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Major Chondroitin Sulfate Proteoglycans Identified in L6J1 Myoblast Culture

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Abstract—The major proteoglycans from L6J1 rat myoblast culture were identified. The proteoglycans were isolated from different constituents of cell culture: culture medium, extracellular matrix (ECM), and myoblasts. To identify their core proteins, the proteoglycans were treated with enzymes specifically digesting chondroitin/dermatan sulfates or chondroitin sulfates. Subsequent electrophoresis and mass spectrometry revealed versican, collagen XII, and inter-α-trypsin inhibitor classified as chondroitin sulfate proteoglycans and biglycan known to be chondroitin/dermatan sulfate proteoglycan. Versican was identified in ECM and the other proteoglycans in the culture medium. Such difference in localization is likely to be a consequence of different biological functions. Versican, collagen XII, and biglycan are synthesized by myoblasts and inter-α-trypsin inhibitor originates from fetal bovine serum (a culture medium component).

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Proteoglycans (PGs) are a large class of complex multifunctional compounds consisting of core protein that is bound to one or more polysaccharide chains – glycosaminoglycans (GAGs) [1]. PGs of skeletal muscles, regulating together with growth factors different myogenesis stages, provoke great interest [2, 3]. Perlecan, versican, biglycan, decorin, betaglycan, syndecans 1-4, and glypican belonging to different PG classes have been identified in muscle tissue [2-4]. These compounds can be synthesized both by muscle and non-muscle cells, for example, by fibroblasts [5]. Myoblast culture can be used to analyze PGs synthesized by myoblasts in particular. In our previous studies we isolated, fractionated, and characterized PGs from rat myoblast culture [6, 7]. It was shown that the main class of PGs and a virtually unique class in the culture medium are chondroitin/dermatan sulfate PGs.

The goal of this study was to identify the main chondroitin sulfate PGs in L6J1 rat myoblast culture.

MATERIALS AND METHODS

Reagents. In this study we used chondroitinases ABC and AC, Alcian blue (Sigma, USA); phenylmethylsulfonyl fluoride, N-ethylmaleimide (Serva, Germany); urea (Vekton, Russia); fetal bovine serum (FBS), Dulbecco modified Eagle's medium, trypsin and EDTA solutions (Biolot, Russia). Reagents for mass spectrometric analysis were obtained from Aldrich (USA) except for trypsin (Promega, USA).

Myoblasts and their cultivation. L6J1 rat transformed myoblasts were obtained from the Russian Cell Culture Collection of the Institute of Cytology of the Russian Academy of Sciences. The cells were cultivated in

Abbreviations: ECM, extracellular matrix; FBS, fetal bovine serum; GAGs, glycosaminoglycans; ITI, inter-α-trypsin inhibitor; PGs, proteoglycans.

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Dulbecco's modified Eagle's medium with 10% FBS. The cells were grown in 175 cm^2 flasks (Sarstedt, Germany). The culture medium was collected and frozen to –20°C in the presence of proteolysis inhibitors – phenylmethylsulfonyl fluoride (1 mM) and N-ethylmaleimide (10 mM). Myoblasts and extracellular matrix (ECM) were treated with buffers containing urea and proteolysis inhibitors and the resulting extracts were frozen to –20°C [6].

Isolation of proteoglycans. PGs from the culture medium, ECM, and myoblasts were isolated and fractionated by ion-exchange chromatography on Q-Sepharose in a NaCl concentration gradient as previously described [6].

Enzymatic treatment of proteoglycans. To isolate core proteins, the PGs were treated with chondroitinases ABC and AC digesting chondroitin/dermatan sulfates and chondroitin sulfates [1, 6].

Electrophoresis. Isolated PGs were separated electrophoretically in 7% or gradient 3-15% or 3-7% SDS-polyacrylamide gels [8]. Samples were dissolved in 60 mM Tris-HCl, pH 6.8, containing 10% glycerol and 0.5 M 2-mercaptoethanol. The gels were treated sequentially with Alcian blue determining acidic polysaccharides and with Coomassie R-250 determining proteins.

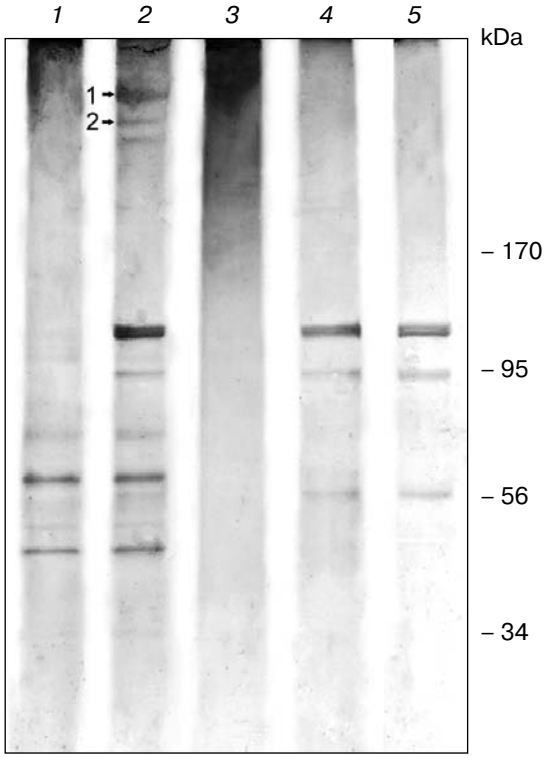

Fig. 1. SDS-PAGE of proteoglycan fractions III and IV from ECM in 3-15% polyacrylamide gel. Lanes: *1*) ECM fraction III (control without enzymatic treatment); *2*) ECM fraction III after treatment with chondroitinase ABC; *3*) ECM fraction IV (control without enzymatic treatment); *4*) ECM fraction IV after treatment with chondroitinase ABC; *5*) chondroitinase ABC. Protein bands depicted by arrows: 1 and 2 are versican core protein.

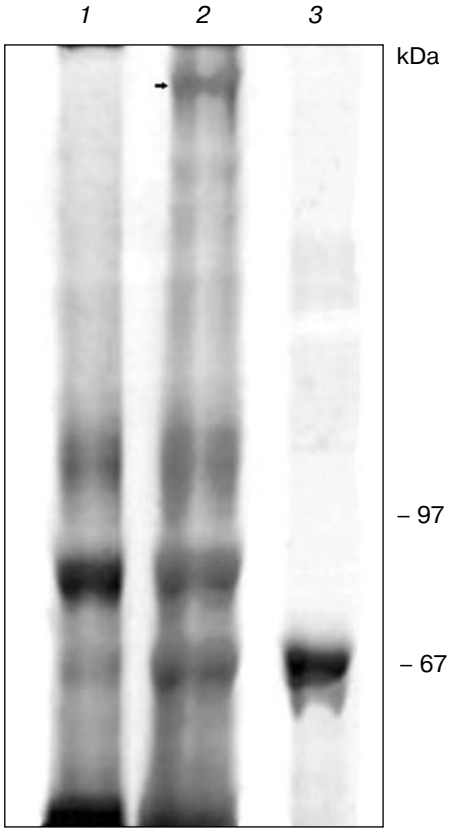

Fig. 2. SDS-PAGE of proteoglycan fraction III from myoblasts in 7% polyacrylamide gel. Lanes: *1*) myoblast fraction III (control without enzymatic treatment); *2*) myoblast fraction III after treatment with chondroitinase AC; *3*) chondroitinase AC. A protein band is depicted by the arrow: unknown core protein of cellular proteoglycans.

Mass spectrometry. Individual PGs were identified using MALDI-TOF mass spectrometry in the Institute of Physical-Chemical Medicine of the Ministry of Health of Russian Federation using a Bruker Ultraflex II mass spectrometer (Bruker Daltonics, Germany) and in the Institute of Bioanalytical Chemistry (Germany) using a Proteomic Analyzer 4700 mass spectrometer (Applied Biosystems, Germany). PG core proteins discovered after treatment of PGs with specific enzymes and subsequent electrophoresis were analyzed. During preparation of samples for mass spectrometry, gel fragments containing proteins were washed with 40% acetonitrile in 0.1 M NH_4HCO_3 to remove the dye. After removing the washing solution, the gel was treated with 15 μg/ml trypsin in 0.05 M NH_4HCO_3 (18 h at 37°C). Then 10% water solution of acetonitrile containing 0.5% CF_3CO_2H was added to the sample. The solution above the gel was used for obtaining MALDI-mass spectra. For this purpose 1 μl of the solution was mixed with 0.3 μl of 20% aqueous ace-

tonitrile solution containing 10 mg/ml 2,5-dihydroxybenzoic acid and 0.5% CF_3CO_2H. The resulting mixture was applied onto a scaffold and dried in air. To control the data of biglycan mass spectrometric analysis, we also used other somewhat different protocols for sample preparation [9]. Mass spectra were registered in positive ion mode using a reflectron within the range m/z = 700-4000. The accuracy of measured peptide weights was 50-100 ppm. Registered mass spectra were analyzed using Mascot software (www.matrixscience.com). The search by "peptide fingerprint" was conducted in the NCBI database of mammalian proteins considering probable methionine oxidation by air oxygen and cysteine modification by acrylamide. Protein identification was regarded positive when acceptance probability was >95%.

RESULTS

Identification of proteoglycan core proteins by electrophoresis. Previously we isolated PGs from L6J1 myoblast culture components – culture medium, ECM, and myoblasts. Isolated PGs were fractionated using ion-exchange chromatography [6]. Fractions of a gradient containing the maximum PG content (fractions III and IV of ECM, fraction III of cells, and fraction IV of culture medium) were analyzed by electrophoresis to detect PG core proteins after preliminary enzymatic degradation of polysaccharide chains and subsequent mass spectrometry. Taking into account the fact that chondroitin/dermatan sulfate PGs form the majority of PGs isolated from myoblast culture (culture medium, 100%; ECM, 67%; cells, 56%) [7], chondroitinases ABC and AC were used for degradation of GAGs. PG core proteins were detected under comparison between data of PGs electrophoretic separation before and after enzyme treatment, which led to dissipation of diffuse gel staining that is typically for PGs, and to the appearance of core protein bands. In this case bands corresponding to chondroitinase AC, chondroitinase ABC, and BSA that the producer adds to the original enzyme sample for its stabilization were registered and excluded (Figs. 1-4).

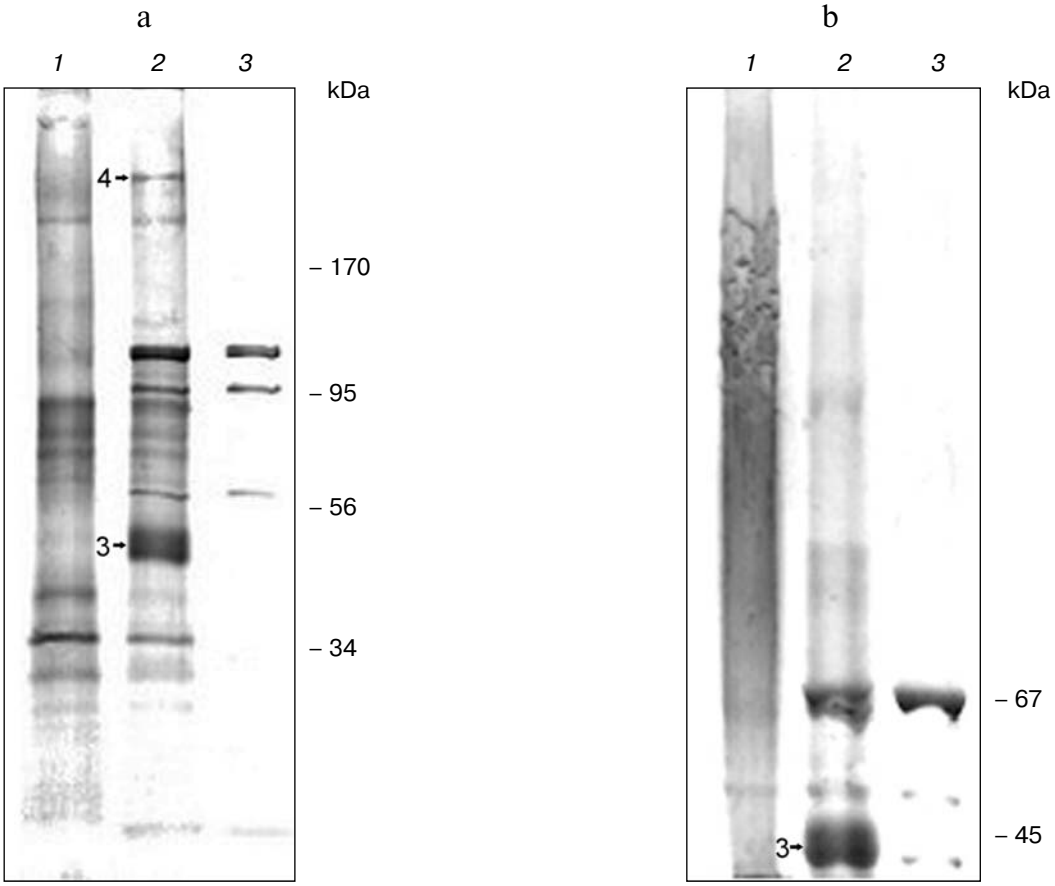

Fig. 3. SDS-PAGE of proteoglycan fraction IV from culture medium in 3-15% polyacrylamide gel (a) or in 3-7% polyacrylamide gel (b). Lanes: *1*) culture medium fraction IV (control without enzymatic treatment); *2*) culture medium fraction IV after treatment with chondroitinase ABC (a), chondroitinase AC (b); *3*) chondroitinase ABC (a), chondroitinase AC (b). Protein bands depicted by arrows: 3, biglycan core protein; 4, collagen XII α-1-chain.

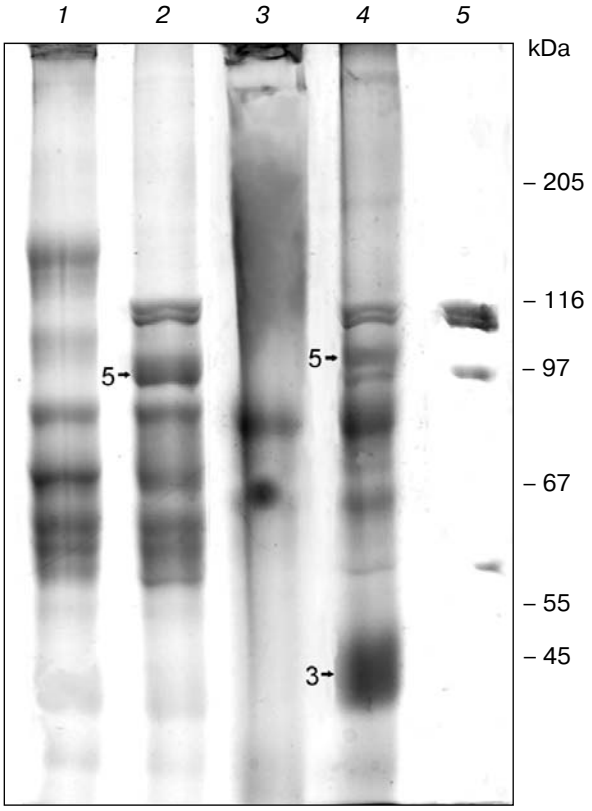

Fig. 4. SDS-PAGE of proteoglycan fraction IV from culture medium and fetal bovine serum (FBS) in 7% polyacrylamide gel. Lanes: *1*) culture medium fraction IV (control without enzymatic treatment); *2*) culture medium fraction IV after treatment with chondroitinase ABC; *3*) FBS fraction IV (control without enzymatic treatment); *4*) FBS fraction IV after treatment with chondroitinase ABC; *5*) chondroitinase ABC. Protein bands depicted by arrows: 3, biglycan core protein; 5, H2 chain of inter-α-trypsin inhibitor.

Seven protein bands, which were absent in corresponding controls (original PG samples and enzymes), were found after electrophoresis of PGs subjected to enzymatic treatment. There were three new protein bands discovered in ECM, one in cells and three in culture medium (Figs. 1-4).

Gradient electrophoresis in 3-15% gel of PGs from ECM fraction III treated with chondroitinase ABC (Fig. 1) revealed three new protein bands with apparent molecular weights of 320, 280, and 260 kDa (Fig. 1, lane *2*). However, we failed to find any PG core protein bands except for the enzyme bands in ECM PG fraction IV (Fig. 1, lane *4*).

The cleavage of myoblast PG fraction III by chondroitinase AC revealed a protein with high molecular weight (>200 kDa) that is absent both in control without enzymatic treatment and in the enzyme sample and which seems to be myoblast PG core protein (Fig. 2, lane *2*).

Electrophoresis of PG fraction IV from culture medium after enzymatic treatment in 3-15% gradient gel revealed two protein bands evidently corresponding to PG core proteins: a large band with molecular weight ~ 40 kDa and a band with molecular weigh >300 kDa (Fig. 3a, lane *2*). Treatment of this fraction with chondroitinase AC with subsequent electrophoresis in 3-7% gradient polyacrylamide gel (Fig. 3b) led to results similar to the those obtained after treatment of this fraction with chondroitinase ABC: diffuse staining disappeared (Fig. 3b, lane *1*) and new protein bands including a major one in the region of 40 kDa appeared (Fig. 3b, lane *2*).

Previously, using chromatography on Q-Sepharose, we showed that FBS PGs are the prevailing PGs in the culture medium [6]. This circumstance required comparative electrophoresis of PGs from culture medium and FBS to determine which PGs are synthesized by myoblasts into the medium. Figure 4 depicts the electrophoresis of fraction IV from culture medium and FBS containing the majority of PGs before and after enzymatic treatment with chondroitinase ABC. In the case of PGs from FBS, enzymatic treatment resulted in disappearance of a band in the region of 130-140 kDa (Fig. 4, lanes *1* and *2*) and in the appearance of one new band (in addition to enzyme bands). Enzymatic cleavage of culture medium PGs resulted in two new bands arising (Fig. 4, lanes *3* and *4*). The band with molecular weight of 100 kDa, which is present in both samples, obviously corresponds to serum PG core protein. A lower molecular weight protein in the region of 40 kDa appearing only in the sample of culture medium seems to be core protein of a PG synthesized by myoblasts into the culture medium.

Mass spectrometric identification of proteoglycans. PG core proteins detected after enzymatic cleavage of carbohydrate chains and subsequent electrophoresis were analyzed by MALDI-TOF mass spectrometry. Core proteins of major PGs from ECM and culture medium were identified (Figs. 1-4, protein bands 1-5).

Mass spectrometric analysis of core proteins of ECM PGs reliably showed that protein bands 1 and 2 with apparent molecular weights 320 and 280 kDa (Fig. 1) are core proteins of versican, a large chondroitin sulfate PG from fibroblasts (table). A fragment of versican core protein with molecular weight 74.4 kDa was identified in both cases. Using BLAST software (http://blast.ncbi.nlm.nih.gov) amino acid sequence of this fragment was compared with all versican splicing forms (V0, V1, V2, and V3). The analyzed fragment had 100% homology with the smallest versican isoform – V3 consisting only of globular domains G1 and G3 that lack GAG chains. The analyzed fragment overlaps with other isoforms, including in addition to globular end domains also central chondroitin sulfate-containing domains [1] in the region of globular domains G1 and G3.

Mass spectrometric analysis of myoblast PG core protein (Fig. 2, lane *2*) did not lead to any reliable data possibly because the analyzed sample was a protein mixture.

Results of mass spectrometric identification of major proteoglycan core proteins in myoblast culture

Number of protein band	PG (core protein)	Identification code in NCBI database	Molecular weight, Da	Mascot Score	Queries matched	Sequence coverage, %
1	*Similar to* [*Segment 1 of 2*] *Versican core protein precursor* [*Rattus norvegicus*]	gi|109465806	74 426	110	14	21
2	*Similar to* [*Segment 1 of 2*] *Versican core protein precursor* [*Rattus norvegicus*]	gi|109465806	74 426	76	12	19
3	*Biglycan* [*Rattus norvegicus*]	gi|1346706	41 613	111	11	33
4	*Procollagen, type XII, alpha 1* [*Rattus norvegicus*]	gi|109485046	338 589	141	46	23
5	*Inter-alpha globulin inhibitor H2 polypeptide* [*Bos taurus*]	gi|148238273	102 821	287	34	33

Analysis of PG core proteins from culture medium reliably revealed three main PGs. They are biglycan (core protein with molecular weight ~40 kDa, Figs. 3 and 4, protein band 3), collagen XII α-1-chain (core protein with molecular weight >300 kDa, Fig. 3a, protein band 4), heavy chain (H2) of inter-α-trypsin inhibitor (core protein with molecular weight ~100 kDa, Fig. 4, protein band 5).

DISCUSSION

During previous studies of PGs from L6J1 myoblast culture, it was shown that chondroitin/dermatan sulfate PGs is a dominant class of these compounds [6, 7]. We attempted to identify the major chondroitin sulfate PGs in L6J1 myoblast culture using mass spectrometry, which together with specific antibodies is widely applied for identification of PGs [10-12]. Four PGs were identified: biglycan, collagen XII, and inter-α-trypsin inhibitor in culture medium and versican in ECM.

The biglycan belongs to the family of "slrps" (small leucine reach PGs) and can be represented by two glycoforms carrying one or two chains of chondroitin/dermatan sulfates [1]. During electrophoresis these glycoforms are detected in the regions of 120-140 and 200-250 kDa [13, 14]. The biglycan detected by us is an electrophoretically very diffuse band in the region of 120-200 kDa (Fig. 4, lane *4*), which corresponds to the glycoform with one carbohydrate chain. The band at the very top of the gel in the region of 250 kDa may represent a second glycoform of biglycan. Therefore, biglycan isolated from culture medium PG fraction IV seems to be represented by two glycoforms. The biglycan is classified as a chondroitin/dermatan sulfate PG [1]. Nevertheless, chondroitinase AC specific only to chondroitin sulfates as well as chondroitinase ABC degrading both chondroitin sulfates and dermatan sulfates effectively cleave this PG. Both enzymes result in formation of biglycan core protein with molecular weight ~40 kDa (Fig. 3, protein band 3). Perhaps this indicates low content of iduronic acid in biglycan GAG chains, which identifies dermatan sulfate class [1] and indicates that biglycan from L6J1 rat myoblasts is predominantly chondroitin sulfate PG.

Collagen XII exists as two splicing forms – "short" and "long" [15]. The "long" collagen XII form is a "part-time" PG containing one chondroitin sulfate chain, while the "short" form does not carry GAG chains. Collagen XII belongs to the FACIT (fibril-associated collagens with interrupted triple helices) class including nonfibrillar collagens associated with collagen fibrils and participating in their correct assembly [1]. Collagen XII consists of three α-1-chains coiled into a triple helix on the C-end region of the molecule [1]. Molecular weights of these chains for two collagen XII forms differ significantly: ~340 kDa for the "long" form and ~220 kDa for the "short" form [16]. According to the literature data, collagen XII in L6J1 myoblasts, which was found during electrophoresis in the region >300 kDa, is the "long" form and therefore belongs to PGs. The intensity of collagen XII α-1-chain staining (Fig. 3b, protein band 4) is significantly lower than staining of the band corresponding to biglycan core protein (Fig. 3b, protein band 3), which indicates its lower content in culture medium in comparison with biglycan.

Inter-α-trypsin inhibitor (ITI) is known to be a component of human blood plasma [17, 18]. ITI contains three different proteins – two heavy chains (H1 and H2 for human) and bikunin covalently bound by one general chondroitin sulfate chain [18]. Bikunin functions as inhibitor of proteases while the function of the heavy chains is unclear [18]. It is known that ITI heavy chains

covalently bound to hyaluronic acid are found in different tissues in some pathologies [17, 18]. It has also been shown that a complex containing only two proteins – H2 and bikunin bound by chondroitin sulfate – is located in human blood plasma together with a full-sized inhibitor molecule [19]. This complex gives a band approximately corresponding to molecular weight 130 kDa under SDS-PAGE analysis [19]. And treatment of the complex with chondroitinase ABC leads to the disappearance of this band and emergence of bikunin and heavy chain H2 bands. We observed a similar picture for FBS PG fraction IV. The band in the region of 130-140 kDa (Fig. 4, lane *1*) faded away, and a new band in the region of 100 kDa (Fig. 4, lane *2*, protein band 5) appeared after cleavage of this fraction by chondroitinase ABC. To some extent it repeats in culture medium PG fraction IV except for the band in the region of 130 kDa, which is not detected due to its screening by biglycan (Fig. 4, lane *3*). In spite of the fact that the bikunin band (~30 kDa) is not detected in FBS and culture medium fractions after enzymatic treatment and electrophoresis, one can state that the H2–bikunin–chondroitin sulfate complex is the third PG from culture medium fraction IV.

Versican belongs to the class of chondroitin sulfates [1]. There are four splicing variants of the versican gene for human, mouse, and bull: V0, V1, V2, and V3 with molecular weights of core proteins of 370(366), 260(262), 182(178), and 72(74) kDa [1, 20], respectively (data for human (mouse) versican). The core protein molecular weights determined by electrophoresis are significantly larger for isoforms V0, V1, and V2 (400, 500, and 550 kDa, respectively) [21, 22] and virtually coincide with theoretical ones only for isoform V3 (~80 kDa) [21]. The authors believe that this discordance can be explained by high degree of N- and O-glycosylation of core proteins in isoforms V0, V1, and V2. Versican synthesized by L6J1 myoblasts appeared as two bands in the region of molecular weights of 320 and 280 kDa during electrophoresis after deglycosylation. These molecular weights are smaller than those obtained experimentally by the authors [21, 22] for versican isoforms V0, V1, and V2. Accordingly, it can be supposed that the noted proteins are the fragments of core proteins of these isoforms. We found the versican electrophoretic separation to be in good agreement with the one obtained for versican isolated from bull tendons [23]. Using antibodies against versican, those authors detected several protein bands with molecular weight >220 kDa, which further were indicated as metabolites of versican isoforms V0, V1, and V2.

Mass spectrometric analysis showed that three identified PGs (biglycan, versican, and collagen XII) have "rat" origin while the inter-α-trypsin inhibitor has "bull" origin (table). This fact indicates that biglycan, versican, and collagen XII are synthesized by L6J1 myoblasts and inter-α-trypsin inhibitor is a component of FBS, which determines its presence in the culture medium. The cells excrete biglycan into the culture medium, while versican is incorporated in ECM, which probably reflects different functions of these PGs. Biglycan is present in trace amounts (compared to the other PGs) in muscle tissue of adult organisms, but its expression increases significantly during regeneration of skeletal muscles [13]. Versican predominates in skeletal muscles during early stages of embryonic myogenesis and during regeneration of damaged muscles [5, 24]. These data may indicate that biglycan and versican are synthesized mainly by myoblasts rather than by differentiated muscle fibers. The functional role of versican and biglycan in muscle tissue regeneration is unclear. Versican belongs to the hyaluronan family, which possesses the important property of interacting with hyaluronic acid [1]. Hyaluronic acid is a main component of cellular glycocalyx (it is associated with its receptor CD44 on the cellular surface). Therefore, ECM versican can act as a connective molecule between ECM and cells. Interestingly, hyaluronic acid is localized mainly on the surface of myoblasts and to only a small extent on the surface of myotubes [25]. These data may suggest that hyaluronic acid together with versican is more important for regulation of myoblasts behavior than for providing life-sustaining activity of differentiated muscle fibers. Perhaps these complexes of versican with hyaluronic acid play a certain role in regulation of myoblast mobility and proliferative activity – characteristics, which distinguish myoblasts from myotubes. Chondroitin sulfate PGs, especially versican, promote myoblast and fibroblast migration [26, 27].

Biglycan excreted by myoblasts into the culture medium may be important in the context of interaction with growth factors. Biglycan binds TGF-β and may be able to modulate its activity in relation to myoblast proliferation [7, 28]. Another member of the "slrps" family, decorin, was detected in skeletal muscles together with biglycan. Decorin has high homology with biglycan and partially repeats its ability to interact with TGF-β and to participate in collagen fibrillogenesis [1, 24]. These two PGs crucially differ only by the presence of additional chondroitin/dermatan sulfate chain in molecule of biglycan [1]. But in contrast to biglycan, which is synthesized mainly by myoblasts, decorin is synthesized by myotubes or muscle fibers [29]. Decorin is considered as a modulator of TGF-β activity. This PG stimulates proliferative action of the factor in non-differentiated muscle cells and inhibits its action in myotubes [30, 31]. The role of biglycan has not been determined so well. Muscle tissue regeneration is almost undisturbed in mice deficient in biglycan, though they are susceptible to osteoporosis [13]. Moreover, an increase in decorin synthesis was detected in skeletal muscles of these mice, which is regarded as a possible mechanism of compensation of biglycan loss. In addition, the interaction between biglycan and dystroglycan (a component of dystrophin–protein complex) was described, which indicates possible participation of bigly-

can in stabilizing this complex that binds cytoskeleton to basal membrane and is essential for muscle tissue integrity [32].

Therefore, the following chondroitin sulfate PGs from L6J1 rat myoblast culture were identified during our study: versican, biglycan, and collagen XII synthesized by myoblasts and inter-α-trypsin inhibitor derived from FBS, a cell culture medium component.

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