfected myotubes, using a high-speed UV confocal laser scanning microscope. A considerable portion of control antibody-transfected myotubes could be triggered to generate calcium spikes, all of which had calcium kinetics identical to wild type myotubes. Upon the expression of anti-HS intrabodies or heparanase, the percentage of excitable myotubes was significantly reduced. Concomitantly, various anomalies in calcium signaling were noticed, whereas wild type calcium spikes were never observed. Excitable hybrid myotubes appeared to differentiate normally, as judged by their
general appearance, the number of nuclei per myotube, the occurrence of spontaneous acetylcholine receptor clustering, and the first occurrence of excitability. In wild type myoblast cultures all excitable myotubes displayed mature calcium kinetics and no aberrant calcium kinetics were observed. Since (for both wild type and hybrid myotubes) poor differentiation results in no excitability at all rather than aberrant calcium kinetics, the anomalies observed in hybrid myotubes are likely to be the result of the affected HS composition, rather than the result of a disturbance in differentiation.

In line with these results are the data obtained from calcium measurements in the GAG-deficient myoblast cell line S27. Although the primary defect is unknown, the S27 cell line is defective in GAG biosynthesis but forms morphologically normal myotubes. The calcium kinetics in S27 myotubes is severely disturbed, further indicating an effect of GAGs in excitation-induced calcium kinetics. Finally, aberrant calcium kinetics are observed in myotubes derived from myoblast cultures from mice carrying targeted disruptions in the gene encoding N-deacetylase/N-sulfotransferase-1, an enzyme involved in HS biosynthesis (Jenniskens et al., submitted). Together, these data indicate a role for HS in skeletal muscle calcium kinetics.

Non-excitable HS-defective myotubes frequently showed spontaneous fluctuations of the [Ca\(^{2+}\)]\(_i\) either as periodical elevations of the resting [Ca\(^{2+}\)]\(_i\) or as autonomously generated calcium spikes, indicating a disturbed calcium regulation. In absence of HS, dihydropyridine receptor-ryanodine receptor coupling appears to be affected and the myotube cell membrane seems to adopt an autonomous membrane oscillatory activity, triggering periodical calcium spikes. The effect of HS on excitation-induced calcium spiking may be mediated through direct or indirect regulatory interactions with ion channels in the cell membrane, like voltage-gated sodium channels or DHPRs, as has been described for extracellular heparin (Knaus et al., 1990; Knaus et al., 1992; Martinez et al., 1996). Lamb and coworkers (1994) found a negative regulatory effect of intracellular heparin, most likely by acting on the DHPR voltage sensor. HS, which is less sulfated than heparin, might act in a similar way. Since specific aberrations in calcium kinetics preferentially occur upon the elimination of certain HS-epitopes, it is tempting to attribute these effects to the HS-sequences involved. For instance, expression of antibody AO4B08, which recognizes HS sequences containing 2-O-sulfated iduronic acid residues, has a different effect than RB4EA12, which preferentially recognizes unsulfated iduronic acid residues. The expression of heparanase brought about all anomalies in calcium kinetics that were observed for individual antibodies, often combined in one recording. This may be due to the relatively broad HS-degrading activity of the enzyme (Vlodavsky and Friedmann, 2001), in contrast to the antibodies that recognize specific HS epitopes. In all cases, calcium spikes occurred both in the presence and in the absence of PHENOTYPIC HEPARAN SULFATE KNOCK OUT MYOTUBES.
extracellular calcium, indicating that released calcium originated from intracellular stores.

Taken together, these data indicate specific functions of HS epitopes in skeletal muscle calcium kinetics. The antibody-based expression systems presented here, add a powerful tool to the repertoire available in the quest for improving our understanding of the HS structure-function relationship. Next to the generation of cell lines or mice defective in HS synthesis, it has now become possible to phenotypically knock out specific HS epitopes, enabling the detailed study of the functions of HS epitopes in cellular physiology in vitro and in the future perhaps in vivo. Using this expression system in skeletal muscle cell culture, we show for the first time a functional role for HS epitopes in myotube excitation-induced calcium spiking.

References


Chapter 7

A Rare Glycosaminoglycan Epitope Present in the Sarcoplasmic Reticulum of Skeletal Muscle is Involved in Calcium Kinetics

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Submitted.
Here we report on the identification of a unique glycosaminoglycan (GAG) epitope, localized in the sarcoplasmic reticulum of mammalian skeletal muscle. Antibody RB4CG9, which interacts with this GAG epitope, was selected by phage display against a GAG preparation derived from skeletal muscle. This antibody reacts with heparin and with the unusual GAG acharan sulfate (AS) but not with other GAGs. AS is a heparan sulfate-like glycopolymer consisting of GlcNAc-IduA2S disaccharides. The antibody does not react with N-sulfated, N-deacetylated, nor with 2-O-desulfated AS, indicating GlcNAc-IduA2S to be of major importance in the epitope. Functional depletion of this GAG epitope in myotubes, by expression of antibody RB4CG9 in C2C12 myoblast cultures, severely affects myotube excitability and alters the kinetics of electrically-induced calcium spikes. They show a significantly lower amplitude of calcium spikes, and frequently undershoots below basal cytosolic calcium level. Moreover, the reuptake rate of cytosolic calcium is strongly increased, arguing for a role of this uniquely located GAG epitope in the kinetics of sarcoplasmic reticulum calcium ATPase.

Introduction

Contraction of skeletal muscle follows upon calcium release from sarcoplasmic stores in response to depolarization of the sarcolemma. This signaling process is called excitation-contraction (e-c) coupling, and depends on intracellular junctions between invaginations of the sarcolemma (the transverse (T) tubules) and cellular calcium stores (the sarcoplasmic reticulum (SR)). These two membranous systems meet in so called triads, where a flattened portion of the T-tubule is flanked on both sides by terminal cisternae of the SR. Dihydropyridine receptors (DHPRs), located in the junctional T-tubular membrane, function as voltage sensors and activate the facing ryanodine receptors (RyRs) in the SR terminal cisternae to release calcium into the sarcoplasm. Following its release, calcium triggers muscle contraction and is pumped back into the SR by calcium ATPases (SERCAs), which are located throughout the SR membrane (Franzini-Armstrong, 1972; Franzini-Armstrong, 1986; Hille, 1992; Flucher and Franzini-Armstrong, 1996).

T-tubules and SR develop simultaneously during myogenesis. Concomitantly, the interactions are formed between the proteins of both membrane systems in the triad (Franzini-Armstrong, 1991; Flucher, 1992; Flucher et al., 1992; 1994). Mature triads contain several proteins that are specifically located in the membrane or lumen of either the T-tubules or the SR. Whereas most of these proteins are thought to be involved in e-
c coupling, few have been studied in detail. The integral SR membrane proteins triadin and junctin, and the SR luminal calcium binding protein calsequestrin are shown to mutually interact and to associate with the RyR, possibly regulating its activity (Guo and Campbell, 1995; Zhang et al., 1997; Ohkura et al., 1998; Caswell et al., 1999; Shin et al., 2000). SERCA activity in skeletal muscle is regulated by two related small SR-transmembrane proteins: phospholamban (in type I fibers) and sarcolipin (in type II fibers; Aubier and Viìres, 1998; East, 2000).

Proteoglycans, consisting of a core protein and a glycosaminoglycan (GAG) moiety, are present in the extracellular matrix and at the surface of virtually all cell types. GAGs are negatively charged polysaccharides, which bind and modulate a large number of proteins and are able to bind cations, notably calcium (Lerner and Torchia, 1986). The large number of modifications in GAG chains makes them a highly versatile group of cell-regulatory molecules (Spillmann and Lindahl, 1994; Bernfield et al, 1999; Turnbull et al., 2001, Gallagher, 2001). To our knowledge, no proteoglycans have been described to be located within the lumen of cellular organelles, except for the Golgi apparatus where the GAG moieties are synthesized and in the nucleus where GAGs are present possibly associated with the process of growth factor internalization.

In this paper, we report the presence of a unique GAG epitope within the SR of the skeletal muscle. We demonstrate that the functional depletion of this epitope results in drastic changes of calcium kinetics. Therefore, we hypothesize a novel role of GAGs in skeletal muscle calcium kinetics.

Materials and Methods

Materials

Synthetic single chain variable fragment (scFv) library #1 (Nissim et al., 1994) was generously provided by Dr. G. Winter (Cambridge University, Cambridge, United Kingdom). Human skeletal muscle samples (diaphragm, m. quadriceps, and m. soleus) were generously provided by Prof. Dr. D. Ruiter (Department of Pathology, University of Nijmegen, Nijmegen, The Netherlands). Frogs (Xenopus laevis, young adult) were a kind gift of Dr. R. Kuiper (Department of Molecular Animal Physiology, University of Nijmegen, Nijmegen, The Netherlands). Creatine kinase knock out mice were provided by Dr. Frank Oerlemans (Department of Cell Biology, University of Nijmegen, Nijmegen, The Netherlands). Anti-SERCA-1/-2 polyclonal rabbit anti-rat antibody was a kind gift of Dr. J. Timmermans (Department of Physiology, University of Nijmegen,
Nijmegen, The Netherlands); mouse monoclonal anti-chicken SERCA-1 IgG (5D2) was a kind gift of Dr. D. Fambrough (Department of Embryology, Carnegie Institution of Washington, Baltimore, MD). Mice (C3H and C57BL/6) and rats (Wistar, male, 10 d; six weeks) were obtained from the University of Nijmegen Central Animal Laboratory. C2C12 (CRL1772) skeletal muscle cell line was purchased from American Type Culture Collection (Rockville, MD).

All chemicals used were from Merck (Darmstadt, Germany), unless stated otherwise. Bacterial media (2xTY and LB), cell culture media, OptiMEM, and LipofectAMIN/ PLUS reagents were obtained from Life Technologies (Paisley, Scotland); tissue culture plastics from Greiner (Frickenhausen, Germany). Chondroitinase ABC (from Proteus vulgaris, EC 4.2.2.4) was from Seikagaku Kogyo Co. (Tokyo, Japan). Heparinase III (from Flavobacterium heparinum, EC 4.2.2.8), heparan sulfate from bovine kidney and from porcine intestinal mucosa, heparin from porcine intestinal mucosa, chondroitin 4-sulfate and chondroitin 6-sulfate from bovine trachea, dermatan sulfate from porcine intestinal mucosa, bovine serum albumin (fraction V), 3,3'diaminobenzidine-4HCl (DAB), antidesmin rabbit monoclonal IgG, and NaN3 were obtained from Sigma (St. Louis, MO). AS was isolated as described (Kim et al., 1996). Anti-c-Myc mouse monoclonal IgG (clone 9E10) and anti-VSV antibody (clone P5D4) were purchased from Boehringer Mannheim (Mannheim, Germany). Anti-c-Myc goat polyclonal IgG (A-14), anti-dihydropyridine receptor goat polyclonal IgG (N-19), and anti-ryanodine receptor goat polyclonal IgG (N-19) were from Santa Cruz Biotechnology (Santa Cruz, CA). Biotin-conjugated donkey-anti-mouse, Vectastain ABC Elite kit was from Vector Laboratories (Burlingame, CA). Alexa 488-conjugated donkey anti-goat IgG and goat anti-mouse IgG, NBD C6-Ceramide (N-1154), Indo-1/AM and Pluronic F127 were purchased from Molecular Probes (Eugene, OR). Mowiol (4-88) was obtained from Calbiochem (La Jolla, CA). Polymerase chain reaction (PCR) chemicals and Taq polymerase (DNA polymerase from Thermus aquaticus) were from Promega (Madison, WI), PCR primers and synthetic oligonucleotides were obtained from Biolegio (Malden, The Netherlands). Eukaryotic expression vector pIREES2-EGFP was obtained from Clontech (Palo Alto, CA). Restriction enzymes and buffers were purchased from New England Biolabs (Beverly, MA). Plasmid DNA isolation kit was purchased from Qiagen (Hilden, Germany). ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit was from PE Applied Biosystems (Norwalk, CT).

All experiments were performed at ambient temperature (21°C), unless stated otherwise.
Selection of antibody RB4CG9

Anti-GAG scFv antibody RB4CG9 was selected against GAG preparations of human skeletal muscle. Selection and characterization of antibody RB4CG9, and preparation of antibodies (periplasmic fraction) were performed as described previously (van Kuppevelt et al., 1998; Jenniskens et al., 2000). Briefly, synthetic scFv library #1 (containing approximately 10⁸ different scFv antibody clones) was subjected to panning against an immobilized skeletal muscle-derived GAG preparation (isolated from human diaphragm, m. quadriceps, and m. soleus). After four rounds of panning, single colonies were picked and the antibodies expressed by individual clones were evaluated for reactivity against various GAGs by ELISA. Subsequently, staining patterns of these antibodies were evaluated by immunohistochemistry on longitudinal cryosections of skeletal muscle.

Cell culture, transfection, and ratiometric measurements of intracellular calcium

Generation of the RB4CG9 expressing pIRES eukaryotic expression vector, cell culture, transfection, and ratiometric calcium measurements were carried out as described (Jenniskens et al., 2002). In short, C₂C₁₂ myoblast cultures were transfected with pIRES expression constructs resulting in the expression of VSV-tagged MPB01 (control) or RB4CG9 antibodies within the Golgi apparatus, concomitant with cytosolic expression of enhanced green fluorescent protein (EGFP). Using video-speed UV confocal laser scanning microscopy, changes in cytosolic calcium concentration upon electric stimulation were monitored as Indo-1 ratios in EGFP positive myotubes. Additional off-line data analysis was performed using the Origin 6.0 software package (Microcal, Northampton, MA).

Pre- and postnatal mouse tissue

C3H mice were bred under standard conditions and monitored for vaginal plugs twice a day. The first day of occurrence of a vaginal plug was taken as day 0 of gestation (E0). Pregnant mice were quarantinised and kept solitary until the litter had reached the indicated E-number (12, 13, 14, 15, 16, 17, 18, and 20 days in utero). Mice were anesthetized, killed by cervical dislocation, and embryos were isolated either with (E12 and 13) or without (E14-E20) the surrounding uteric tissue, rinsed in PBS, snap-frozen in liquid nitrogen-cooled isopentane, and stored at -80°C. Birth occurred on average at day E20 (P0). Mice of postnatal day 2 (P2) were anesthetized prior to freezing. Adult muscle specimens were isolated from the pregnant females that were killed. For each
developmental stage, at least three individual mice, derived from two separate litters, were studied.

**Immunohistochemistry**

Skeletal muscle tissue specimens were snap-frozen in liquid nitrogen-cooled isopentane and stored at -80°C. 3 or 5 μm thick (muscle specimens) or 5 to 10 μm thick (mouse embryos and newborns) cryosections were cut, mounted on slides, dried thoroughly, and stored at -80°C until use. Tissue cultures were washed three times with PBS, dried overnight, and stored at -80°C. Cryosections or tissue cultures were stained as described previously (Jenniskens et al., 2000; Jenniskens et al., 2002). Confocal images were made on a Nikon Diaphot inverted microscope attached to a BioRad MRC1024 ES confocal laser scanning microscope (Hemel Hepingstead, UK). Digital images were processed using Confocal Assistant 4.02 and Adobe Photoshop 6.0 software.

**Electron microscopy**

Frogs (*Xenopus laevis*) were anesthetized with MS222 and perfused with 0.9% NaCl, followed by Bouin fixative (9% (v/v) paraformaldehyde, 3% (w/v) picric acid, 5% (v/v) acetic acid). Skeletal muscle samples were taken and immersed overnight in Bouin fixative at 4°C. After washing in 0.1 M phosphate buffer, pH 7.4 (2 times 1 h), 75 μm sections were cut using a Leica VT1000S vibratome. Sections were rinsed three times in PBS, pH 7.4 (20 min each), incubated for 60 min in PBS containing 0.1% (w/v) BSA, followed by free floating overnight incubation with VSV-tagged anti-GAG antibody RB4CG9. After rinsing three times in PBS, sections were incubated with anti-VSV antibody P5D4 for 90 min, rinsed three times, and incubated with biotin-conjugated donkey-anti-mouse antibody for 60 min. Subsequently, sections were incubated in a solution of avidin and biotinylated horseradish peroxidase for two hours, followed by a 0.02% DAB solution in 0.05 M Tris/3% H₂O₂ for 10 min. The substrate reaction was stopped by rinsing twice in PBS (5 min each), followed by 0.1 M phosphate buffer (1 h). As a control, a phage display-derived antibody that does not react with HS was used (MPB01). Next, sections were osmicated for one hour in 1% osmiumtetroxide and rinsed again in 0.1 M phosphate buffer. Sections were dehydrated in a grade series of ethanol, embedded in epon 812 via propylene oxide and mounted in epon 812. After polymerization, a Reichert Ultracut-E was used to cut 80 nm thick sections, which were examined under a Jeol 1010 electron microscope.
Results

Selection of the RB4CG9 antibody

Antibody RB4CG9 was obtained by phage display as described (Jenniskens et al., 2000). Four rounds of panning were performed against a GAG preparation derived from human skeletal muscle (combined diaphragm, m. quadriceps, and m. soleus). Selection resulted in the isolation of two identical scFv antibodies designated RB4CG9. RB4CG9 is a member of VH family 4 (DP segment 67) and bears the complementarity determining region 3 (a major determinant in antigen specificity) amino acid sequence LRSVSPGY.

RB4CG9 recognizes a rare GAG epitope

In ELISA, antibody RB4CG9 showed no reactivity with K5 (heparosan, the precursor polysaccharide of heparan sulfate (HS)), chondroitin sulfate (CS), and dermatan sulfate (DS). Immunoreactivity was seen with GAG preparations from mammalian skeletal muscle and with heparin. Surprisingly, RB4CG9 also showed reactivity with acharan sulfate (AS), a HS-like GAG isolated from the giant African snail (Achatina fulica), which mainly consists of GlcNAc-IduA2S disaccharides (Kim et al., 1996).

Table 1. Evaluation of RB4CG9 specificity by ELISA

<table>
<thead>
<tr>
<th>GAG preparation</th>
<th>Reactivity</th>
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<tr>
<td>K5 capsular polysaccharide (E. coli)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-</td>
</tr>
<tr>
<td>AS (Achatina fulica)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>++</td>
</tr>
<tr>
<td>N-sulfated AS</td>
<td>-</td>
</tr>
<tr>
<td>N-deacetylated AS</td>
<td>-</td>
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<tr>
<td>2-O-desulfated AS</td>
<td>-</td>
</tr>
<tr>
<td>CS (bovine trachea)</td>
<td>-</td>
</tr>
<tr>
<td>DS (porcine intestinal mucosa)</td>
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<td>GAG preparation (human m. quadriceps)</td>
<td>+</td>
</tr>
<tr>
<td>GAG preparation (rat diaphragm)</td>
<td>++</td>
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<tr>
<td>GAG preparation (rat m. quadriceps)</td>
<td>+</td>
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<tr>
<td>GAG preparation (rabbit diaphragm)</td>
<td>++</td>
</tr>
<tr>
<td>GAG preparation (rabbit m. quadriceps)</td>
<td>+</td>
</tr>
<tr>
<td>GAG preparation (mouse total skeletal muscle)</td>
<td>++</td>
</tr>
<tr>
<td>AS after heparinase treatment&lt;sup&gt;c&lt;/sup&gt;</td>
<td>+</td>
</tr>
<tr>
<td>Heparin after heparinase treatment&lt;sup&gt;c&lt;/sup&gt;</td>
<td>±</td>
</tr>
<tr>
<td>GAG preparations after heparinase treatment&lt;sup&gt;c&lt;/sup&gt;</td>
<td>+</td>
</tr>
<tr>
<td>GAG preparations after Chondroitinase ABC treatment</td>
<td>++</td>
</tr>
</tbody>
</table>

RB4CG9 antibodies were applied to GAG preparations immobilized on microtiter plates. Bound antibodies were detected using anti-c-Myc mouse monoclonal antibody 9E10, followed by alkaline phosphatase-conjugated rabbit anti-mouse IgG, after which enzymatic activity was measured using p-nitrophenyl as a substrate. Reactivity: ++, strong; +, moderate; ±, low; -, absent (n=5).

<sup>a</sup> Similar to the HS precursor polysaccharide (heparosan)

<sup>b</sup> AS, acharan sulfate; a HS-like GAG

<sup>c</sup> Digested with heparanase I, II, and III
RB4CG9 had no reactivity with N-sulfated, deacetylated, or 2-O-desulfated AS, or with HS from sources other than muscle tissue (Table 1). Treatment of GAG preparations with chondroitinase ABC (digests CS and DS) did not interfere with immunoreactivity. Treatment with a combination of heparinases I (digests HS/ heparin at H_{N,STY}-I_2S), II (digests AS and HS/ heparin at H_{N,STY-G/I_2S}), and III (digests HS/ heparin at H_{NAc-I}/ H_{N,STY-G} (X, sulfated or unsubstituted; Y, sulfated or acetylated)) diminished reactivity, arguing for a HS-like nature of the RB4CG9 GAG-antigen.

**Unique location of the RB4CG9 epitope in skeletal muscle**

In immunohistochemistry, antibody RB4CG9 reacted only with skeletal muscle tissue. Studies on longitudinal cryosections of muscle specimens of various species (frog, rat, mouse, rabbit, and human) revealed a distinct cross-striation after staining with the RB4CG9 antibody. RB4CG9 reactivity appeared in bands, approximately 2 μm interspersed, transversely oriented to the longitudinal direction of muscle fibers (Fig. 1a).

**Figure 1.** Immunolocalisation of GAG epitope RB4CG9 in adult and developing skeletal muscle. Longitudinal cryosections of adult mouse m. soleus (a), cross cryosections of m. quadriceps (b), and cryosections of myogenic regions of fetal mice at embryonic days E14 (c and e) and E20 (birth; d and f) were stained for GAG epitope RB4CG9. Regular cross striation, approximately 2 μm interspersed, was observed (a). In muscle specimens composed of different fiber types, a differential staining was observed (b). Cross (c and d) and longitudinal (e and f) cryosections of mouse fetal myogenic regions showed GAG epitope RB4CG9 to be present near the sarcolemma of primary myotubes in a punctuate pattern at E14 (c and e), and cross striated throughout muscle fibers at E20 (d and f). Scale bar, 30 μm (a); 500 μm (b); 50 μm (c-f).
Colocalization studies in various postural muscles (*m. extensor digitorum longus; m. soleus; m. quadriceps*) with antibodies against myosin fast, SERCA-1, and SERCA-1/-2 showed that antibody RB4CG9 has a strong preference for slow myofibers (type I; oxidative), as compared to fast myofibers (type II; glycolytic; Fig. 1b).

**Figure 2.** Immunolocalisation of GAG epitope RB4CG9 in skeletal muscle. Longitudinal cryosections of rat skeletal muscle were stained for GAG epitope RB4CG9 (a1-c1), RyR (a2), SERCA (b2), and desmin (c2). Merged images are shown in color (a3-c3), of which enlargements are shown in the far right panel (a4-c4). GAG epitope RB4CG9 (large arrows to the left of panels 4) is flanked by RyR (small arrows to the right of panels a4), and alternates with bands with no staining, suggesting the presence of this GAG epitope over either the longitudinal or the fenestrated SR. Note the double cross-striated bands when stained for RyR (a2), due to the presence of two triads per sarcomere. An overlap in location with SERCA (small arrows to the right of panel b4) and with desmin (small arrows to the right of panel c4) confirms the location of this GAG epitope over the fenestrated SR. Scale bar, 20 μm (1-3); 2.5 μm (4).
In cryosections of whole mouse embryos RB4CG9 reacted with myogenic regions only (Fig. 1). At embryonic day 12, antibody RB4CG9 stained the periphery of developing primary myotubes. Ongoing myogenesis and the concomitant development of the muscle triad membranous systems resulted in a punctuate staining pattern with antibody RB4CG9 near the sarcolemma of primary myotubes at E14 (Fig. 1c and 1e), which developed to a cross-striation of the entire muscle fiber at E20 (Fig. 1d and 1f) and in further stages of development.

GAG epitope RB4CG9 was moderately detectable in cultured C2C12 myotubes; small intracellular granules could be stained, together with a general cytoplasmic staining. Under the culturing conditions used, C2C12 myotubes did not reach a differentiation stage in which RB4CG9 cross-striation became visible (data not shown).

**Immunohistochemical localization of GAG epitope RB4CG9**

Staining of skeletal muscle with antibody RB4CG9 resulted in a highly ordered pattern of transversely oriented bands. To investigate whether this GAG epitope occurs associated with either of the membrane systems of the triad, we performed colocalization studies of RB4CG9 with sarcomeric proteins and with several ion channels of the e-c coupling system. Skeletal muscle of mammals contains two triads per sarcomere, located at the A-I junction (reviewed in Franzini-Armstrong, 1972). In rat RB4CG9 did not colocalize with the DHPR nor with the RyR (fig. 2a1-a4). With SERCA, an overlap was found (fig. 2b1-b4), whereas RB4CG9 staining also colocalized with desmin, indicating a distribution over the Z-line (fig. 2c1-c4). A sarcomeric band that was flanked by RyR and negative for desmin did not stain with antibody RB4CG9. Similar staining patterns were obtained for mouse, rabbit, and human skeletal muscle. In frog skeletal muscle only one triad is present per sarcomere, which is located at the Z-line. Here, RB4CG9 staining overlapped with SERCA (data not shown).

Since colocalization studies suggested the presence of GAG epitope RB4CG9 in muscle SR, we looked into greater detail to the subcellular location. Immuno electron microscopy on frog skeletal muscle using RB4CG9 indicated the presence of the GAG epitope within the sarcoplasmic reticulum (Fig. 3b). In mammalian muscle, however, we could not achieve electron microscopic immunostaining with this antibody. We therefore performed immunostaining on skeletal muscle from mice double mutant for the mitochondrial and cytosolic creatine kinase loci (creatinine kinase/−; Steeghs et al., 1997). Creatine kinase−/− muscle displays longitudinally oriented tubular aggregates consisting of SR-terminal cisternae, which were strongly stained with antibody RB4CG9 (Fig. 3a).
Figure 3. GAG epitope RB4CG9 is located in the SR of skeletal muscle. Longitudinal cryosections of m. soleus of creatine kinase-knock out mouse (a) were stained for GAG epitope RB4CG9. RB4CG9 staining results in an ordered pattern of transversely oriented bands as well as longitudinally oriented tubular structures that represent aggregates of SR-terminal cisternae, which are present in skeletal muscle of creatine kinase-knock out mice (Steeghs et al., 1997; arrows in a). Immuno-electron microscopical localization of GAG epitope RB4CG9 in frog skeletal muscle (b) confirms that GAG epitope RB4CG9 is present within the sarcoplasmic reticulum. SR, sarcoplasmic reticulum; T, T-tubule. Scale bar, 20 μm (a); 0.1 μm (b).

Phenotypic knock out of GAG epitope RB4CG9 leads to aberrant calcium kinetics in cultured myotubes

The SR location of GAG epitope RB4CG9 prompted us to study its possible involvement in calcium kinetics in C2C12 myotubes. For this, we used a pIRES-based eukaryotic expression system adapted for expression of anti-HS antibodies within the Golgi apparatus, simultaneously with cytosolic expression of EGFP (Jenniskens et al., 2002). Previously, we have shown that the expression of anti-HS antibodies can be used to phenotypically knock out HS epitopes, resulting in myotubes with a defective HS composition. In transfected myoblasts, RB4CG9 antibodies were detected within the Golgi apparatus, as was confirmed with C6-Ceramide, and in cytosolic vesicles (Fig. 4a, insert). Transfected myoblasts fused with non-transfected neighboring myoblasts to form EGFP-positive myotubes, which could be used for Indo-1 ratiometric measurements of the intracellular calcium concentration ([Ca2+]), using a high-speed UV confocal laser scanning microscope. Under the conditions used in this study, 32% of the myotubes transfected with vectors encoding EGFP, or EGFP plus control antibody MPB01 was excitable (Table 2). Upon the expression of anti-GAG antibody RB4CG9, the amount of excitable EGFP-positive myotubes was significantly reduced to 8%.
Table 2. The effect of endogenous expression of anti-GAG antibody RB4CG9 on 3 day-differentiated C_{2}C_{12} myotubes.

<table>
<thead>
<tr>
<th>Transfected myotube</th>
<th>n</th>
<th>Excitability (%)</th>
<th>n</th>
<th>Amplitude (ratio)</th>
<th>Rate constant μ (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C_{2}C_{12} Control</td>
<td>35</td>
<td>32</td>
<td>12</td>
<td>2.04 ± 0.34</td>
<td>0.30 ± 0.04</td>
</tr>
<tr>
<td>C_{2}C_{12} RB4CG9</td>
<td>37</td>
<td>8*</td>
<td>8</td>
<td>1.50 ± 0.25**</td>
<td>0.14 ± 0.03**</td>
</tr>
</tbody>
</table>

Ratiometric measurements of [Ca^{2+}]_{i} were performed upon electric stimulation of individual EGFP-positive C_{2}C_{12} myotubes. The overall percentage of myotubes that responded to stimuli by generating calcium spikes (i.e., excitable) was determined in transfected cultures. The kinetics of individual calcium spikes were analyzed for a number of spikes from separate measurements of multiple recording sessions. n represents the number of individual cells of which calcium transients were monitored (overall) or the number of individual calcium spikes that were analyzed (calcium spike kinetics).

* Expressing either cytosolic EGFP alone, or cytosolic EGFP and control antibody MPB01
** P value <0.001

Calcium spikes of RB4CG9-expressing excitable myotubes (Fig. 4a) had significantly lower amplitudes as compared to control myotubes and frequently showed undershoots during decline to basal level (Fig. 4b and 4c). Moreover, individual calcium spikes differed in amplitude when compared to each other and occasionally failed to be generated upon stimulation. Spontaneous fluctuations of the resting [Ca^{2+}]_{i} were frequently observed in RB4CG9-expressing myotubes, in contrast to control-transfected myotubes. Electric stimulation often triggered RB4CG9-expressing myotubes to generate a series of oscillatory calcium spikes. Spontaneous generation of calcium spikes was seldom seen, as in control myotubes. Removal of extracellular calcium neither affected the occurrence nor the amplitudes of induced calcium spikes (data not shown).

Calcium spikes of control-transfected myotubes were selected from individual measurements of multiple recording sessions, and averaged out to generate a mean wild type calcium spike (Fig. 4c). Similarly, a mean RB4CG9 calcium spike was derived from measurements of RB4CG9 expressing myotubes. Individual analysis of the spikes showed that upon electrical stimulation maximum [Ca^{2+}]_{i} levels were reached within 99 ms (three data points), after which they rapidly declined to basal level. In control myotubes a significantly higher rise in Indo-1 ratio (2.04±0.34) was observed, as compared to RB4CG9-expressing myotubes (1.50±0.25 (p<0.003); Table 2). The decline of [Ca^{2+}]_{i} to basal level could be described by a mono-exponential function \( Y_t = Y_0 + Ae^{-t/\mu} \) (\( Y_t = \) ratio as a function of time; \( Y_0 = \) ratio at t=0 (basal ratio); \( A = \) scaling factor; \( t = \) time; \( \mu = \) decay constant)), for both control and RB4CG9 expressing myotubes, using the Levenberg-Marquard algorithm (Press, 1995). The small difference between the experimental trace and the exponential fit showed this description to be valid (R^2=0.99). These mono-exponential kinetics suggest one main calcium reuptake process (Lieste et al., 1998; Koop-
man et al., 2001; de Groof et al., 2001), described by a decay constant $\mu$, which is inversely proportional to the rate of $[\text{Ca}^{2+}]$, decline (Table 2). Fitting of the $[\text{Ca}^{2+}]$ decline of the individual recordings resulted in an average decay constant for control myotubes of $0.30s \pm 0.04$ ($n=12$) and for RB4CG9 expressing myotubes of $0.14s \pm 0.03$ ($n=8$), indicating a significant ($p<0.001$) decrease of the decay constant upon depletion of this GAG epitope (Table 2). Following decline to basal $[\text{Ca}^{2+}]$ level, RB4CG9-transfected myotubes showed an undershoot that gradually returned to basal level. When plotting the derivative of the decay phase of the mean calcium spike Indo-1 ratios ($d[\text{Ca}^{2+}] / dt$) against the normalized Indo-1 ratio (Fig. 4d), the decline of the linear fit through the data points correlates with the $[\text{Ca}^{2+}]$ removal speed. Since the decline of this linear fit is more steep for RB4CG9 transfected myotubes, it can be concluded that the $[\text{Ca}^{2+}]$ removal speed its increased upon depletion of GAG epitope RB4CG9.

**Figure 4.** Ratiometric measurements of cytosolic calcium in control and RB4CG9 transfected C2C12 myotubes. C2C12 myoblasts were transfected with
expression vectors, encoding control antibody MPB01 or anti-GAG antibody RB4CG9, combined with cytosolic EGFP. The insert in panel a shows the expression of VSV-tagged antibodies within the Golgi apparatus (dark staining). Upon confluency, C2C12 myotubes were differentiated for 3 days. Regions of interest (ROIs) were drawn on EGFP images (488 nm) of the myotubes (exemplified by the ROI in a). Changes in intracellular calcium were measured in ROIs, corrected for background, and plotted against time as ratio values (405 nm/485 nm; b); bars below ratio traces indicate time points of electrical stimulation. Individual 405 nm and 485 nm signals are depicted for the underlined part of each trace. The physiological relevance of the ratio traces is demonstrated by the rise of the 405 nm signal (calcium-bound Indo-1), simultaneous with the decline of the 485 nm signal (calcium-unbound Indo-1). Excitable myotubes transfected with control-antibody MPB01 showed wild type calcium spikes upon stimulation (b; Control). Myotubes transfected with anti-GAG antibody RB4CG9 had substantially lower amplitudes and showed undershoots in returning to basal [Ca\(^{2+}\)]. (b; RB4CG9). Calcium spikes of control and RB4CG9-expressing myotubes were selected from individual measurements of multiple recording sessions and averaged to generate mean calcium spikes (c). By plotting the derivative of the decay-phase (insert) of the mean calcium spikes against the normalized Indo-1 ratio, it can be illustrated that the mono-exponential rate constant \(\mu\) decreases upon depletion of GAG epitope RB4CG9 (d; the insert indicates the data points (taken from c) used for analysis). Since \(\mu\) is inversely proportional to the calcium removal speed, the intracellular calcium removal kinetics are accelerated in the absence of GAG epitope RB4CG9.

**Discussion**

Antibody RB4CG9 was selected by phage display against GAG preparations derived from human skeletal muscle. In ELISA, the antibody was reactive with GAG preparations from skeletal muscle, with heparin (a highly sulfated form of HS), and with AS. AS is a HS-like GAG, composed of GlcNAc-IduA2S as basic disaccharide units, of which about 10% is N-sulfated. Fully N-sulfated AS, however, does not react with antibody RB4CG9, nor do N-deacetylated and 2-O-desulfated AS. This indicates that GlcNAc-IduA2S sequences are involved in the formation of the epitope defined by antibody RB4CG9. Reactivity with skeletal muscle GAG preparations, heparin, and AS in ELISA was diminished by heparanase digestion, indicating the HS-like nature of the antigen.
In immunofluorescence studies, the antibody reacted exclusively with skeletal muscle cells. Colocalization studies in various adult postural muscles showed GAG epitope RB4CG9 to be predominantly present in type I (oxidative; ‘slow-twitch’) muscle fibers, as compared to type II (glycolytic; ‘fast-twitch’) fibers. The distinct distribution of RB4CG9 argues for an involvement of this GAG epitope in muscle-specific properties, possibly by fine-tuning the physiological requirements.

During in vivo myogenesis, RB4CG9 initially stained the periphery of developing primary myotubes, eventually resulting in cross-striation of full-grown muscle fibers. Colocalization studies of GAG epitope RB4CG9 with sarcomeric proteins and with several ion channels of the e-c coupling system in mammals and frog, studies on creatine kinase-β mice, and immuno electron microscopy studies all indicated GAG epitope RB4CG9 to be present in the SR. More specific, GAG epitope RB4CG9 is located in the fenestrated SR and the terminal cisternae (Fig. 5).

The unusual subcellular distribution of GAG epitope RB4CG9 partially overlaps with the recently published locations of γ-sarcoglycan in the SR membrane (Ueda et al., 2001). This sarcoglycan isoform is a type II transmembrane glycoprotein and a member of the dystrophin complex. γ-sarcoglycan is located in the SR terminal cisternae and throughout the fenestrated SR. Mutations in γ-sarcoglycan are causal for severe childhood autosomal recessive muscular dystrophy (Noguchi et al., 1995; McNally et al., 1996). δ-sarcoglycan is restricted to the SR terminal cisternae and is mutated in autosomal recessive limb-girdle muscular dystrophy (Jung et al., 1996; Nigro et al., 1996). However, we could not locate any putative heparin/HS binding motif in neither δ- nor γ-sarcoglycan, leaving us with no clue as to a possible interaction of GAG epitope RB4CG9 with these proteins.

The strict location of GAG epitope RB4CG9 within the SR may indicate a function for GAGs in this organelle. This GAG epitope may be involved in maintaining the integrity of the SR lumen against mechanic or osmotic forces, as was proposed for CS in T-tubules (Davis and Carlson, 1994). Being negatively charged, GAGs may be involved in the sequestering of calcium within the SR, as was shown for the SR-resident protein calsequestrin (MacLennan and Wong, 1971; MacLennan et al., 1983; Lerner and Torchia, 1986). Interestingly, one of the proposed functions of AS in the giant African snail (Achatina fulica) is the binding of bivalent cations such as calcium (Kim et al., 1996). Possibly, by affecting the local calcium concentration near regulatory SR-luminal sites of RyRs or SERCAs, GAGs may regulate the activity of these proteins, similar to the previously proposed mechanism for heparin-stimulated RyR activity (Men’shikova et al., 1986; Bezprozvanny et al., 1993). Another possibility is the involvement of GAG epitope RB4CG9 in e-c coupling via direct or indirect interactions with SERCA or ion channels. Such interactions have been shown for heparin in DHPR activity (Knaus et al., 1990; Martinez et al., 1996) and for the SR-resident proteins calsequestrin and triadin in.
their regulation of the RyR (Guo and Campbell, 1995; Ohkura et al., 1998).

To evaluate possible interactions between GAGs and SR-proteins, we screened several SR-resident proteins and ion channels involved in e-c coupling for putative HS/ heparin-binding consensus motifs. Amino acid sequences of calsequestrin (accession number P07221), junctate (NP_004309), junctin (AAG16983), phosphoglucomutase (P00949), d-sarcoglycan (AAC50921), g-sarcoglycan (AAC50269), sarcoplasmic reticulum glycoprotein (AAA60730), triadin (AF220558, AJ243303, AJ243304), DHPR-alpha1 (AAB37235), DHPR-alpha2/delta (NM_000722), RyR1 (NM_000540), RyR3 (NM_001036), SERCA1 (A48849), and SERCA2 (NM_001681) were analyzed for the occurrence of HS/ heparin-binding consensus motifs: XBBXB in β-strands and XBBBXXBX in α-helices (Cardin and Weintraub, 1989), XBBXBBXXXXBX (Sobel et al., 1992), and TXXBXXTXBXXTXB (Hileman et al., 1998; (X, any amino acid; B, basic amino acid (H, K, R); T, turn)). Multiple XBBXB and XBBBXXBX motifs were present in most of the proteins mentioned above, except for the sarcoglycans. XBBXBBXXXXBB and TXXBXXTXBXXTXB motifs were not found in any of these proteins. In SERCA1, a XBBXB motif was found starting at amino acid 682, located in a turn in the cytosolic phosphorylation domain. RyR1 and RyR3 contain multiple XBBXB and XBBBXXBX motifs, but these are located at the cytosolic side. It will be interesting to study interactions between GAG epitope RB4CG9 and SR resident proteins in future studies.
Previously, we have shown that expression of anti-HS antibodies in cell culture leads to the phenotypic knock out of the HS-epitopes involved (Jenniskens et al., 2002). Endogenous expression of anti-GAG antibody RB4CG9 in transiently transfected C2C12 myoblast culture resulted in a marked decrease in the number of excitable myotubes and in altered kinetics of electrically induced calcium spikes. Depletion of GAG epitope RB4CG9 resulted in 50% reduced amplitudes of calcium spikes and a doubled \([\text{Ca}^{2+}]_r\) removal speed. Since calcium removal kinetics could be fitted with a mono-exponential function, the transport of calcium from the cytosol to the SR is most likely mediated by one main removal process, namely SERCA activity. The anomalies induced by the absence of GAG epitope RB4CG9 may be explained by an involvement of this GAG epitope in mediating SERCA activity, possibly in combination with phospholamban or sarcolipin. One can envisage the binding of calcium to the negatively charged GAG molecules after its arrival in the SR. An increase in GAG-bound calcium in the vicinity of the SERCAs may be sensed and may result in a lower pumping speed. In absence of GAG epitope RB4CG9, this negative feedback mechanism is absent, leading to unrestrained calcium pumping, eventually resulting in an undershoot of \([\text{Ca}^{2+}]_r\) below the basal level. The mechanism responsible for the return of \([\text{Ca}^{2+}]_r\) from the undershoot to basal level remains unclear, but may reflect an intrinsic activity of RyR2 to balance the resting \([\text{Ca}^{2+}]_r\).

In this paper we describe a unique HS-like GAG epitope, likely containing GlcNAc-IduA2S sequences, that specifically localizes in the skeletal muscle fenestrated SR and the terminal cisternae. To our knowledge for the first time, a tissue-specific GAG epitope is shown to be located within an organelle other than the nucleus or Golgi apparatus. Depletion of GAG epitope RB4CG9 by endogenous expression of the RB4CG9 antibody results in altered calcium kinetics in myotubes in vitro, indicating a role for this GAG epitope in skeletal muscle calcium kinetics.

References


Chapter 8

Survey and Summary
Background and aim of the study

Heparan sulfate proteoglycans (HSPGs) are major components of the skeletal muscle cell surface and of the basal lamina (BL) surrounding muscle cells. HSPGs consist of a protein core to which multiple heparan sulfate (HS) chains are attached. Individual HSPGs, notably agrin and perlecan, have been intensely studied in recent years. However, these studies focused on the protein core, ignoring the HS moiety mainly because of a lack of appropriate tools. Previously, it was shown that antibodies specific for unique HS epitopes can be selected by means of phage display. The aim of the study described here was to select antibodies against skeletal muscle HS, to investigate the occurrence of specific HS epitopes in muscle, and to analyze the involvement of HS in muscle development and physiology.

Spatiotemporal occurrence of HS epitopes in skeletal muscle

Anti-HS antibodies were selected by phage display against HS from skeletal muscle, as described in chapter 2. Using these antibodies, several distinct HS epitopes were shown to be differentially distributed in skeletal muscle. Whereas some HS epitopes were generally present in muscle BL, others had a more restricted distribution, especially with regard to the NMJ. Both in situ and in vitro, some HS epitopes colocalized with acetylcholine receptor clusters.

In chapter 3 the epitopes recognized by anti-HS phage display antibodies are characterized in more detail. The topological distribution of HS epitopes is tightly regulated and antibodies recognize chemically different HS epitopes, the position of sulfate groups being of major importance.

Chapter 4 describes in detail the distribution of HS epitopes in skeletal muscle during embryogenesis in the mouse. The spatiotemporal expression of various HS epitopes changes markedly during muscle development. This dynamic regulation may create a specific extracellular microenvironment, necessary for the correct sequence of events in myogenesis and synaptogenesis, and argues for roles of individual HS epitopes in underlying developmental processes.

A functional role for HS in muscle calcium kinetics

Chapter 5 describes changes in calcium kinetics in cultured primary myotubes of mice carrying a targeted disruption of a gene encoding an
enzyme involved in HS synthesis. Muscle cells of mice knock out for N-deacetylase/N-sulfotransferase-1 show changes in HS composition, as determined by immuno staining, as well as a significant change in the kinetics of calcium spikes. Upon electrical excitation, a lower flux of calcium over the SR membrane and a slower decay to basal [Ca²⁺]ᵢ were observed as compared to wild type myotubes. The relationship demonstrated between changes in HS and in calcium kinetics is in accordance with the data presented in the following two chapters.

Apart from their obvious use in immunohistochemistry, phage display-derived antibodies can be used in expression studies. In chapter 6 novel eukaryotic expression systems are described, in which anti-HS antibodies are expressed as intrabodies in the C₂C₁₂ myoblast cell line. Targeting of anti-HS antibodies or the HS-degrading enzyme heparanase to the secretory pathway through the Golgi apparatus results in a phenotypic knock out of HS at the cellular surface. Myoblasts expressing either antibodies or heparanase form myotubes that are defective in HS. These myotubes are seriously affected in their calcium kinetics.

A rare HS-like glycosaminoglycan (GAG), which is unique for skeletal muscle tissue and which is located in the sarcoplasmic reticulum, is described in chapter 7. Antibodies against this GAG were selected against skeletal muscle GAG preparations by means of phage display. Using the previously described expression systems (chapter 6), this molecule can be knocked out in cultured myotubes, resulting in dramatic changes of the kinetics of electrically induced calcium spikes. This GAG is unique in its muscle specificity, in its intracellular location, and in the effects of its role in calcium kinetics. The data presented in these chapters argue for the involvement of HS in skeletal muscle calcium kinetics.

**Future research**

The results presented in this thesis offer new insights in the diversity, topology and function of HS epitopes. The preferential binding of anti-HS antibodies to defined synthetic oligosaccharides were established. It remains to be elucidated whether these HS epitopes are unique for specific core proteins. Moreover, it is a major challenge to elucidate the monosaccharide sequences of the HS epitopes recognized by the antibodies.

Knocking out of HS at the cellular surface by the expression of epitope-specific anti-HS intrabodies or by the HS-degrading enzyme heparanase offers a new and powerful tool to investigate the roles of HS. The use of universal eukaryotic expression vectors with strong viral promoters allows the general application of these systems in many fields in which GAG are involved. These systems may prove to be valuable in many in vitro studies in which the roles of HS or specific HS epitopes are studied
apart from the core protein to which they are attached.

An antibody expression system that can be induced to various levels \textit{in vitro}, is described in chapter 6. This system may also prove to be useful \textit{for in vivo} HS research. The inducible expression of anti-HS intrabodies or heparanases by the oral addition of the inducing agent ecdyson, may enable the temporal knock out of HS epitopes \textit{in vivo} at any time point. Such studies may attribute to a better insight in the roles of HS epitopes in developmental or physiological processes. Similar techniques may also be used to study the function of other GAGs.

The identification of a rare, muscle-specific GAG species, which is located within the sarcoplasmic reticulum and has a function in cellular physiology, seems in contradiction with the current view of GAGs being present in the extracellular environment only (except for the presence of HS in the nucleus). It would be interesting to further investigate the nature of this GAG, and to elucidate its mechanism of action in muscle calcium kinetics.

The functional properties of the cellular HS epitopes should be analyzed in more detail. Next to the developmental and physiological functions described in this study, the involvement of the cellular HS constitution in pathological conditions is of special interest. Since HSPGs have been involved in interstitial myopathies (e.g. Schwartz-Jampel syndrome, Silverman-Handmaker dyssegmental dysplasia, Simpson-Golabi-Behmel syndrome), it will be interesting to investigate the role of the HS moieties in these disorders. The antibodies and expression systems described in this thesis may facilitate new approaches for this purpose.
Chapter 9

Samenvatting in het Nederlands
Achtergrond en doelstelling van het onderzoek

Spieren zijn organen die zijn gespecialiseerd in het ontwikkelen van de mechanische kracht die tot beweging leidt. Skeletspieren zijn aan beide uiteinden door middel van pezen bevestigd aan de botten van het skelet. De gecoördineerde bewegingen van een organisme worden mogelijk gemaakt doordat individuele spiervezels worden geïnnerveerd door motorneuronen in zogenaamde neuromusculaire junctions (NMJs).

Tussen de individuele vezels waaruit skeletspier is opgebouwd bevindt zich een laag vezel-achtig materiaal; de extracellulaire matrix (ECM). Deze matrix vormt een structureel verbindingssweefsel dat belangrijke functies heeft bij: (a) het ontstaan van spierweefsel tijdens de embryonale ontwikkeling, (b) het herstel van spierweefsel na beschadiging en (c) de verdeling van de mechanische krachten die op de spiervezels komen te staan bij contractie. Een specifieke structuur binnen de ECM is de basale lamina (BL), die de individuele spiervezels omgeeft. De BL is continu over het gehele oppervlak van de spiervezel, ook in NMJs waar deze structuur de ruimte tussen de uitloper van het motorneuron en de spiervezel vult.

Heparan sulfaat-proteoglycanen (HSPGs) zijn belangrijke componenten van de BL en het celopervlak van de skeletspier. HSPGs bestaan uit een eiwitketen met daaraan gekoppeld een of meer heparan sulfaat (HS) moleculen. Individuele HSPGs, met name agrine en perlecan, zijn in de afgelopen jaren intensief bestudeerd. Deze studies richtten zich echter op de eiwitketens van deze moleculen, aangezien er nauwelijks methoden waren om de HS moleculen te onderzoeken. In voorgaand onderzoek is aangetoond dat het mogelijk is om antilichamen te selecteren die specifiek bepaalde epitopen op HS moleculen herkennen. Het doel van het in dit proefschrift beschreven onderzoek was om antilichamen te selecteren tegen HS geïsoleerd uit skeletspier, de distributie van specifieke HS epitopen in spier BL te onderzoeken en de betrokkenheid van HS bij de ontwikkeling en de fysiologie van de spier te analyseren.

De distributie van HS-epitopen in skeletspier

In hoofdstuk 2 is beschreven hoe met behulp van ‘phage display’ epitopesspecifieke antilichamen zijn geselecteerd tegen HS geïsoleerd uit skeletspier. Gebruikmakend van deze antilichamen werd aangetoond dat individuele HS-epitopen verschillend voorkomen in spier BL. Sommige HS epitopen zijn aanwezig in de gehele BL, terwijl andere HS epitopen slechts beperkt voorkomen, in het bijzonder met betrekking tot de NMJ. Verschillende HS epitopen colocaliseren met acetylcholine receptor clusters zowel in situ als in vitro.
In hoofdstuk 3 worden de epitopen bestudeerd die worden herkend door de anti-HS antilichamen. De verschillende HS epitopen vertonen een gereguleerde topologische verdeling en zijn ook chemisch verschillend. De positie van de sulfaatgroepen is het belangrijkst.

Hoofdstuk 4 beschrijft in detail het voorkomen van HS epitopen in skeletspier tijdens de embryogenese in de muis. Er wordt aangetoond dat gedurende de ontwikkeling van de spier de spatio-temporele expressie van de verschillende HS epitopen in hoge mate is gereguleerd. Deze dynamische distributie van HS epitopen kan een specifiek extra-cellulair microklimaat creëren, dat nodig is voor de verschillende processen tijdens de myogenese en synaptogenese.

Een rol voor HS in de calciumhuishouding van spier

In hoofdstuk 5 worden veranderingen beschreven in de calciumkinetiek van gekweekte primaire spiercellen van muizen met een defect in een gen coderend voor een eiwit dat is betrokken bij de synthese van HS. Spiercellen van muizen knock-out voor het enzym N-deacetylase/N-sulfotransferase-1 vertonen duidelijk minder reactie met anti-HS antilichamen. Ook worden significante veranderingen in de kinetiek van calciumtransienten waargenomen. Na electrische stimulatie is er een verminderde flux van calcium over de membraan van het sarcoplasmatisch reticulum en een langzamere daling naar de basaalwaarde vergeleken met wildtype spiercellen. In overeenstemming met de data gepresenteerd in het volgende hoofdstuk wordt een verband beschreven tussen veranderingen in HS in de spier en in de calciumkinetiek.

In hoofdstuk 6 worden nieuwe expressiesystemen beschreven, waarin anti-HS antilichamen als zogenaamde intrabodies tot expressie worden gebracht in een C2C12 myoblast-cellijn. Door endogene expressie van anti-HS antilichamen of van het HS-abbrekende enzym heparanase kan worden voorkomen dat HS epitopen aan het cellulaire oppervlak tot expressie komen. Als gevolg hiervan ontstaan er spiercellen die aangedaan zijn in hun oppervlakte HS. Deze cellen vertonen verschillende afwijkingen in hun calciumhuishouding.

Een zeldzaam HS-achtig glycosaminoglycan (GAG), dat specifiek is voor skeletspier en is gelocaliseerd in het sarcoplasmatisch reticulum wordt beschreven in hoofdstuk 7. Een antilichaam hiertegen werd geselecteerd tegen GAGs geïsoleerd uit skeletspier. Dit molecuul kan deficiënt gemakkelijker in gekweekte C2C12 spiercellen door expressie van het antilichaam met behulp van de eerder beschreven expressiesystemen. Het gevolg hiervan is dat de kinetiek van electrisch-geïnduceerde calciumfluxen significant verandert.

De in deze hoofdstukken beschreven resultaten wijzen op een rol voor HS bij de calciumhuishouding in skeletspier.
### List of abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ACh</td>
<td>acetylcholine</td>
</tr>
<tr>
<td>AChE</td>
<td>acetylcholine esterase</td>
</tr>
<tr>
<td>AChR</td>
<td>acetylcholine receptor</td>
</tr>
<tr>
<td>AP</td>
<td>action potential</td>
</tr>
<tr>
<td>AS</td>
<td>acharan sulfate</td>
</tr>
<tr>
<td>BL</td>
<td>basal lamina</td>
</tr>
<tr>
<td>[Ca^{2+}]_{i}</td>
<td>cytosolic Ca^{2+} concentration</td>
</tr>
<tr>
<td>CDR3</td>
<td>complementarity determining region 3</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary</td>
</tr>
<tr>
<td>CK</td>
<td>creatine kinase</td>
</tr>
<tr>
<td>CS</td>
<td>chondroitin sulfate</td>
</tr>
<tr>
<td>DHPR</td>
<td>dihydrophyridine receptor</td>
</tr>
<tr>
<td>DS</td>
<td>dermatan sulfate</td>
</tr>
<tr>
<td>e-c coupling</td>
<td>excitation-contraction coupling</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>EDL</td>
<td>extensor digitorum longus</td>
</tr>
<tr>
<td>EGFP</td>
<td>enhanced green fluorescent protein</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme linked immunosorbent assay</td>
</tr>
<tr>
<td>EPP</td>
<td>endplate potential</td>
</tr>
<tr>
<td>GAG</td>
<td>glycosaminoglycan</td>
</tr>
<tr>
<td>Gal</td>
<td>D-galactose</td>
</tr>
<tr>
<td>GalNAc</td>
<td>N-acetyl-D-galactosamine</td>
</tr>
<tr>
<td>GlcA</td>
<td>D-glucuronic acid</td>
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<tr>
<td>GlcNAc</td>
<td>N-acetyl-D-glucosamine</td>
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<td>HA</td>
<td>hyaluronic acid</td>
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<tr>
<td>Hep</td>
<td>heparin</td>
</tr>
<tr>
<td>HS</td>
<td>heparan sulfate</td>
</tr>
<tr>
<td>HS2ST</td>
<td>heparan sulfate 2-O-sulfotransferase</td>
</tr>
<tr>
<td>HS6ST</td>
<td>heparan sulfate 6-O-sulfotransferase</td>
</tr>
<tr>
<td>HSPG</td>
<td>heparan sulfate proteoglycan</td>
</tr>
<tr>
<td>IduA</td>
<td>L-iduronic acid</td>
</tr>
<tr>
<td>IFA</td>
<td>immunofluorescence assay</td>
</tr>
<tr>
<td>KS</td>
<td>keratan sulfate</td>
</tr>
<tr>
<td>MuSK</td>
<td>muscle-specific kinase</td>
</tr>
<tr>
<td>NDST</td>
<td>N-deacetylase/N-sulfotransferase</td>
</tr>
<tr>
<td>NMJ</td>
<td>neuromuscular junction</td>
</tr>
<tr>
<td>3-OST</td>
<td>heparan sulfate glucosaminyl 3-O-sulfotransferase</td>
</tr>
<tr>
<td>ROI</td>
<td>region of interest</td>
</tr>
<tr>
<td>RyR</td>
<td>ryanodine receptor</td>
</tr>
<tr>
<td>sBL</td>
<td>synaptic basal lamina</td>
</tr>
<tr>
<td>scFv</td>
<td>single chain variable fragment</td>
</tr>
<tr>
<td>SERCA</td>
<td>sarcoplasmic reticulum calcium ATPase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>SR</td>
<td>sarcoplasmic reticulum</td>
</tr>
<tr>
<td>T-tubule</td>
<td>transverse tubule</td>
</tr>
<tr>
<td>VgNa’Ch</td>
<td>voltage gated Na’channel</td>
</tr>
<tr>
<td>Xyl</td>
<td>D-xylose</td>
</tr>
</tbody>
</table>
List of publications


Dankwoord

...en toen zat ik op een mooie dag voor mijn laptop op mijn werkplek hier op het MIT; ongeveer drie maanden geleden ruilde ik mijn vertrouwde Nijmeegse omgeving in voor nieuwe uitdagingen in en rond Boston. Met veel plezier denk ik terug aan mijn promotieperiode in Nijmegen. Nu mijn ‘boekje’ bijna klaar is wil ik graag de mensen bedanken die betrokken zijn geweest bij de totstandkoming van dit proefschrift.

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Bovenal ben ik mijn ouders heel erg dankbaar voor dik dertig jaar onbegrensde steun en welgemeende interesse.

Cambridge (MA, USA), augustus 2002
Guido John Jenniskens was born on Thursday November 18, 1971 in Roermond (The Netherlands). In 1991, he graduated from 'Scholengemeenschap St. Ursula' in Horn (The Netherlands), after which he studied biology at the University of Nijmegen (Nijmegen, The Netherlands). In 1997, he graduated on major subjects in Biochemistry (Prof. Dr. J. Veerkamp and Dr. T. van Kuppevelt) and in Molecular Biology (Prof. Dr. R. Konings and Dr. L. Lubsen), and a subsidiary subject at Organon (Oss, The Netherlands; Prof. Dr. W. Olijve and Dr. S. Mosselman).

In the same year, he started his Ph.D. project at the Department of Matrix Biochemistry (Prof. Dr. J. Veerkamp and Dr. T. van Kuppevelt) of the University Medical Center (UMC) St. Radboud at the Nijmegen Center for Molecular Life Sciences (NCMLS).

During his Ph.D. project, he supervised three trainees and one undergraduate student. Also, he attended three international symposia in which he participated by poster presentations: the 'Wenner Gren Foundations International Symposium on Heparan Sulfate Proteoglycans' (Stockholm, April 1999), the 'Association Francaise contre les Myopathies 2000 International Congress of Myology' (Nice, March 2000; where he was rewarded a poster prize), and the 'Gordon Research Conference on Muscle Excitation-Contraction Coupling' (New London, NH, June 2000).

In July 2002, he presented the results of his Ph.D. work in a presentation at the 'Gordon Research Conference on Proteoglycans' in Andover, NH.

Since April 2002, Guido Jenniskens is working as a Postdoctoral Fellow at the Division of Bioengineering and Environmental Health (Prof. Dr. R. Sasisekharan) at the Massachusetts Institute of Technology (MIT), Cambridge, MA, USA.
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In juli 2002 presenteerde hij de resultaten van zijn promotie-onderzoek tijdens de ‘Gordon Research Conference on Proteoglycans’ te Andover, NH.

Sinds april 2002 is Guido Jenniskens als PostDoc verbonden aan de afdeling Bioengineering and Environmental Health (Prof. Dr. R. Sasisekharan) van het Massachusetts Institute of Technology (MIT) te Cambridge, MA, USA.