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

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The basal lamina (BL) 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 (HS) 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.

Key words: heparan sulfate proteoglycan; glycosaminoglycan; basal lamina; neuromuscular junction; myogenesis; synaptogenesis

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 (N MJ) 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 (Chen et al., 1995), and 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.
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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 μg/ml coating solution), and allowed to bind for 90 min. Alternatively, scFv antibodies were preincubated overnight with the test molecule (10 μg/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, and heparan sulfate 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, after which enzymatic activity was measured using p-nitrophenol phosphate as a substrate. Substrate affinity: 1+, very strong; 2+, strong; 3+, moderate; 4+, weak; 5, absent (n = 5).

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-nitrophenol phosphate as a substrate. Substrate affinity: 1+, very strong; 2+, strong; 3+, moderate; 4+, weak; 5, absent (n = 5).

Selection of antibodies against skeletal muscle GAGs

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

### 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>
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<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>
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<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>
<tr>
<td>Heparin, N-desulfated and N-acetylated</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-nitrophenol phosphate as a substrate. Substrate affinity: 1+, very strong; 2+, strong; 3+, moderate; 4+, weak; 5, absent (n = 5).

1Similar to the HS precursor polysaccharide.

2HS fraction eluting from anion exchange column at the NaCl concentration indicated.

3Inhibition-ELISA.

### RESULTS

#### Selection of antibodies against skeletal muscle GAGs

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

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immunofluorescence. Forase deficient; pgs glucuronosyltransferase-deficient; N different CHO cell lines [wild type, 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).

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 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 toward 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).

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 fully confluent of the S27 cell line (Gordon and Hall, 1989) and confluent cultures of different CHO cell lines [wild type, N-acetylg glucosaminyl- 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).

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

Figure 2. Staining of heparinase III-treated skeletal muscle cryosections with anti-HS scFv and anti-HS stub antibodies. Nontreated (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 α-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.
antibodies were visualized by incubation with rabbit polyclonal anti-c
neuromuscular junction, T. marmarota

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Table 3. Immunostaining patterns of anti-HS antibodies

<table>
<thead>
<tr>
<th>Tissue</th>
<th>AO4B05</th>
<th>AO4B08</th>
<th>AO4F12</th>
<th>RB4CB9</th>
<th>RB4CD12</th>
<th>RB4EA12</th>
<th>RB4EG12</th>
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</thead>
<tbody>
<tr>
<td>Mature human, rat, and mouse skeletal muscle</td>
<td></td>
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<tr>
<td>Extrasynaptic basal lamina</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>
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<td></td>
<td></td>
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<tr>
<td>C2C12 skeletal muscle cell line</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>
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<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>AChR clusters</td>
<td>++*</td>
<td>+*</td>
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<td>+*</td>
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<td>S27 cell line (defective in proteoglycan synthesis)</td>
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<tr>
<td>Cell surface (on contact places)</td>
<td>–</td>
<td>–d</td>
<td>–d</td>
<td>–d</td>
<td>–c</td>
<td>–d</td>
<td>–d</td>
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<tr>
<td>Basal lamina (during differentiation)</td>
<td>–</td>
<td>–d</td>
<td>–d</td>
<td>–d</td>
<td>–c</td>
<td>–d</td>
<td>–d</td>
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<tr>
<td>CHO cell line (wild type)</td>
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<tr>
<td>Cell surface</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–c</td>
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<tr>
<td>CHO cell line (677 mutant)</td>
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<tr>
<td>Cell surface</td>
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<td>–d</td>
<td>–c</td>
<td>–c</td>
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<td>–d</td>
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<tr>
<td>CHO cell line (F17 mutant)</td>
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<tr>
<td>Cell surface</td>
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<td>–d</td>
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<td>–c</td>
<td>–c</td>
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<tr>
<td>CHO cell line (745 mutant)</td>
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<td>Cell surface</td>
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</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 electocytes, 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)].

aStaining intensity decreased with differentiation level.
bStaining intensity increased with differentiation level.
cSmall granules within the cytosol: + + .
dSmall granules around the nucleus: + + +.

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. marmarota electric organ

Because the anti-HS antibodies showed differential staining patterns with regard to nerve- and muscle-derived (extrasyn-
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 both muscular and neural 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 both muscular and neural 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.
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).

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, Figs. 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, on alignment and fusion (processes that trigger BL formation), the entire myotube surface was stained. AChR clusters, which develop on the surface of multinucleated 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 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 that were predominantly present near the nuclei (Fig. 10).

Incubation of cryosections of rat skeletal muscle 11 d 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).

**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 S27 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

![Figure 5](image-url)

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 α-bungarotoxin (a2–d2). Double-label micrographs (a3–d3) show in yellow the colocalization of the HS epitopes bound by the scFv 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 abundance 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.
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 such as 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 synapsec-
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 extra-synaptic 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 investi-
Aneurally grown C2C12 myoblasts start aligning when they reach C2C12 skeletal muscle cells at various stages of differentiation. These HS epitopes argue for such a regulatory mechanism. The distinct distribution in both time and space of cytokines to specific HS sequences, as reviewed recently by Lyon (RB4CB9, RB4CD12, RB4EA12, and RB4EG12), especially at sites of synaptogenesis. Local binding of growth factors and 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. How bryonic muscular development, as may be expected on the basis of their staining patterns in mature skeletal muscle tissue. How- 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 argue 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 (Portier 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 threefold 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 glypicans 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 accompa-
Heparan sulfate (HS) epitopes separate from their core protein. 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 coworkers (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, provide a basis for investigating the specific functions 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


