Extracellular matrix histone H1 binds to perlecan, is present in regenerating skeletal muscle and stimulates myoblast proliferation

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Summary

Heparan sulfate chains of proteoglycans bind to and regulate the function of a wide variety of ligands. In myoblasts, heparan sulfate proteoglycans modulate basic fibroblast growth factor activity and regulate skeletal muscle differentiation. The aim of this study was to identify endogenous extracellular ligands for muscle cell heparan sulfate proteoglycans.

[^{35}S]heparin ligand blot assays identified a 33/30 kDa doublet (p33/30) in detergent/high ionic strength extracts and heparin soluble fractions obtained from intact C_2C_{12} myoblasts. p33/30 is localized on the plasma membrane or in the extracellular matrix where its level increases during muscle differentiation. Heparin-agarose-purified p33/30 was identified as histone H1. In vitro binding assays showed that histone H1 binds specifically to perlecan. Immunofluorescence microscopy showed that an extracellular pool of histone H1 colocalizes with perlecan in the extracellular matrix of myotube cultures and in regenerating skeletal muscle. Furthermore, histone H1 incorporated into the extracellular matrix strongly stimulated myoblast proliferation via a heparan-sulfate-dependent mechanism.

These results indicate that histone H1 is present in the extracellular matrix of skeletal muscle cells, where it interacts specifically with perlecan and exerts a strong proliferative effect on myoblasts, suggesting a role for histone H1 during skeletal muscle regeneration.

Key words: Histone H1, Proteoglycans, Myogenesis, Extracellular matrix, Skeletal muscle regeneration

Introduction

The identification of proteins that bind to the glycosaminoglycan (GAG) chains of proteoglycans has allowed a greater understanding of the wide variety of functions that these macromolecules exert on cell behavior (Bernfield et al., 1999; Iozzo, 1998). Especially relevant examples are proteins that bind to heparin, an analogue of heparan sulfate, which is the GAG attached to most membrane-anchored and basal lamina proteoglycans. Functional heparin-binding domains have been identified in constituents of the extracellular matrix (ECM) (Barkalow and Schwarzbauer, 1991; Kouzi-Koliakos et al., 1989), heparin-binding growth factors (Bernfield et al., 1999) and proteins present on the surface of bacteria, parasites and viruses (Rostand and Esko, 1997). In addition to these examples, a number of diverse extracellular proteins display affinity for heparan sulfate, including proteins involved in morphogenesis, cell adhesion, proliferation, migration, tissue remodeling and blood coagulation (Bernfield et al., 1999).

We are interested in the role that heparan sulfate proteoglycans play during skeletal muscle formation. In this process, committed myogenic progenitor cells, or myoblasts, are maintained in a proliferative, undifferentiated state until appropriate signals trigger their conversion to multinucleated myotubes. The differentiation of muscle cells is controlled in a negative manner by specific heparan-sulfate-binding mitogens, such as basic fibroblast growth factor (FGF-2), hepatocyte growth factor and transforming growth factor-β (Brunetti and Goldfine, 1990; Heino and Massague, 1990; Takayama et al., 1996).

Sodium chlorate, a specific inhibitor of proteoglycan sulfation, has previously been shown to decrease the deposition and assembly of ECM components in cultured C_2C_{12} myoblasts that are induced to differentiate. This results in the inhibition of cell fusion (Melo et al., 1996; Olwin and Rapraeger, 1992; Osses and Brandan, 2001) and FGF-2-dependent suppression of myoblast differentiation (Larraín et al., 1998). We have also shown that the expression of heparan sulfate proteoglycans during the terminal skeletal muscle differentiation of C_2C_{12} cells is highly regulated. The expression of glypican, a glycosylphosphatidylinositol-anchored heparan sulfate proteoglycan that is also released into the ECM, increases (Brandan et al., 1996; Campos et al., 1993), whereas expression of perlecan, an ECM proteoglycan, and the transmembrane proteoglycans syndecan-1 and -3 decreases during differentiation (Fuentealba et al., 1999; Larraín et al., 1997a; Larraín et al., 1997b). Furthermore, heparan sulfate proteoglycans may regulate muscle physiology. We have demonstrated that expression of syndecan-1 (Larraín et al., 1998) and syndecan-3 (Fuentealba et al., 1999) inhibit

the differentiation of cultured myoblasts in an FGF-2-dependent manner. On the other hand, the presence of perlecan in muscle basement membrane has been suggested to participate in cell-ECM interactions (Villar et al., 1999), neuromuscular junction formation (Jacobson et al., 2001) and muscle regeneration (Gulati et al., 1983).

In the present study we searched for endogenous skeletal muscle cell ligand(s) for heparan sulfate proteoglycans that had not previously been described. We did this on the premise that knowledge of such proteins might aid in elucidating the role(s) that these macromolecules exert in muscle physiology. Here, we demonstrate that an extracellularly occurring histone H1 colocalizes with the heparan sulfate proteoglycan perlecan in the ECM of myotube cultures and in regenerating skeletal muscle. In vitro assays show that histone H1 stimulates myoblast proliferation via a heparan-sulfate-dependent mechanism. We propose that this extracellular heparan sulfate proteoglycan-binding protein may play a functional role during muscle regeneration.

Materials and Methods

Materials

The C_2C_{12} cell line was purchased from ATCC, Manassas, VA. The JIE7 antibody (against *Escherichia coli* β-galactosidase) developed by T. L. Mason and J. A. Partaledis and the F1.652 antibody (against embryonic myosin) developed by Helen Blau were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA. Dulbecco's modified Eagle's (DMEM)/Ham's F12 medium (1:1), chicken embryo extract, horse serum, fetal calf serum and Hank's balanced salt solution were obtained from Life Technologies, Inc., Boston, MA. Creatine kinase (CK) and lactate dehydrogenase (LDH) assay kits were from Valtek, Santiago, Chile. Heparin, chondroitin sulfate, dermatan sulfate, N-desulfated heparin, heparin-agarose, laminin, fibronectin, fluorescein-isothiocyanate-conjugated and tetramethyl-rhodamine-5(and 6)-isothiocyanate-conjugated anti IgG were from Sigma, St Louis, MO. Histone H1 and anti-human histone H1 monoclonal antibody (clone AE-4) were from Upstate Biotechnology, Lake Placid, NY. The C17 polyclonal anti-histone H1 antibody was from Santa Cruz Biotechnology, Inc., Santa Cruz, CA. SuperSignal ULTRA Chemiluminiscent substrate was from Pierce, Rockford, IL. Macro-Prep DEAE was from Bio-Rad, Hercules, CA. Cell death detection ELISA^{PLUS} kit was from Roche, Mannheim, Germany. Heparitinase and anti-Δ-heparan sulfate antibody were from Seikagaku, Tokyo, Japan. [^{35}S]H_2SO_4 carrier-free (1050-1600 Ci/mmol) and [methyl-^{3}H]thymidine (79 Ci/mmol) were obtained from NEN Life Science Products, Boston, MA. Tissue Freezing Medium gel was from Triangle Biomedical Sciences, Dr Durham, NC. Cellulosenitrate was from Schleicher and Shuell, Dassel, Germany.

Cell culture

The mouse skeletal muscle cell line C_2C_{12} was grown and induced to differentiate as previously described (Larraín et al., 1997b). In some experiments, cells were cultured and maintained during differentiation in the presence of sodium chlorate at a final concentration of 30 mM (Melo et al., 1996). MST cells were grown as previously described (Montgomery et al., 1992).

Protein extractions and [^{35}S]heparin ligand blot assay

For sequential extractions, C_2C_{12} myotubes were rinsed twice with phosphate buffered saline (PBS) and harvested by scraping in the same buffer. The cells were homogenized and a PBS-soluble extract was obtained by centrifuging for 10 minutes at 13,000 ***g*** and 4°C. The resulting pellet was homogenized in TX-100 buffer (0.05 M Tris-HCl, pH 7.4, 0.5% Triton X-100, 0.15 M NaCl) and centrifuged under the same conditions. The final pellet was homogenized in TX-100/KCl solution (0.05 M Tris-HCl, pH 7.4, 0.5% Triton X-100, 0.5 M KCl). For heparin displacement of extracellular proteins from intact muscle cells, myoblasts or differentiating myoblasts were washed three times for 10 minutes with cold PBS containing 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$ under gentle agitation at 4°C. The cells were then incubated in 2 mg/ml heparin in the same buffer for 20 minutes. The PBS-heparin extract was clarified by centrifugation at 13,000 ***g*** for 2 minutes at 4°C. Acid extraction of nuclear proteins was carried out essentially as described previously (Schmiedeke et al., 1989).

For ligand blot assays, [^{35}S]heparin was biosynthetically labeled and isolated from MST cells as previously described (Montgomery et al., 1992). Proteins in the various muscle cell extracts were separated by SDS-PAGE and electrotransferred onto nitrocellulose membranes at 100 V for 4 hours. Membranes were then blocked with 5% bovine serum albumin (BSA) in PBS for 1 hour and incubated overnight with 2×10^6 cpm/ml [^{35}S]heparin at 4°C under gentle agitation. After washing the membranes, heparin-binding proteins were then visualized by autoradiography.

Heparin-agarose chromatography

Myotubes were subjected to the sequential extraction described above. TX-100/KCl extracts were dialyzed against 0.05 M NaCl, 0.05 M Tris-HCl, pH 7.8 and loaded onto a 0.5 ml heparin-agarose column pre-equilibrated in 0.3 M NaCl, 0.05 M Tris-HCl, pH 7.8, and 1 mM PMSF (Brandan and Inestrosa, 1984). After rinsing the column extensively with equilibration buffer, bound material was eluted using a linear gradient of 0.3 to 1 M NaCl in 0.05 M Tris-HCl, pH 7.8. Total, unbound and eluted fractions were analyzed by SDS-PAGE followed by Coomassie blue staining.

Identification of p33/30

Identification of p33/30 was carried out at HHMI Biopolymer and W. M. Keck Biotechnology Resource Laboratory, Yale University, CT, USA. Briefly, molecular masses of in-gel digested p33 peptides were obtained by matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) carried out on a Research Grade, VG Tofspec SE instrument. Resulting peptides were further purified on a C-18 reverse-phase HPLC column and a single peptide subjected to conventional Edman degradation for amino-acid sequencing using an Applied Biosystems sequencer. Immunoblots were developed as previously described (Brix et al., 1998) using anti-histone H1 antibody and visualized by enhanced chemiluminescence (ECL).

Binding of heparan sulfate proteoglycans to histone H1

Histone H1 (0.5 mg) was separated by SDS-PAGE and electrotransferred onto a nitrocellulose sheet. After brief staining with 0.5% Ponceau S red, 5% acetic acid, histone H1-containing nitrocellulose pieces were excised and then blocked with 5% dry non-fat milk, 0.05 M Tris-HCl, pH 7.4, 0.15 M NaCl. A proteoglycan-enriched fraction obtained from myotube-conditioned media was partially purified on a 1 ml DEAE column pre-equilibrated in 0.15 M NaCl, 0.05 M Tris-HCl, pH 7.4 and eluted with 1 M NaCl prepared in the same buffer. The nitrocellulose immobilized-histone H1 was incubated overnight with the dialyzed proteoglycan-containing fraction in 2% BSA at 4°C and washed with the same buffer. Heparan sulfate proteoglycans bound to immobilized histone-H1 were then eluted with 8 M urea, 0.05 M Tris-HCl, 0.15 M NaCl, pH 7.4. Total, unbound and bound fractions were dialyzed and treated with

heparitinase as described (Brandan et al., 1996). The core proteins of heparan sulfate proteoglycans were visualized by western blot using the anti-Δ-heparan sulfate monoclonal antibody and polyclonal anti-perlecan specific antisera, as previously described (Fuentealba et al., 1999; Larraín et al., 1997a).

Induction of rabbit skeletal muscle regeneration

The tibialis anterior muscle of adult hamsters were injected intramuscularly, under ketamine/xylazine anesthesia, with 0.25 ml of an aqueous solution of $BaCl_2$ (1.2% w/v) (Caldwell et al., 1990). Four and five days later the injected muscle area was dissected out of the anesthetized animal and the tissue quickly frozen in isopentane previously cooled in liquid nitrogen. Frozen tissue was embedded in Tissue Freezing Medium gel and a series of 6 μm cryostat sections was obtained.

Immunofluorescence

Immunofluorescence microscopy of C_2C_{12} cultures and skeletal muscle cryosections was carried out essentially as described previously (Riquelme et al., 2001). In some experiments, 95% ethanol/5% acetic acid was used for fixation and permeabilization of cells.

Proliferation assays

Myoblast proliferation was assayed by measuring [^{3}H]thymidine incorporation, as described previously (Currie et al., 1997). For some assays, skeletal muscle ECM was obtained after detaching myotubes differentiated during six days by incubating with 10 mM EDTA in PBS at 37°C for 10 minutes, as described previously (Andress, 1995). Anti-histone H1 (C17) or anti-β galactosidase antibodies were diluted 1:100 for an overnight preincubation of the ECM-containing dishes and 1:200 through the proliferation assay.

Others

Adhesion assays were performed as described previously (Chernousov et al., 1996). DNA and protein content determinations were performed as described previously (Riquelme et al., 2001). Cell death was determined by measuring histone-complexed DNA fragments (mono and oligonucleosomes) in conditioned media (necrosis) and cell extracts (apoptosis) using specific ELISA assays as described by the supplier.

Results

An extracellular p33/30 protein doublet binds to heparin and its level increases during skeletal muscle differentiation

In order to identify heparin-binding proteins in C_2C_{12} cultures, proteins that were soluble in PBS, TX-100 and TX-100/KCl were obtained by sequential extraction and subjected to ligand blot assays using [^{35}S]heparin as a probe. As shown in Fig. 1A, two proteins of 33 and 30 kDa (named p33/30 hereafter), which were present only in the TX-100/KCl extract, showed significant [^{35}S]heparin binding. Similar ligand blot assays using chondroitin and dermatan sulfate GAG chains as competitors suggested that p33/30 bound specifically to heparin (Fig. 1C).

To determine whether p33/30 was present in the extracellular space, intact C_2C_{12} cell monolayers on various days of differentiation were incubated with a heparin solution. The solubilized material was analyzed by [^{35}S]heparin ligand

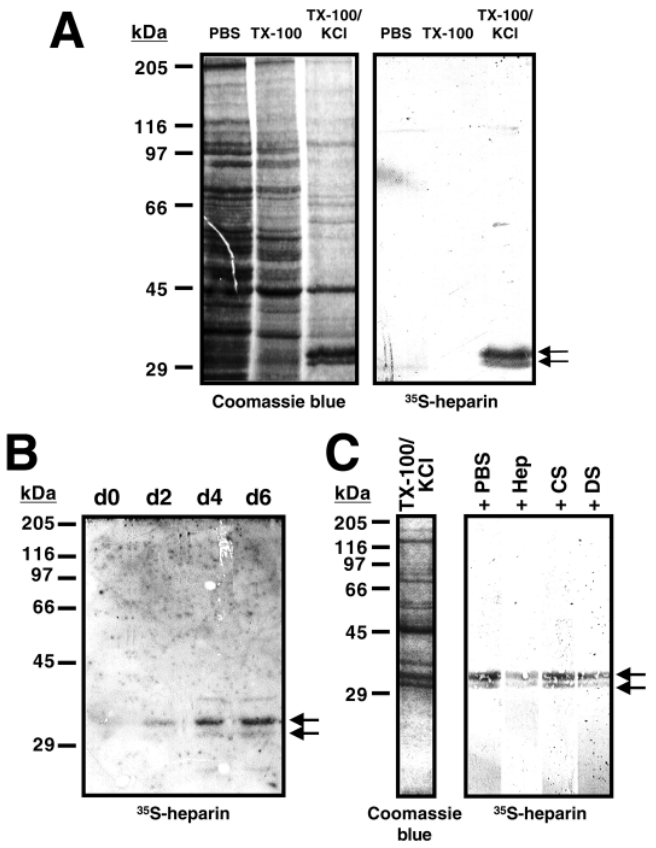

Fig. 1. Skeletal muscle cells synthesize an extracellular heparin-binding protein that increases in concentration during differentiation. (A) Proteins soluble in PBS, TX-100 and TX-100/KCl were sequentially extracted from C_2C_{12} myoblasts. Proteins were analyzed by SDS-PAGE and stained with Coomassie blue (left panel) or subjected to a [^{35}S]heparin ligand blot assay (right panel). (B) Heparin-solubilized proteins obtained from intact C_2C_{12} cultures on various days of differentiation (days 0-6) were analyzed by SDS-PAGE. Gel loading was normalized on the basis of equivalent amounts of DNA per lane. The presence of the p33/30 doublet was detected by a [^{35}S]heparin ligand blot assay. (C) Proteins solubilized in TX-100/KCl obtained from myoblasts were analyzed by SDS-PAGE and stained by Coomassie blue (left panel) or analyzed in a competition [^{35}S]heparin ligand blot assay (right panel). [^{35}S]heparin was co-incubated with 2 mg/ml of unlabeled heparin (Hep), chondroitin sulfate (CS) or dermatan sulfate (DS). An equivalent amount of total protein was loaded per lane. Molecular weight markers are shown on the left. Arrows on the right indicate the migration of p33/30.

Table 1. Small amounts of intracellular molecules are detected in PBS-heparin extracts obtained from intact myotubes

	CK (UI/ng DNA)	DNA (μg/ml)	LDH (UI/ng DNA)
Crude extract	9.95±1.29	9.73±0.42	15.26±0.94
PBS-heparin	0.11±0.02 (1.11±0.20)	0.05±0.01 (0.51±0.10)	0.09±0.01 (0.59±0.07)

PBS-heparin extracts were obtained from C2C12 cultures that had differentiated for 6 days. The table shows the content of CK, DNA and LDH determined in PBS-heparin and 0.5% Triton X-100 extracts (crude extract). All the values correspond to the average±s.d. of two different experiments performed in triplicate. The percentage of each determination in PBS-heparin with respect to crude extracts is shown in parentheses.

blot assay. As shown in Fig. 1B heparin was able to solubilize p33/30 from intact cells. In control experiments CK, LDH and DNA were poorly detected in the heparin extracts under these conditions (Table 1), demonstrating that the permeability of the cell monolayers was unaffected by heparin treatment. Furthermore, as shown in Fig. 1B, the binding of [^{35}S]heparin to heparin-solubilized p33/30 increased during skeletal muscle differentiation. These data strongly suggest that p33/30 binds to heparan sulfate, is present on the cell surface or in the extracellular space of muscle cell cultures, and increases during differentiation.

p33/30 is histone H1

In order to purify p33/30, TX-100/KCl-soluble proteins obtained from differentiated myotubes were subjected to heparin-agarose chromatography. Proteins were eluted by a linear NaCl gradient, analyzed by SDS-PAGE and stained with Coomassie blue. Fig. 2A shows a 30 and 33 kDa doublet that was almost completely retained by the column and was eluted between 0.55 and 0.75 M NaCl. The identity of this p33/30 doublet was determined by MALDI-MS analysis of heparin affinity-purified fractions. The highest probability scores were obtained with histone H1 from different species (data not shown). Moreover, N-terminal sequencing of an HPLC-purified tryptic peptide from p33 showed highest identity with different subclasses of histone H1 from at least four species (Fig. 2B). This identification was confirmed by western blot analysis using an anti-histone H1 monoclonal antibody. As shown in Fig. 2C, an immunoreactive 33/30 kDa doublet was detected in the fractions shown above to contain p33/30 by the [^{35}S]heparin ligand blot assay. The high specificity of the antibody for histone H1 was confirmed by specific staining of a nuclear-acid-soluble protein fraction containing nuclear skeletal muscle histones (Fig. 2C). Furthermore, like p33/30, histone H1 was specifically solubilized from myoblasts by heparin, unlike other GAGs (Fig. 2D). Collectively, these data allow the identification of p33/30 as a subclass of histone H1.

Extracellular histone H1 binds specifically to perlecan in skeletal muscle ECM

To study a possible interaction between histone H1 and heparan sulfate proteoglycan(s), a solid-phase assay was performed by incubating a partially purified total proteoglycan

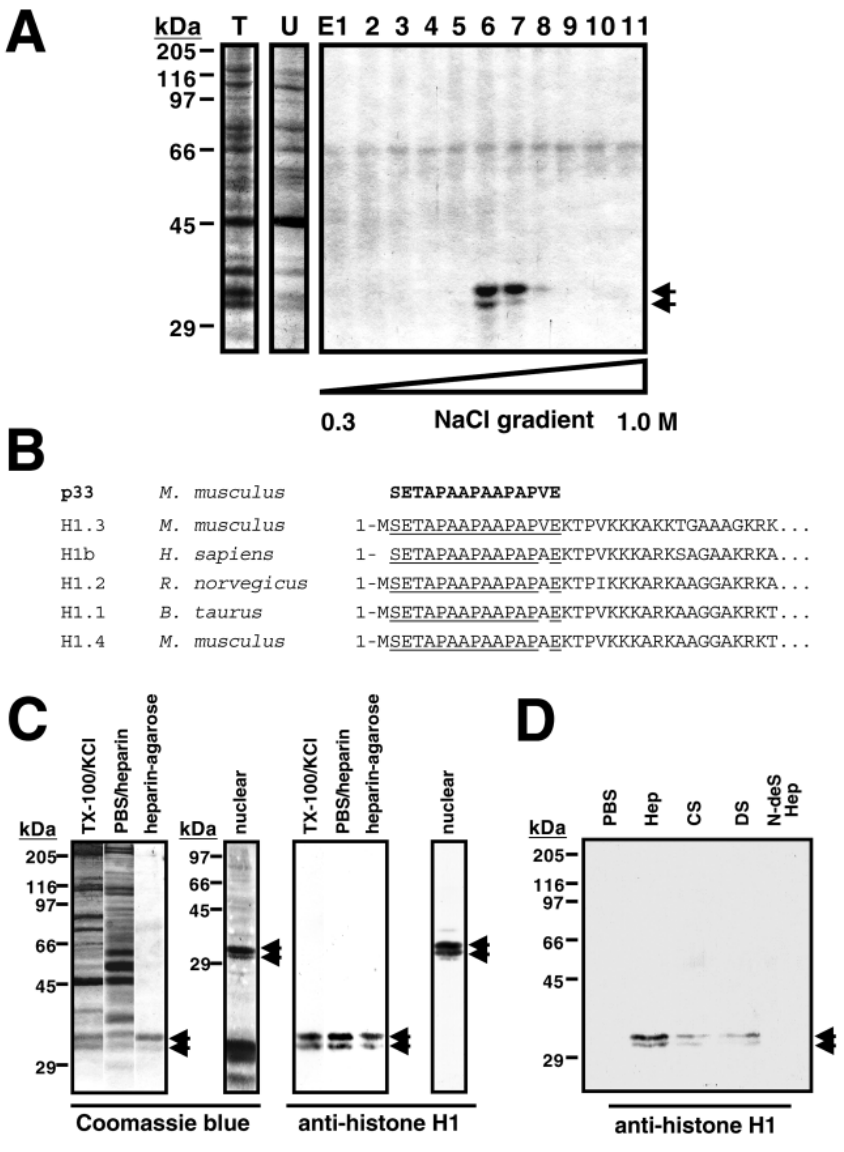

Fig. 2. The p33/30 purified by heparin-affinity chromatography from myotubes is histone H1. (A) Triton X-100/KCl fractions (T) were subjected to heparin-affinity chromatography, and the resulting unbound (U) and NaCl-eluted fractions (E1-11) were analyzed by SDS-PAGE and stained with Coomassie blue. The molecular weight markers and the NaCl gradient are shown. Arrows on the right indicate the migration of p33/30. (B) A 15 amino acid sequence was obtained from a HPLC-purified p33-derived peptide. High homology with sequences of various subclasses of histone H1 from mouse, human, rat and bovine are shown for the peptide. Sequences are given in the one-letter code for amino acids. Underlined amino acids correspond to identities with the p33 sequence (in bold). (C) TX-100/KCl, PBS/heparin extracts, heparin-affinity purified p33/30 and an acid-soluble nuclear fraction obtained from myotubes were separated by 12.5% (20% in the case of nuclear extract) SDS-PAGE and stained with Coomassie blue (left panel). Similar SDS gels were transferred onto nitrocellulose membranes, stained with a specific monoclonal anti-histone H1 antibody and detected by ECL (right panel). (D) C_2C_{12} myoblasts were extracted in the presence of 2 mg/ml heparin (Hep), chondroitin sulfate (CS), dermatan sulfate (DS) or N-desulfated heparin (N-deS Hep). Equivalent amounts of total proteins were analyzed by western blot with a specific monoclonal anti-histone H1 antibody, and detected by ECL. Molecular weight markers are shown on the left and the migration of p33/30 is indicated (arrows) on the right of each panel.

fraction obtained from C_2C_{12} myotube-conditioned media with immobilized histone H1. Total, unbound and histone-H1-bound proteoglycans were analyzed by western blot using anti-Δ-heparan sulfate monoclonal antibody, which recognizes heparan sulfate proteoglycan core proteins after heparitinase treatment (Fig. 3A), and a polyclonal anti-perlecan-specific antibody (Fig. 3B). Although several core proteins of heparan sulfate proteoglycans, including glypican, were detected with the anti-Δ-heparan sulfate antibody in the total sample, only perlecan was observed to bind to the immobilized histone H1. In a negative control experiment, heparan sulfate proteoglycans did not bind to a blank nitrocellulose sheet (data not show). These in vitro data strongly suggest that histone H1 binds specifically to perlecan.

To evaluate the extracellular localization of histone H1 in skeletal muscle cells cultured in vitro, dual immunofluorescent staining was performed on non-permeabilized C_2C_{12} myotubes. As shown in Fig. 4 (left and middle panels), anti-histone H1 antibody (H1) produced an extracellular filamentous staining pattern that colocalized extensively with the heparan sulfate proteoglycans perlecan (per) and glypican (gly). Interestingly, as is the case with perlecan, the extracellular histone H1 distribution was restricted to the ECM in myotubes, whereas glypican was also present on the plasma membrane. Control experiments in permeabilized myotubes showed, as expected, anti-histone H1 antibody staining primarily in cell nuclei (*p*H1).

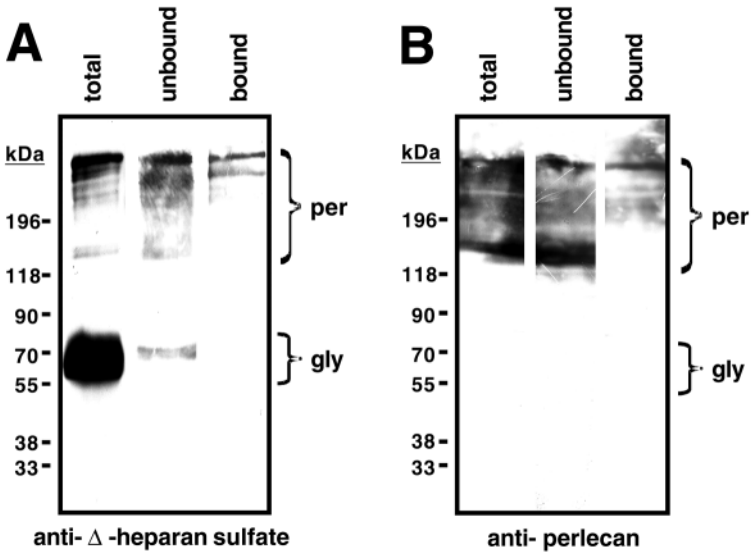

Fig. 3. Histone H1 binds specifically to perlecan secreted by myotubes. A total proteoglycan-enriched fraction obtained from the conditioned media of myotubes was incubated with histone H1 immobilized on nitrocellulose membranes and then eluted with 8 M urea. Total, unbound and histone-H1-bound fractions were incubated with heparitinase and separated by SDS-PAGE. Western blots were performed using (A) an anti-Δ-heparan sulfate monoclonal antibody to reveal the core proteins of heparan sulfate proteoglycans and (B) specific anti-mouse perlecan antisera, and detected by ECL. The migration of perlecan (per) and glypican (gly) are indicated on the right, and molecular weight markers on the left of each panel.

To investigate the relation between heparan sulfate proteoglycans present in the ECM and the extracellular localization of histone H1, C_2C_{12} cultures were treated with sodium chlorate, a metabolic inhibitor of heparan sulfate synthesis (Fig. 4, right panel). Chlorate treatment resulted in fewer and shorter myotubes and a strong disorganization of the ECM (Melo et al., 1996), as revealed by phase-contrast microscopy as well as anti-perlecan (per) and anti-glypican (data not shown) staining. Under these conditions, the immunoreactivity of extracellular histone H1 was strongly diminished, suggesting that heparan sulfate proteoglycans are essential for its extracellular localization in the ECM.

The extracellular localization of histone H1 was evaluated in hamster skeletal muscle that had been induced to regenerate after barium chloride injection. As observed by hematoxilyn/eosin staining (Fig. 5A), four days after the injection, abundant mononucleated cells – probably both inflammatory and muscle precursor cells – were encountered together with degenerating myofibers and remnants of the original basement membranes. Newly formed centrally nucleated myotubes could be observed at this stage, but they were more evident and abundant five days after the injection. Fig. 5B shows that in normal adult skeletal muscle (ctrl) only nuclear histone H1 staining was observed. However, in regenerating muscle at day four after barium chloride injection, a pool of extracellular histone H1 was detected in a filamentous pattern between myofibers and mononucleated cells (H1, insert) that does not colocalize with nuclei stained with Hoechst 33258 (Ho, insert). Histone H1 extracellular staining colocalized with anti-perlecan antibody staining in the basement membrane of degenerated fibers (per, insert). At the fifth day of regeneration, extracellular histone H1 staining was weaker and was found mostly associated with the basal lamina surrounding the newly formed regenerating myotubes, where again it colocalized with perlecan. Similar myotubes can be detected by indirect immunofluorescence anti-embryonic myosin (em), a transient early skeletal muscle differentiation marker in similar regenerating foci of adjacent cryosections. No staining was observed with this antibody in control skeletal muscle (data not shown).

Taken together, these results demonstrate that a pool of extracellular histone H1 binds specifically to perlecan in skeletal muscle ECM.

Cell death may be a source of histone H1 in the ECM

To determine whether cell lysis could account for the presence of histone H1 in the ECM, LDH activity was assayed in conditioned media collected over 2 day intervals following induction of cell differentiation. Fig. 6A shows that LDH was released into the medium during the 6 days after induction of differentiation, with the highest level of LDH activity detected within the first two days. In these experiments, necrotic and apoptotic cell death were also evaluated using ELISA assays. As shown in Fig. 6B, necrosis was the main cause of cell death and showed a similar temporal pattern to LDH release during differentiation, whereas very little apoptotic cell death was detected.

The cumulative LDH release, calculated using the data in Fig. 6A (Fig. 6A, insert), closely paralleled the accumulation

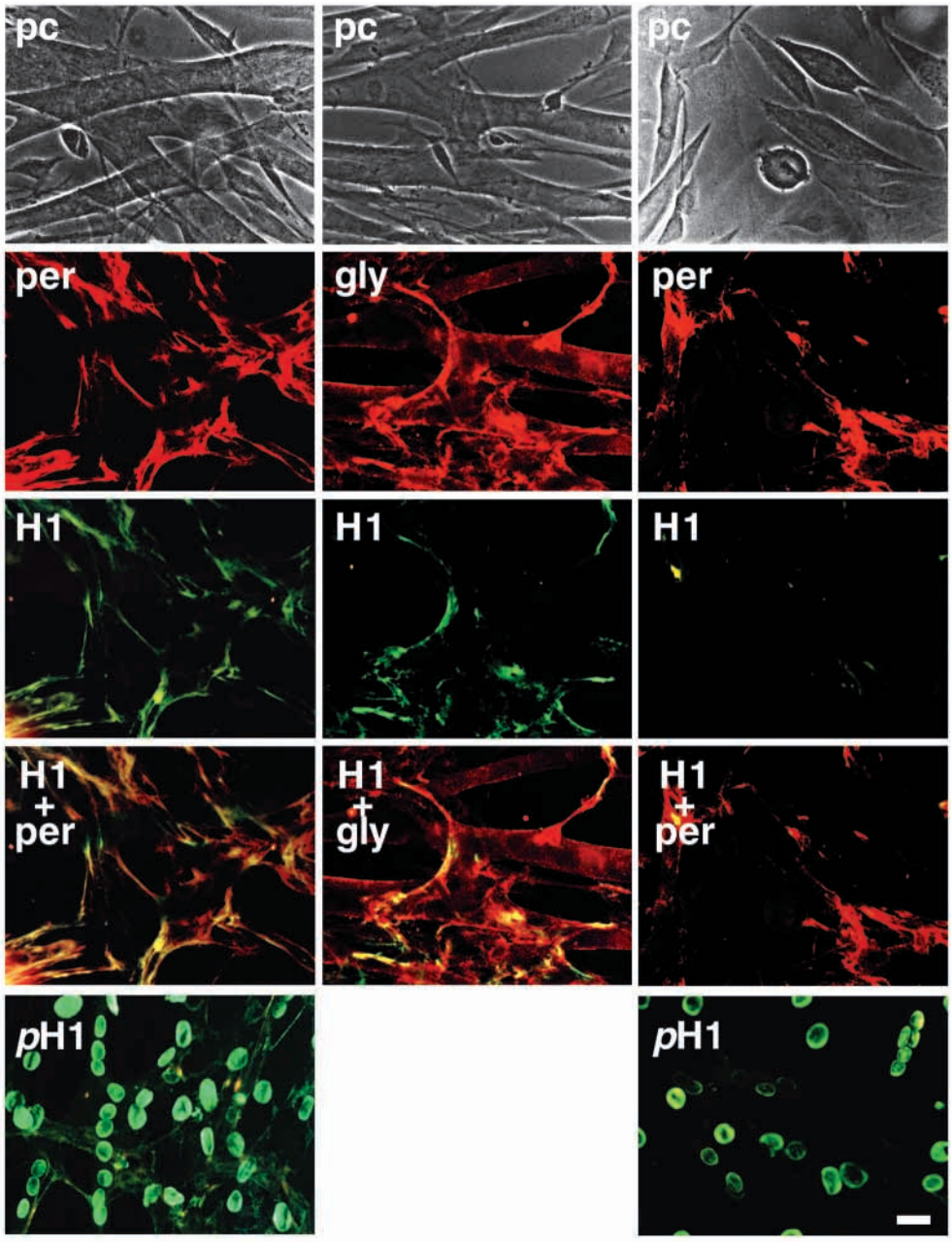

Fig. 4. A pool of histone H1 is localized to the myotube ECM and colocalizes with heparan sulfate proteoglycans. Inhibition of proteoglycan sulfation reduces ECM localization of histone H1. (Left and middle panels) Non-permeabilized myotubes were double-stained with anti-mouse perlecan (per) or anti-rat glypican (gly) antibodies together with an anti-human histone H1 (H1). Phase contrast micrographs of respective fields are also shown (pc). Merged images (*H1*+per and *H1*+gly) are also shown. As a control, permeabilized myotubes were stained with the anti-human histone H1 (*p*H1). TRITC-conjugated anti-rabbit IgG and FITC-conjugated anti-mouse IgG were used as secondary antibodies. (Right panel) C_2C_{12} cultures were treated with sodium chlorate during differentiation and processed as described for the left panel. Bar, 10 μm.

of extracellular heparin-displaced histone H1 detected during the differentiation of C_2C_{12} cells (Fig. 6C, Fig. 1B). These findings support the possibility of cell death as the source of the pool of histone H1 in the ECM of skeletal muscle cells.

Histone H1 stimulates the proliferation of myoblasts

To study the possible functions of extracellular histone H1, myoblasts were plated on dishes coated with histone H1 purified from myoblasts by heparin-affinity chromatography. Myoblast attachment and spreading was monitored by phase contrast microscopy. Fig. 7A (upper panel) shows that 2 hours after plating myoblasts on histone H1-coated dishes assumed a flattened polygonal morphology characteristic of C_2C_{12} myoblasts. For comparison, laminin, fibronectin and BSA were also used as attachment substrates. Myoblasts were well spread on laminin but not BSA. Interestingly, 21 hours after plating (Fig. 7A, lower panel), there was a large increase in the number of cells on histone-H1-containing dishes. Laminin showed a similar apparent increase in cell number, whereas fibronectin and BSA failed to produce such an effect. This result led us to examine a possible role for extracellular histone H1 in myoblast proliferation.

Cell proliferation, assayed by measuring [^{3}H]thymidine incorporation, was seen to increase upon addition of exogenous histone H1 in a time- and concentration-dependent manner, reaching a maximal value at 10 μg/ml (Fig. 7B,C). To evaluate the involvement of heparan-sulfate-like molecules in the proliferative effect observed for histone H1, myoblasts were incubated with increasing concentrations of histone H1 in the

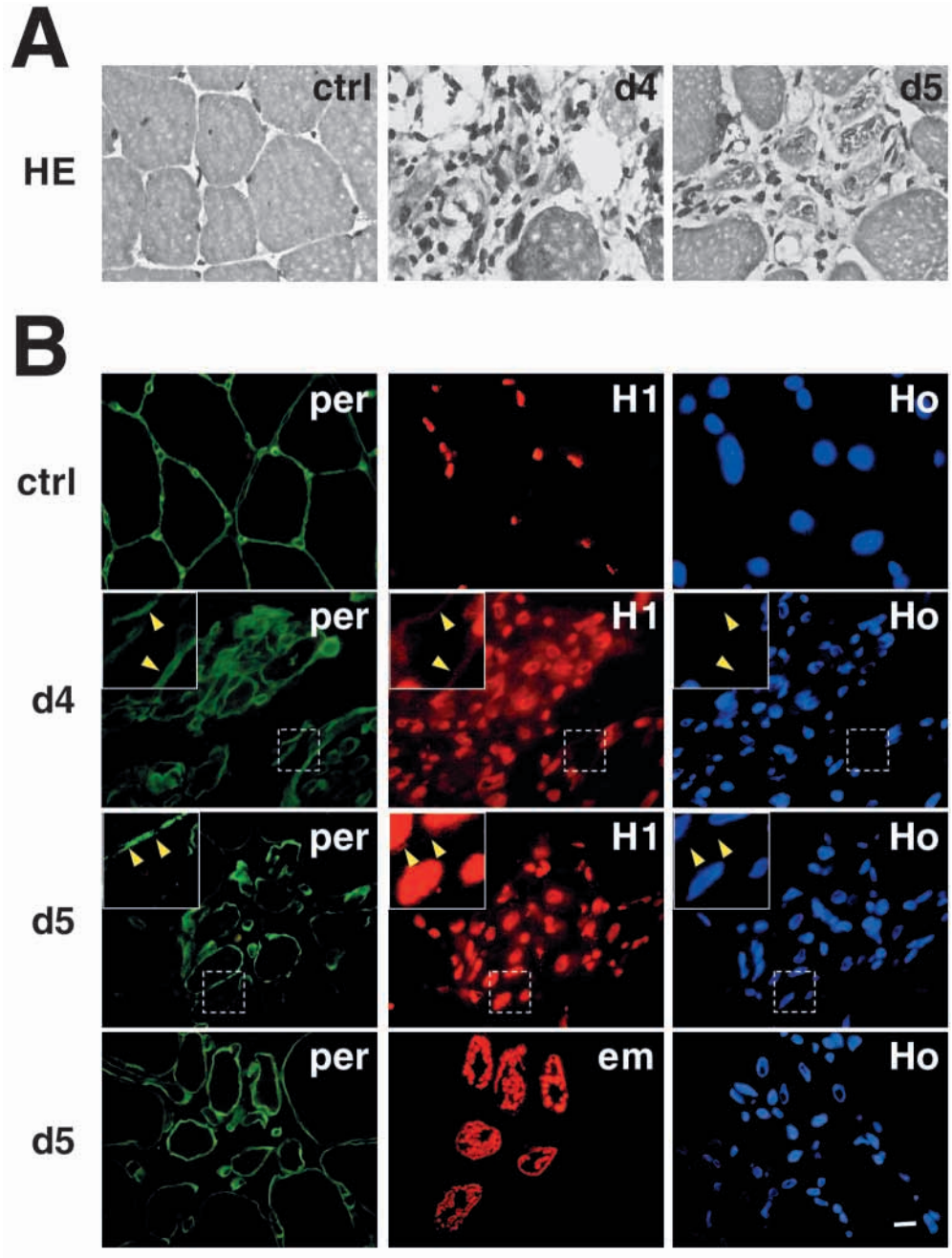

Fig. 5. Histone H1 colocalizes with perlecan in regenerating skeletal muscle. Hamster tibialis anterior muscle was induced to regenerate for four (d4) or five days (d5) after a barium chloride injection. (A) Serial cryosections were stained with hematoxylin/eosin to visualize morphological progression. (B) Similar sections were double stained with monoclonal antibodies against histone H1 (H1) or embryonic myosin (em) together with a polyclonal anti-perlecan (per) antibodies. TRITC-conjugated anti-mouse IgG and FITC-conjugated anti-rabbit IgG were used as secondary antibodies. Nuclei in regeneration foci were visualized by Hoechst 33258 nuclear staining (Ho). As a control, cryosections of non-injected hamster muscle (ctrl) were similarly analyzed. Inserts correspond to the regions marked with a dotted square in the respective sections. Arrows indicate the colocalization of anti-histone H1 and anti-perlecan antibodies in the ECM. Bar, 25 μm.

absence or presence of heparin. Fig. 7D shows that histone H1-dependent [^{3}H]thymidine incorporation was increased in cells that were also treated with heparin. Moreover, the incorporation of [^{3}H]thymidine induced by histone H1 was almost completely abolished in cells treated with sodium chlorate (data not shown). To directly evaluate the function of histone H1 externalized by C_2C_{12} cells, [^{3}H]thymidine incorporation was determined on myoblasts seeded on myotube ECM, which were prepared by detaching the myotube monolayer. This histone H1-containing ECM (data not shown) was previously incubated with anti-histone H1 (C17) and the antibody was maintained during the assay. As a control, an unrelated anti-β galactosidase antibody was also used. Anti-histone H1 antibody caused a 40% reduction of the proliferative effect induced by the myotube ECM (Fig. 7E). Collectively, these results strongly suggest that endogenously released histone H1, which is bound to the ECM, stimulates myoblast proliferation via a mechanism dependent on heparan sulfate chains.

Discussion

The results presented in this study provide novel evidence that a pool of histone H1, a nuclear protein bound to linker DNA connecting adjacent nucleosomes in eukaryotic chromatin, becomes associated with the ECM of a skeletal muscle cell line and regenerating skeletal muscle. The amount of this extracellular histone H1, which colocalized with heparan

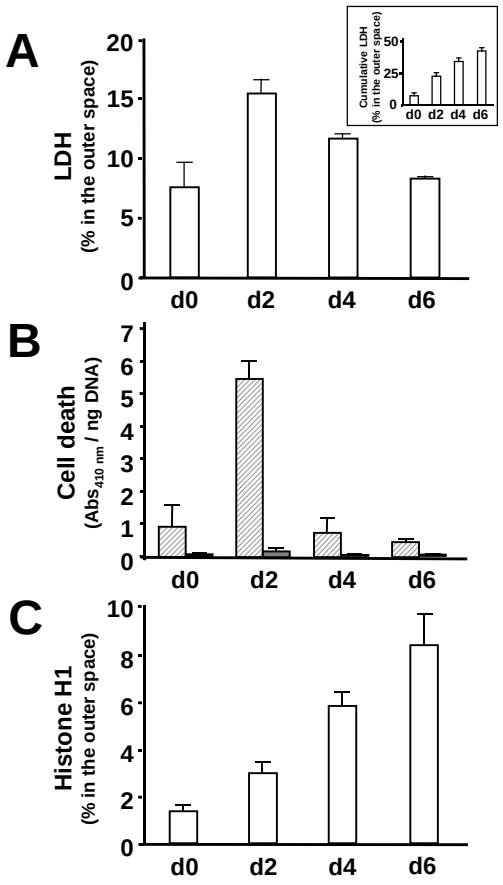

Fig. 6. Cell death may be related to accumulation of extracellular histone H1. **(**A) C_2C_{12} cells were induced to differentiate. The culture medium was changed every 2 days. LDH activity was quantified in conditioned media harvested on the indicated days and in cell extracts. Extracellular LDH (in the extracellular space) is expressed as a percentage of the total enzyme. The insert shows cumulative LDH release, calculated using the data in Fig. 6A. (B) Necrosis (dashed bars) and apoptosis (closed bars) were determined by specific ELISA assays. Data are expressed as the absorbance at 410 nm per ng of DNA. (C) Heparin-displaced proteins from intact cells and TX-100/KCl extracts from the corresponding cells were subjected to [^{35}S]heparin ligand blot assay. Densitometric analysis of heparin-displaced histone H1 is shown as the percentage of total histone H1. Data are plotted as a function of the differentiation period (days 0-6). Results correspond to the average and s.d. of two independent experiments.

sulfate proteoglycans, increases during muscle differentiation in culture. Moreover, our results demonstrate that histone H1 binds to and colocalizes specifically with perlecan, an ECM heparan sulfate proteoglycan expressed by muscle cells (Larraín et al., 1997a; Murdoch et al., 1994). We also showed that histone H1 promoted the proliferation of myoblasts via a mechanism that was stimulated by exogenous heparin, which acts as a functional and structural analogue of heparan sulfate chains.

Previous studies have shown that nucleosome-complexed core histones, namely H2A, H2B, H3 and H4 (Schmiedeke et al., 1989) as well as histone-like proteins (Kohnke-Godt and Gabius, 1991), may bind to basement membranes and particularly to cell surface heparan sulfate proteoglycans (Watson et al., 1999). Extracellular nucleosomes have been linked either to the formation of auto-antibodies in the pathogenesis of systemic lupus erythematosus (Schmiedeke et al., 1989) or the stimulation of the synthesis of immunoglobulins and other immunomodulatory factors (Emlen et al., 1992).

Our data unequivocally demonstrate that the extracellular 33/30 kDa doublet, which was displaced from intact cell monolayers by heparin, is a subclass of histone H1, as determined by MALDI-MS, N-terminal sequencing and immunological analyses. During this investigation, extracellular histone H1 was never found complexed to DNA or other core histones.

Histone H1 is expressed in a wide variety of isoforms (Schulze et al., 1994) and shows different degrees of post-translational modification (Spencer and Davie, 1999). Nuclear histone H1 functions not only as a structural protein but also acts as a positive and negative regulator of gene transcription (Wolffe et al., 1997). Indeed, genes controlling muscle differentiation are known to be selectively repressed by histone H1 (Steinbach et al., 1997). Cytoplasmic pools of histone H1 have also been described in mammalian cells (Zlatanova et al., 1990), and an extracellular histone H1 was identified at the cell surface of a macrophage cell line, where it acts as a thyroglobulin-binding protein that mediates its endocytosis via a mechanism that is inhibited by heparin (Brix et al., 1998). Moreover, addition of histone H1 to different cell types has been correlated with cell division (Kundahl et al., 1981), differentiation (Okabe-Kado et al., 1981) and cytotoxicity (Class et al., 1996). These studies have led to the notion that histone H1 may act as a multifunctional protein, which is consistent with the findings described in this study.

The present data suggest that among several GAGs, histone H1 binds specifically to heparan sulfate chains. This interaction seems to be dependent on GAG sulfation, given that N-desulfated heparin failed to solubilize histone H1 from myoblasts. More specifically, a biochemical approach showed that, among the whole population of heparan sulfate proteoglycans externalized by myotubes, histone H1 exclusively binds to perlecan. Immunohistological studies in non-permeabilized myotubes showed that histone H1 colocalizes extensively with perlecan in the ECM, even though glypican is also present on the cell surface. Similarly, a filamentous pattern of extracellular histone H1 was observed in skeletal muscle regeneration foci. This pattern was also observed for the immunostaining of heparan sulfate proteoglycan core proteins (data not shown), and the specific colocalization of histone H1 with perlecan suggests that it is deposited in the ECM of regenerating skeletal muscle.

The specific interaction of histone H1 with perlecan is an intriguing finding given that many extracellular ligands bind to the heparan sulfate chains of different proteoglycans with similar affinities (Bernfield et al., 1999; Tumova et al., 2000). Moreover, we have demonstrated the presence of at least perlecan and glypican in the ECM of differentiating myotubes (Brandan et al., 1996; Larraín et al., 1997a). However, there is an increasing body of evidence that suggests a role for specific heparan sulfate sequence motifs in ligand specificity (for a review, see Turnbull et al., 2001). Alternatively, the expression of multiple isoforms of perlecan (Iozzo, 1998) might influence its specific binding to histone H1.

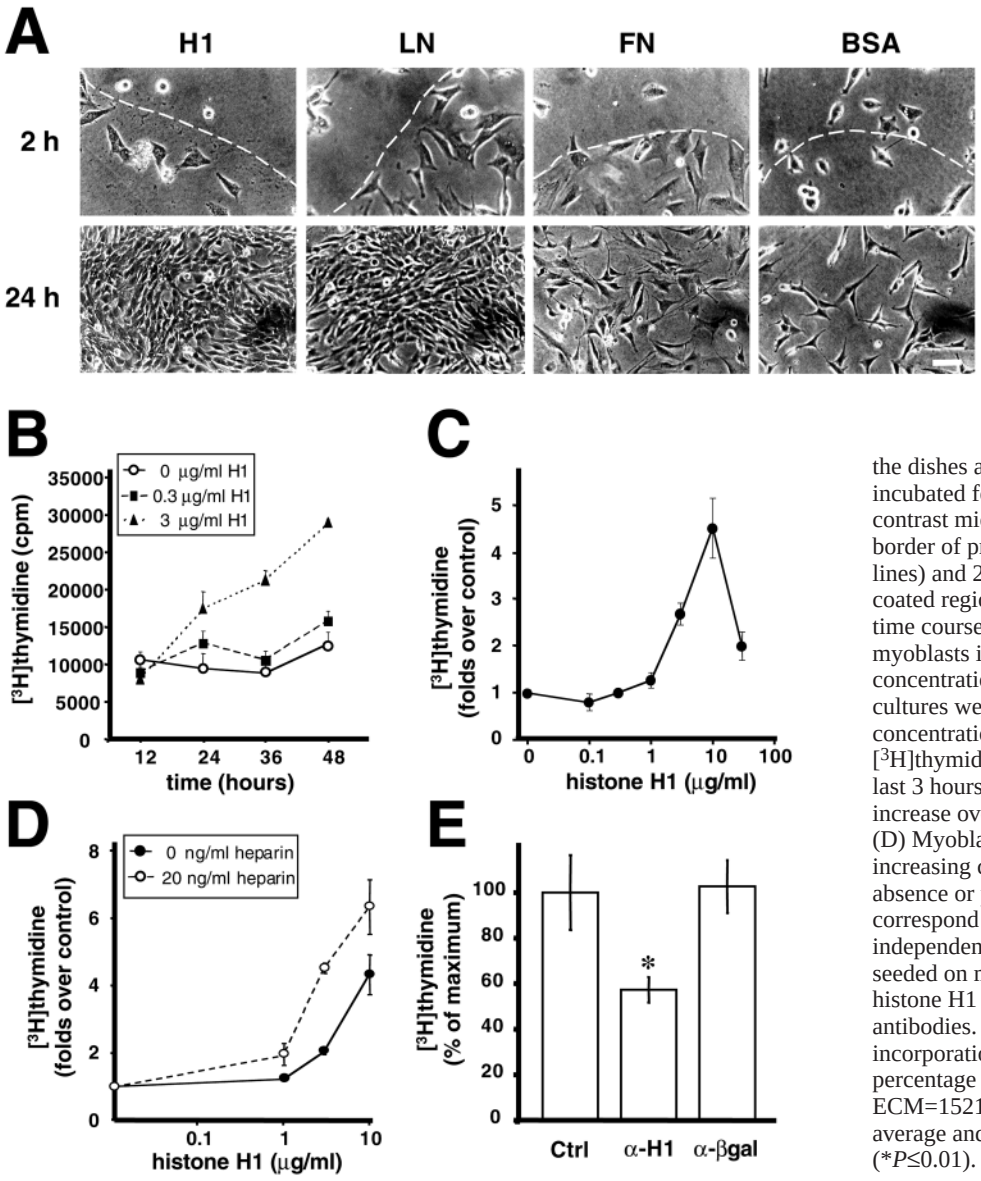

Fig. 7. Histone H1 induces myoblast proliferation via a heparan-sulfate-dependent mechanism. (A) Culture dishes were coated with nitrocellulose, and 10 μl droplets containing 2 μg of affinity-purified histone H1 obtained from myotubes (H1), laminin (LN), fibronectin (FN) or BSA were applied to the surface of the dishes and dried. C_2C_{12} myoblasts were then incubated for 2 hours and examined by phase contrast microscopy. Photographs were taken at the border of protein-coated areas (upper panel, dashed lines) and 21 hours later, at the center of the protein-coated regions (lower panel). Bar, 10 μm. (B) The time course of the [^{3}H]thymidine incorporation in myoblasts induced by the addition of the indicated concentrations of exogenous histone H1. (C) C_2C_{12} cultures were incubated with increasing concentrations of histone H1 for 24 hours. [^{3}H]thymidine incorporation was determined in the last 3 hours of incubation and expressed as fold increase over controls (without histone H1). (D) Myoblast cultures incubated for 24 hours with increasing concentrations of histone H1 in the absence or presence of heparin (20 ng/ml). Results correspond to the average and s.d. of two independent experiments. (E) Myoblasts were seeded on myotube ECM preincubated with anti-histone H1 (α-H1) or anti-β galactosidase (α-βgal) antibodies. After 24 hours, [^{3}H]thymidine incorporation was determined and expressed as a percentage of maximum effect (control ECM=1521±250 cpm). The results correspond to the average and s.d. of two independent experiments (*P≤0.01).

The results of this study point to a functional role for extracellular histone H1 in cell proliferation. We observed a five- to seven-fold increase in [^{3}H]thymidine incorporation in histone-H1-treated muscle cells, which was correlated with an increase in cell number. Moreover, an anti-histone H1 antibody caused a 40% reduction of the proliferation induced by the ECM obtained from differentiated skeletal muscle cells. Heparan sulfate proteoglycans have been extensively characterized as co-receptors of mitogenic factors, with the FGF-heparan sulfate interaction constituting an archetype example (Bernfield et al., 1999). We have previously demonstrated that the myogenic inhibitory activity of FGF-2 is potentiated by the transmembrane heparan sulfate proteoglycans syndecan-1 and -3 (Fuentealba et al., 1999; Larraín et al., 1998). Extracellularly occurring heparan sulfate proteoglycans such as perlecan or shed syndecan-1 may have the ability to inhibit signaling by sequestering the growth factor (Aviezer et al., 1994b; Kato et al., 1998). Nevertheless, perlecan has also been described as a positive regulator of FGF-2 activity in some conditions (Aviezer et al., 1994a), suggesting that cellular context may be important in determining functional roles.

As a potential mechanism for the observed effect of histone H1 on myoblast proliferation, the entry of extracellular histone H1 to the nucleus, which can be increased by the addition of heparin (Villeponteau, 1992), has been shown previously to generate new initiation sites for DNA replication (Kundahl et al., 1981). Although our results do not provide direct evidence, it is tempting to suggest that heparan sulfate may physiologically mediate the internalization of histone H1, as heparin addition was found to increase histone-H1-induced myoblast proliferation. In further support of this hypothesis, perlecan has been reported to mediate the internalization of different ligands (Fuki et al., 2000), in addition to its localization on the surface of proliferative myoblasts (Larraín et al., 1997a).

On the basis of the present novel data, we suggest that the presence of histone H1 in the ECM may play a role in muscle

physiology. The regenerative capacity of skeletal muscle arises mainly from the activation of a small population of quiescent mononucleated cells, called satellite cells, located between the basal lamina and the sarcolemma of mature muscle fibers (Mauro, 1961). After injury, satellite cells, from which C_2C_{12} cells are derived (Andres and Walsh, 1996), proliferate and fuse in response to extracellular signals in order to regenerate the damaged muscle fibers. Several well-known heparin-binding mitogenic factors, such as FGF-2, are released from muscle cells following injury (Clarke et al., 1993), and here we provide evidence suggesting that cell death may be involved in histone H1 externalization. Infiltrating leukocytes accumulate early at the site of damage and phagocytose the necrotic tissue. However, empty muscle fiber basement membrane structures and specific molecular components, including type IV collagen, laminin and heparan sulfate proteoglycan, are initially preserved (Gulati et al., 1983) and are repopulated by proliferating myoblasts. The persistence of basement membranes has been shown to accelerate muscle regeneration (Caldwell et al., 1990) and favor the reinnervation at original synaptic sites (Sanes et al., 1978). In this context, our results support the idea that after damage histone H1 may act as an externalized growth signal in regenerating muscle that can be stored by heparan sulfate proteoglycan ECM components, such as perlecan, to subsequently induce the proliferation of satellite cells. Indeed, during inflammatory processes, active growth-factor–heparan-sulfate complexes have been shown to be released from ECM via controlled proteolytic cleavage (Benezra et al., 1993; Whitelock et al., 1996).

In summary, our results demonstrate that histone H1 localizes to the ECM of cultured muscle cells and in regenerating skeletal muscle, where it binds specifically to perlecan. Our results also suggest that extracellular histone H1 may stimulate the proliferation of satellite myoblasts during skeletal muscle regeneration.

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