Phenotypic knock out of heparan sulfates in myotubes impairs excitation-induced calcium spiking[1]

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SPECIFIC AIMS

Heparan sulfates (HS) are anionic polysaccharides that bind and modulate a large number of proteins but are difficult to analyze due to their saccharide nature. To study the roles of HS apart from the core proteins to which they are usually attached, we devised eukaryotic expression systems that enabled us to phenotypically knock out the HS moiety without interfering with the expression of the core proteins.

PRINCIPAL FINDINGS

1. Generation of anti-HS antibody expression constructs

DNA encoding the anti-HS antibodies (single-chain variable fragments) was excised from the original pHEN plasmid and provided with an immunoglobulin leader sequence to allow the antibody to enter the secretory pathway. It was reasoned that the antibodies may bind to their HS epitopes in the Golgi apparatus, the site of HS synthesis, thereby inactivating the specific HS epitope. The antibody encoding DNA was cloned into eukaryotic expression vectors pIRES2-EGFP (bicistronic expression together with cytosolic-enhanced green fluorescent protein, EGFP) and pIND (an ecdysone inducible expression vector). Likewise, the HS-degrading enzyme heparanase was cloned.

2. Anti-HS antibodies can be expressed as intrabodies and localize to the Golgi apparatus

Constructs were transiently transfected in CHO, COS-7, and C_2C_{12} (myoblast) cells. A polarized, perinuclear expression was observed that colocalized with NBD C_6-ceramide staining, a selective dye for the Golgi network. Transfection efficiency was 15–20% in C_2C_{12} myoblasts. Transfected myoblasts fused with neighboring wild-type myoblasts to form morphologically normal hybrid myotubes.

3. Expression of anti-HS intrabodies results in phenotypic HS knockout myotubes

Endogenous expression of a specific VSV-tagged anti-HS antibody blocks cell surface expression of the epitope recognized by this antibody (**Fig. 1**). This was studied in transiently transfected C_2C_{12} myoblast cultures. The endogenously expressed antibodies could be visualized using an antibody against the VSV tag (green in Fig. 1). Cell surface HS epitopes were visualized by an antibody against the same HS epitope, but tagged with a c-*Myc* tag (red in Fig. 1). The HS epitope in question could not be detected in the ECM of transfected cells, indicating the effective depletion of this epitope by the intrabodies. The occurrence and location of two HS proteoglycan core proteins (syndecan and perlecan) were not affected. Phenotypic knock out of one specific HS epitope did not necessarily affect other epitopes, providing evidence for the presence of individual epitopes on separate HS molecules. Expression of heparanase resulted in significantly diminished staining for all HS epitopes studied.

4. Excitation-induced calcium spiking is disrupted in HS knockout myotubes

Expression of anti-HS antibodies resulted in a decrease of the number of electrically excitable myotubes (control antibody, 29%; anti-HS antibody, 0–21%); the percentage of excitable cells varied depending on the antibody used. Calcium kinetics were studied by ratiometric measurements of cytosolic calcium ($[Ca^{2+}]_i$) using a high-speed UV confocal laser scanning microscope. Myotubes transfected with anti-HS antibodies display aberrant calcium kinetics (**Fig. 2**), whereas myotubes transfected with control antibodies show wild-type kinetics. The anomalies observed include skipping of calcium spikes, slow decay, differences in amplitudes, and spontaneous generation of calcium spikes (Fig. 2). Expression of heparanase often resulted in a combi-

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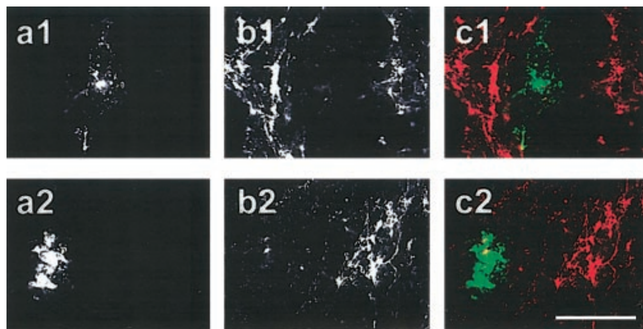

Figure 1. Expression of anti-HS intrabodies blocks the expression of the involved HS epitope at the cell surface. C_2C_{12} myoblasts were transfected with ecdysone-inducible expression vectors encoding VSV-tagged anti-HS antibody AO4B05 (a1-2). Antibodies were detected in cytosolic granules, most likely complexed with the HS epitope to which they bind. Counter-staining for cell surface HS using c-*Myc*-tagged AO4B05 (b1-2) reveals the presence of these HS epitopes at the surface of nontransfected cells, but not at the surface of transfected cells. The merge of images *a* and *b* (c1-2) 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.

nation of these anomalies (Fig. 2). All calcium spikes were independent of the presence of calcium in the extracellular environment, indicating that the calcium released originated from intracellular stores. Myotubes from the C_2C_{12}-derived myoblast cell line S27 were hardly excitable. The S27 cell line is defective in glycosaminoglycan biosynthesis (shorter, undersulfated HS chains are formed). S27 myotubes that could be triggered to form a calcium spike (~2%), however, generated only minor spikes.

CONCLUSIONS AND SIGNIFICANCE

For molecules like HS, which are not genetically encoded, it has been difficult to generate dominant negative in vitro or in vivo research systems. With the generation of a panel of antibodies that recognize specific HS epitopes in skeletal muscle, we recently developed tools appropriate for studying the physiological roles of HS epitopes. In this paper, we report that intracellular expression of these antibodies results in a phenotypic knock out of HS.

Expression of anti-HS intrabodies (and heparanase) was accomplished in various expression systems, including the ecdysone-inducible eukaryotic expression system. Using this system, we anticipate possible future implementation of the system in a transgenic mouse model, thus enabling the regulation of expression of the antibodies in vivo in time. The expression systems presented here add to the small repertoire of tools available to study the physiological roles of HS and of glycosaminoglycans in general.

We have shown that the calcium kinetics of HS-defective myotubes are severely affected, indicating that HS epitopes have a role in the proper functioning of excitation-induced calcium spiking in skeletal muscle. Since certain aberrations in calcium kinetics preferentially occur upon the elimination of specific HS epitopes (Fig. 2), it is tempting to attribute these effects to the HS sequences involved. For instance, the expression of antibody AO4B08, which recognizes HS sequences containing 2-*O*-sulfated iduronic acid residues, has a different effect than the expression of antibody RB4EA12, which preferentially recognizes unsulfated iduronic acid residues.

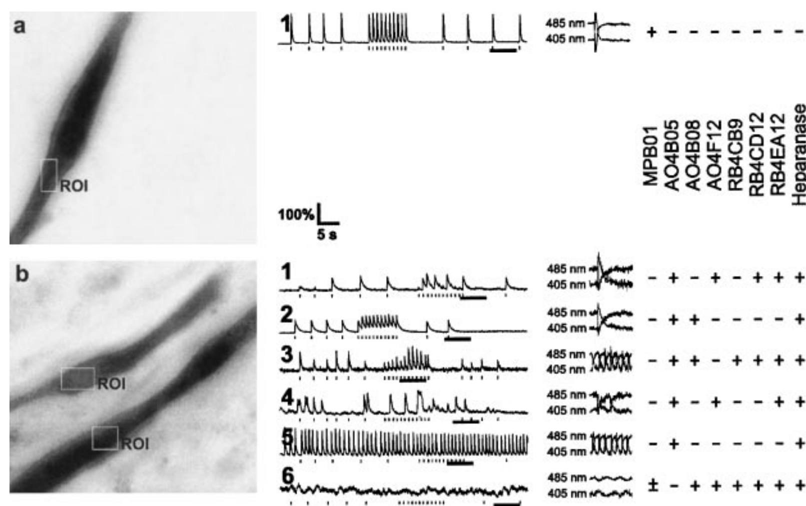

Figure 2. Ratiometric measurements of cytosolic calcium in transfected C_2C_{12} myotubes. C_2C_{12} myoblasts were transfected with a bicistronic expression vector encoding control antibody MPB01 and cytosolic EGFP (*a*) or with an expression vector encoding an anti-HS antibody or heparanase combined with cytosolic EGFP (*b*). Upon confluency, C_2C_{12} myotubes were differentiated for 3 days. Regions of interest (ROIs) were drawn on EGFP images (488 nm) of the myotubes (exemplified by the ROIs, *a, 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 heparanase on no occasion showed 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 $[Ca^{2+}]_i$. The table in the right panel depicts the occurrence of the anomalies for each expressed protein. Individual 405 and 485 nm signals are depicted for the underlined part of each trace. 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.

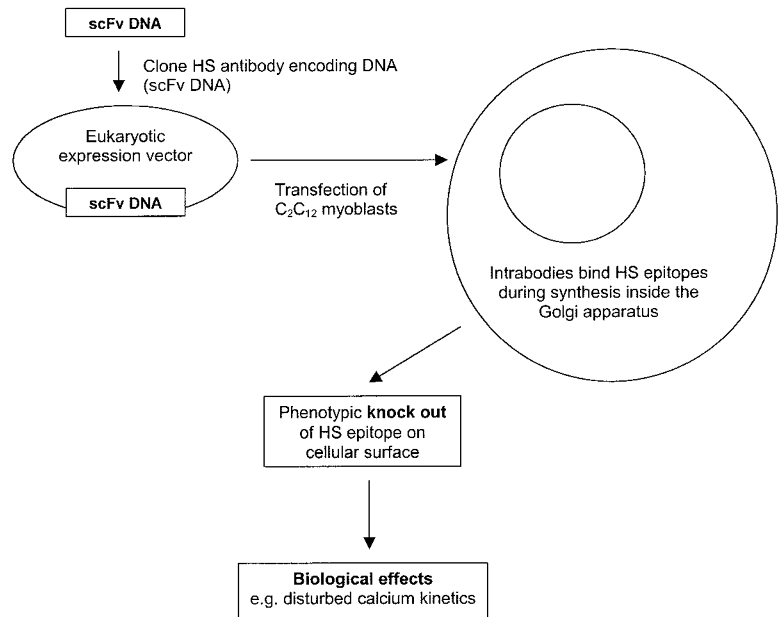

Figure 3. Schematic representation of the expression of anti HS intrabodies using the expression systems described in this paper.

The effect of HS on calcium kinetics may be direct, through the binding of calcium (HS is an excellent binder of calcium) or through interaction with ion channels, or indirect, by binding to growth factors and their receptors. It has been shown that extracellular heparin, a highly sulfated form of HS, interacts with the dihydropyridine receptor, an L-type calcium channel in skeletal muscle, and profoundly affects its kinetic properties. In vitro heparin activates the ryanodine receptor, a calcium channel present in the sarcoplasmic reticulum membrane. Moreover, in Schwartz-Jampel syndrome, a human myotonic disorder, the HS proteoglycan perlecan is mutated, implicating a function of HS proteoglycans in muscle excitability in men.

In conclusion, we present newly devised expression systems in which HS epitopes can be phenotypically knocked out by the endogenous expression of epitope-specific anti-HS antibodies or heparanase. Concomitantly, we report on a novel role for HS in skeletal muscle calcium kinetics. FJ