methods

Sandwich immunoassay for the measurement of murine syndecan-4

Vincent Rioux, Reiko Y. Landry, and André Bensadoun[1]

Division of Nutritional Sciences, Savage Hall, Cornell University, Ithaca, NY 14853

Abstract A sensitive and specific immunoassay for mouse syndecan-4 is described. The assay is a non-competitive direct sandwich enzyme-linked immunosorbent assay based on the production of an affinity-purified polyclonal antibody directed against syndecan-4 extracellular domain, used both as a trapping and detecting antibody. The standard curve is constructed with recombinant 6-His-tagged syndecan-4 extracellular domain. The assay allowed quantification of syndecan-4 core protein in the partially purified proteoglycan fraction from adipocyte 3T3-F442A cells, as well as in the cellular whole and clarified lysates. Removal of glycosaminoglycan chains on syndecan-4 core protein is required for maximal epitope exposure. The standard curve ranged between 7 and 70 fmol per well. For the 14 standard curves run on different days, the absorbance at 490 nm for 35.5 fmol of recombinant syndecan-4 was 0.610 ± 0.110 (n = 14) with a corresponding blank absorbance of 0.089 ± 0.030. At low (5.7 fmol) and high (42.8 fmol) levels of whole cell syndecan-4, the intra-assay and inter-assay coefficients of variation were 4.9 and 15.3 percent, and 3.3 and 9.7 percent, respectively. A survey of the syndecan-4 expression in various mouse tissues shows that syndecan-4 is highly expressed in the kidney, brain, testes and liver, but can also be measured in several different adipose tissue sites.—Rioux, R., R. Y. Landry, and A. Bensadoun. **Sandwich immunoassay for the measurement of murine syndecan-4.** ***J. Lipid Res.*** **2002.** 43: **167–173.**

Supplementary key words enzyme-linked immunosorbent assay • quantification • syndecan-4 • heparan sulfate proteoglycans • adipose tissue

Syndecan-4 is a widely expressed transmembrane heparan sulfate proteoglycan (HSPG) (1). The syndecan family of HSPGs contains four members (syndecan-1 to syndecan-4) with homologous transmembrane and cytoplasmic domains, and distinctive extracellular domains (1, 2). Their structure is characterized by hybrid glycosylation with long unbranched glycosaminoglycan (GAG) chains, containing heparan sulfate (HS) and chondroitin sulfate (CS) chains (3). The length of GAG chains and the degree of sulfation are not uniform, resulting in considerable heterogeneity in molecular weight and charge (4). Because of the polyanionic nature of GAG chains, syndecans interact with numerous extracellular ligands, thereby affecting a large spectrum of biological activities in the cells (1, 2, 5). These activities include modulation of growth factor activities, anticoagulation, cell adhesion, extracellular chaperone activities, and lipoprotein metabolism. Syndecans are also known to be shed from the surface of epithelial and endothelial cell lines and by a proteolytic cleavage near the plasma membrane that releases their extracellular domain outside of the cell (6, 7). In addition to the activity shared by all syndecans, syndecan-4 can bind and activate protein kinase C (8). The recent generation of syndecan-4 deficient mice (9) demonstrated that, in cultured fibroblasts, focal adhesion formation is inhibited only in specific conditions. Syndecan-4 deficiency was also shown to result in increased degeneration of fetal vessels in the placenta (10) and in delayed wound repair (11).

Syndecan-4 mRNA expression has been studied during mouse embryogenesis (12) and measured in various tissues and cultured cells by Northern blot analysis (13–15), semi-quantitative RT-PCR analysis (15–17) and in situ hybridization (10, 14). Due to the relative complexity of its structure, syndecan-4 core protein expression has been less studied than mRNA expression, although western blotting analyses (15, 17, 18) and immunohistochemistry (10, 14, 19) have been performed. Quantitative measurements of syndecan-4 protein have been complicated by the presence of GAG chains, poor solubility in the absence of detergents, and the propensity for the core protein to self-associate (1).

In the present study, a specific sandwich immunoassay has been developed for the accurate determination and quantification of the mouse syndecan-4 in adipocyte 3T3-F442A cell and other mouse tissue extracts. The assay is based on the production of an affinity-purified polyclonal

Abbreviations: CL, clarified lysate; CS, chondroitin sulfate; GAG, glycosaminoglycan; HRP, horseradish peroxidase; HS, heparan sulfate; HSPG, heparan sulfate proteoglycan; PBS-T, PBS-0.05% Tween 20; PVDF, polyvinylidene difluoride; syndecan-4 ED, syndecan-4 extracellular domain; WL, whole lysate.

[1] To whom correspondence should be addressed.
e-mail: ab55@cornell.edu

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antibody directed against the extracellular domain of the mouse syndecan-4. The ELISA described here is very sensitive and repeatable, and shows for the first time that syndecan-4 is expressed in adipocyte cells. Using this method, we examined the distribution of syndecan-4 in various mouse tissues.

MATERIALS AND METHODS

3T3-F442A cell culture

The 3T3-F442A mouse pre-adipocytes were obtained as a generous gift from Dr. H. Green (20). The cells were cultured in DMEM containing 10% calf serum, 2 mM L-glutamine and 10 mM HEPES, at 37°C in 10% CO_2, until 2 days after reaching confluence. The cells were then treated to induce their differentiation with DMEM containing 10% fetal bovine serum, 5 μg/ml insulin, 2 mM L-glutamine and 10 mM HEPES.

Production of the recombinant 6-His-tagged syndecan-4 extracellular domain

The mouse syndecan-4 cDNA was obtained by a combination of PCR and hybridization screening of a mouse 3T3-L1 cDNA library in the λZAP vector (Stratagene, La Jolla, CA) generously provided by Dr. Daniel Lane. The library was amplified and the lysates from ten 150 mm dishes were analyzed by PCR with the following primers 5′-CCTCCCGGACGATGAAGAC-3′, and 5′-AGGAAAACGGCGAAGAGGATGC-3′. The sequences of these oligonucleotides were taken from the published rat syndecan-4 sequence (21). Following two rounds of PCR screening, positive clones were isolated by screening of the positive lysates with a random primed α-^{32}P-labeled probe. The probe consisted of the PCR product generated by the two oligonucleotides listed above. The longest clone isolated extended from nucleotide-107 to 1219 in which the 25th nucleotide is the translation start site. The syndecan-4 cDNA sequence between nucleotide 118 and 435 (22) was subcloned into pQE30 (Qiagen, Valencia, CA) and transfected into DH5α Escherichia Coli. Following culture and centrifugation of the transformed E. Coli, the bacterial pellet was lysed in 6 M guanidine-HCl, 0.02 M Tris-HCl, 0.1 M NaCl, pH 8.0. The lysate was sonicated six times at 100 W, stirred for 30 min at room temperature and clarified by centrifugation (12,000 g, 10 min, 4°C). The supernatant was then mixed with Talon metal affinity resin (Clontech, Palo Alto, CA). After 1 h of shaking at room temperature, the resin was washed six times with 8 M Urea, 0.02 M Tris-HCl, 0.01 M NaCl, pH 8.0. The recombinant 6-His-tagged protein was then eluted with a gradient of imidazole (0 to 100 mM) in 8 M Urea, 0.02 M Tris-HCl, 0.01 M NaCl, pH 8.0. The recombinant protein has 129 amino acids. The sequence includes a 12 amino acid extension on the amino-terminus (MRGSHHHHHHGS) of the protein and 11 residue addition at the C-terminus (SRVDLQPSLIS). The calculated molecular mass is 14.03 kDa.

Affinity-purified antibody against mouse syndecan-4 extracellular domain

The anti-syndecan-4 antibody was generated by immunizing a rabbit with the recombinant 6-His-tagged syndecan-4 extracellular domain (syndecan-4 ED). In order to purify the immunoglobulins, 5 mg of the recombinant 6-His-tagged syndecan-4 ED were coupled to Affi-prep 10 support (BioRad, Hercules, CA) to generate an affinity column. Anti-serum was diluted 4-fold in the equilibrium buffer (0.2 M Tris, 0.5 M NaCl, pH 8.0) and applied to the affinity column. After washing the column with the equilibrium buffer, the immunoglobulins were eluted with 0.2 M glycine, 0.5 M NaCl, pH 2.8. The collected samples were pooled, dialyzed overnight against PBS containing 0.02% NaN_3 and concentrated.

Partial purification of HSPGs from 3T3-F442A cells

3T3-F442A cells were used at different stages of their differentiation. Most of this work was performed on cells collected 12 h after the addition of the differentiation medium. The medium was taken off, the cell layers were rinsed twice with ice-cold PBS and harvested with the aid of a rubber policeman in PBS. Cell suspensions were centrifuged at 800 g for 10 min. Cells from five dishes (100 mm) were usually pooled for each experiment. One ml of lysis buffer (0.75 M NaCl, 10 mM HEPES, 1 mM EDTA, 1% Triton X-100) containing a protease inhibitor mixture (final concentrations, 1 μg/ml leupeptin, 1 μg/ml antipain, 10 μg/ml benzamidine, 10 IU/ml trasylol, 1 μg/ml chymostatin, 1 μg/ml pepstatin A) was added and the cell pellet was sonicated two times successively (20 s, 100 W) to obtain a whole cellular lysate (WL). The lysate was cleared by centrifugation at 10,000 g for 30 min. After removing the fat layer and pellet, the cellular clarified lysate (CL) was diluted with 4 volume. of H_2O to obtain a final concentration of 0.15 M NaCl, and applied to a DEAE-sephacel column (bed volume, 0.5 ml) equilibrated in 0.15 M NaCl, 1 mM EDTA, 10 mM Tris-HCl, pH 7.4, 0.1% Triton X-100. After washing with several column volumes, bound proteoglycans were eluted with 4 M guanidine-HCl, 1 mM EDTA, 50 mM sodium acetate, 0.6% CHAPS, pH 6.0 (23). The partially purified HSPGs from the cells (cell HSPGs), and the cellular WL and CL fractions obtained previously were dialyzed overnight against 50 mM NaCl, 4 mM $CaCl_2$, 20 mM Tris, pH 7.4, and freeze-dried. The concentrated samples were resuspended in 250 μl heparitinase buffer (3 mM Ca acetate, 10 mM EDTA, 10 mM N-ethylmaleimide, 10 mM HEPES, pH 7.0) containing protease inhibitor mixture. Triton X-100 (0.05% final concentration) was added in the cell HSPG fraction. The protein contents were determined (24) and 50 μl of the samples were incubated at 37°C for 4 h in a mixture of 80 mU chondroitin sulfate ABC lyase (chondroitinase ABC, EC 4.2.2.4; Sigma, St. Louis, MO) and 5 mU heparin sulfate lyase (heparinase III, EC 4.2.2.8; IBEX Technologies, Inc., Montreal, Canada).

SDS-PAGE and Western blot

Samples from 3T3-F442A cells were diluted in Laemmli sample buffer, separated by SDS-PAGE on 10% acrylamide resolving gels, and transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore, Bedford, MA). After blocking in PBS containing 0.05% (w/v) Tween 20 (PBS-T) and 5% (w/v) non-fat dry milk for 2 h at room temperature, the membrane was incubated overnight at 4°C in the same solution supplemented with 0.1 μg/ml affinity-purified antibody against syndecan-4 ED. The membrane was then washed with PBS-T and incubated for 2 h at room temperature in PBS-T containing 5% non-fat dry milk and 1:9000 diluted goat anti-rabbit IgG conjugated to peroxidase (BioRad). After washing with PBS-T, the secondary antibody was detected by chemiluminescence (West-Pico, Pierce, Rockford, IL). Molecular weights were estimated using prestained standards (Sigma Chemical Company, St. Louis, MO) and the protein marker (cat #BOA001, MoBiTec, Marco Island, FL).

ELISA procedure for mouse syndecan-4

A microtiter plate (Nunc, Roskilde, Denmark) was coated overnight at 4°C with 1 μg per well of affinity-purified polyclonal antibody against syndecan-4 ED, diluted in 200 μl 0.1 M carbonate-bicarbonate buffer pH 9.6, containing 0.02% (w/v) NaN_3. After three washes with PBS-T, the wells were blocked with 300 μl of 1% (w/v) BSA in PBS-T for 2 h at 37°C. The recombinant 6-His-

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tagged syndecan-4 ED was diluted with 1% BSA, 2 M NaCl, 0.05% Tween 20, 10 mM phosphate pH 7.4, to generate standard solutions containing 7.1 to 71 fmol syndecan-4 ED per 200 μl. Cell extracts from 3T3-F442A adipocytes were diluted in the same solution. After three washes with PBS-T, 200 μl standards and samples were added per well and incubated overnight at 4°C. The affinity-purified antibody, conjugated to horseradish peroxidase (HRP) as previously described (25), was diluted (1:5000) in 1% BSA, PBS-T. After three washes with PBS-T, 200 μl of the diluted conjugate were added per well and incubated overnight at 4°C. Following six washes with PBS-T, 200 μl substrate solution (0.4 mg/ml o-phenylenediamine in 0.01% H_2O_2, 0.1 M citrate-phosphate buffer, pH 5.0) was added to each well. After incubation at room temperature for 15 min, the reaction was stopped by addition of 50 μl 2.5 M H_2SO_4 to each well, and absorbance was read at 490 nm.

Extraction from tissues

Tissues from three-month-old male mice (strain 129) were homogenized in an extraction solution containing 4 M guanidine-HCl, 1% (v/v) Triton X-100, 1 mM EDTA, 50 mM Na actetate, 1 mM PMSF, 10 mM N-ethylmaleimide, 1 μg/ml leupeptin, 1 μg/ml antipain, 10 μg/ml benzamidine, 10 IU/ml trasylol, 1 μg/ml chymostatin, 1 μg/ml pepstatin A, pH 6.0. The homogenates were stirred at 4°C for 4 h. Insoluble residues were removed by centrifugation at 2,000 g, and the supernatants were dialyzed overnight against 50 mM NaCl, 4 mM $CaCl_2$, 20 mM Tris, pH 7.4. The samples were then concentrated, resuspended in heparitinase buffer and deglycosylated by the addition of chondroitinase ABC and heparinase III, as previously described above for 3T3-F442A cell extracts.

RESULTS

Specificity of the affinity-purified antibody against syndecan-4 extracellular domain

The specificity of the affinity-purified anti-syndecan-4 antibody was first examined by western-blotting analyses on samples from 3T3-F442A cells harvested 12 h after exposure to differentiation medium. The cellular whole and clarified lysates (WL and CL) and the partially purified HSPGs from cells (cell HSPGs) were separated by SDS-PAGE, before or after deglycosylation with heparinase III and chondroitinase ABC. **Figure 1** shows that after digestion of the GAG chains with both enzymes, the antibody yields in Western blots a single band at about 32,000, either with the partially purified cell HSPGs, the CL fraction, or the WL fraction. When GAG chains are not released, no immunoreactive bands are observed. This may be due to the broad spectrum of molecular weights of native syndecan-4 and the low efficiency of the transfer of these molecules to Immobilon-P membranes. With polyclonal antibodies directed against the extracellular domain (14) or the cytoplasmic domain (26) of mouse syndecan-4, molecular weights of approximately 35,000, higher than the predicted 21,500, were reported. It is unlikely that the antibody recognizes other syndecans since syndecan-1, syndecan-2, and syndecan-3 give protein bands on SDS-PAGE migrating at an Mr of 80,000, 48,000 and 120,000, respectively (27). The bands in Fig. 1 are somewhat broad. It is unlikely that this is due to incomplete digestion of GAG chains, since the recombinant protein produces also a broad band.

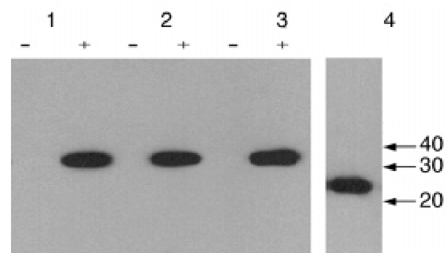

Fig. 1. Western blotting analyses of syndecan-4 in 3T3-F442A cells. Proteins from whole lysate (53.2 μg, lane 1), clarified lysate (37.4 μg, lane two), partially purified cell HSPGs (10.0 μg, lane three), and the recombinant syndecan-4 ED (0.5 ng, lane four) were subjected to SDS-PAGE and analyzed by Western blot using the affinity-purified syndecan-4 antibody. Each cellular sample was either treated with heparinase III and chondroitinase ABC (+) or left untreated (−). Molecular weights of standard protein markers are indicated at the right.

To verify that the bands seen in Fig. 1 represent syndecan-4, a whole lysate sample from 3T3-F442A cells (100 μg of protein) and the recombinant syndecan-4 (1 ng) were separated on SDS-PAGE and transferred to Immobilon-P membrane as described in Materials and Methods. The membrane was stained with Ponceau S and each lane was cut in half. After blocking the membrane, the right side of each lane was incubated overnight with 0.05 μg/ml anti-syndecan-immunoglobulins and the left side was incubated with the same concentration of antibodies supplemented with 5 μg/ml recombinant syndecan-4. The competition was very effective. The portions of the membrane (**Fig. 2**, lanes one and three) incubated with excess syndecan-4 showed no detectable bands. These results show that the antibody is specific and that the removal of GAG chains

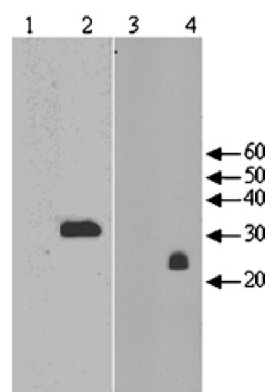

Fig. 2. Specificity of the anti-syndecan-4 immunoglobulins. A deglycosylated whole lysate sample from 3T3-F442A cells (100 μg protein, lanes 1and 2) and recombinant syndecan-4 (1 ng, lanes 3 and 4) were subjected to SDS-PAGE and transferred to Immobilon-P membrane as described in Materials and Methods. The membrane was stained with Ponceau S and each lane was cut in half. The right side of each sample (lanes two and four) was incubated overnight with 0.05 μg/ml anti-syndecan-4 antibody and the left sides (lanes one and three) with the same incubation solution supplemented with 5 μg/ml recombinant syndecan-4 ED. Incubations with the secondary antibody were carried out as described in Materials and Methods.

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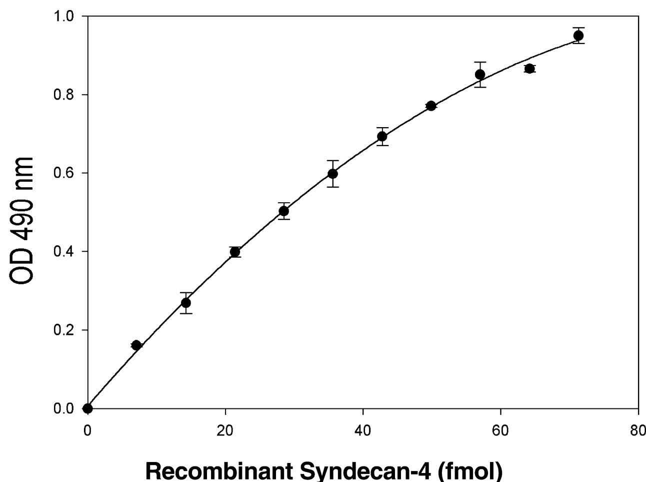

Fig. 3. Standard curve obtained by ELISA for the recombinant 6-His-syndecan-4 extracellular domain. The wells were coated with 1 μg anti-syndecan-4 antibody and then incubated with increasing amounts of 6-His-tagged syndecan-4 extracellular domain polypeptide. Each point represents the mean ± SD of four replicates. The generated curve was fitted to a quadratic equation.

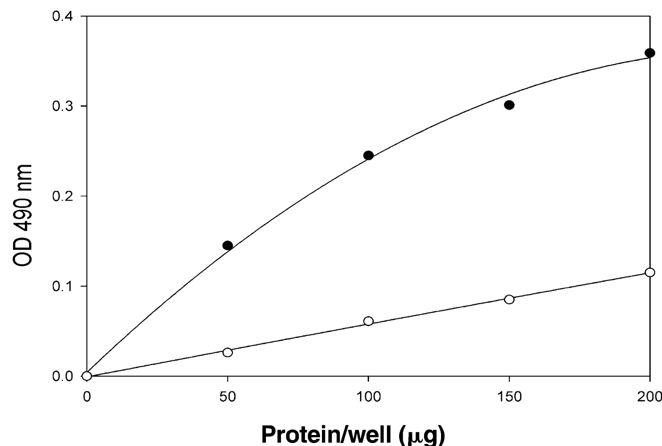

Fig. 4. Syndecan-4 protein trapped in two successive incubations. Wells of an ELISA plate were coated with 1 μg anti-syndecan-4 antibody and incubated with increasing amounts of proteins from a deglycosylated whole lysate sample from 3T3-F442A cells. After 24 h incubation, the samples were removed from the first plate and transferred to a second plate. Both plates were developed as described in Materials and Methods. Closed triangle: first incubation; open circle: second incubation.

on syndecan-4 ED is required for maximal exposure of epitopes to the antibody.

Optimal ELISA conditions

For calibration of the ELISA, the recombinant 6-His-tagged syndecan-4 ED polypeptide was used as a standard. The conditions for the assay (amount of the trapping antibody, antigen concentration and dilution of the detecting antibody conjugated to HRP) were established by various titrations. A typical standard curve obtained with the assay, for amounts of recombinant 6-His-tagged syndecan-4 ED ranging from 7.1 to 71 fmol, is presented in **Fig. 3**. The standard curve was adequately fitted by a quadratic equation. The mean r^2 for 14 standard curves was 0.998 ± 0.001 with an OD_{490} of 0.610 ± 0.110 for 35.5 fmol of recombinant syndecan-4. A coating concentration for anti-syndecan-4 antibody of 1 μg IgG per well and a dilution of 1:5000 for the HRP-conjugated antibody were enough for maximum color development and minimal blank values. Various salt concentrations (0.15 M to 2.0 M NaCl) were also evaluated for standard and sample preparation. A high salt level of 2 M NaCl did not affect the absorbance (data not shown) and was therefore chosen to decrease the potential non-specific binding. The average chemical blank value (n = 14) was 0.089 ± 0.030.

Amount of syndecan-4 trapped in the ELISA

Wells in an ELISA plate were coated with 1 μg of anti-syndecan-4 immunoglobulins and incubated with increasing amounts of proteins from a deglycosylated whole lysate sample from 3T3-F442A cells. After 24 h of incubation at 4°C, the samples were removed from the first plate and transferred on a second plate coated as the first plate. The two plates were developed as described in Materials and Methods. **Figure 4** shows that in the range of proteins loaded, between 80% and 85% of the total syndecan-4 in the sample is trapped and detected after the first development. Increasing the amount of anti-syndecan immunoglobulins in the wells above 1 μg/well does not increase the yield. This is illustrated in **Fig. 5** where the amount of antibody was varied between 0.5 μg and 2 μg. The samples analyzed were deglycosylated whole lysate, clarified lysate, and partially purified HSPGs.

Analysis of native syndecan-4 in 3T3-F442A cell extracts

Antigen saturation curves were constructed using the conditions described above, with increasing amounts of samples from 3T3-F442A cells, after or without deglycosylation with heparinase III and chondroitinase ABC. The

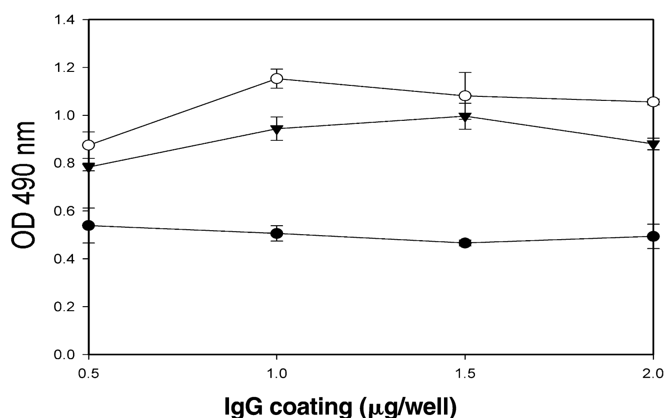

Fig. 5. Effect of increasing amounts of trapping anti-syndecan-4 antibody. An ELISA plate was coated with increasing amounts of anti-syndecan-4 antibody and incubated with deglycosylated proteins from whole lysate (266 μg/well, closed inverted triangle), clarified lysate (187 μg/well, open circle) and partially purified HSPGs (30 μg/well, closed circle) from 3T3-F442A cells. Each point represents the mean and standard deviation of three replicates.

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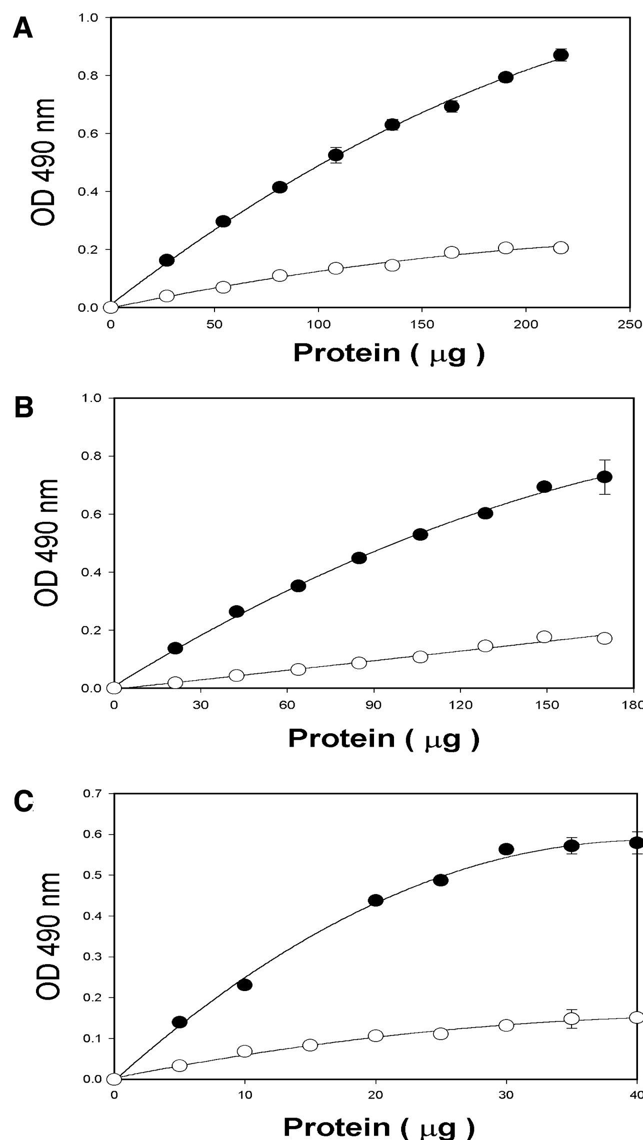

Fig. 6. Assay of syndecan-4 in different fractions from 3T3-F442A cells whole lysate (A), clarified lysate (B), partially-purified HSPGs (C). The wells were coated with 1 μg anti-syndecan-4 antibody and then incubated with increasing amounts of each sample. Samples were either treated with heparinase III and chondroitinase ABC (closed circle) or analyzed directly (open circle). Each point represents the mean ± SD of triplicate assays. All curves were fitted with quadratic equations.

heparitinase buffer and enzymes did not contribute to the absorbance at 490 nm. **Figure 6** shows a good correlation between the total amounts of proteins and the syndecan-4 detected in each analyzed cellular fraction (WL, CL, and cell HSPGs). In each of the fractions tested, deglycosylation with heparinase III and chondroitinase ABC greatly increased the absorbance. To further increase the absorbance, we also tried to pre-incubate the standards and samples in the presence of 10 mM SDS for 1 h at 37°C, before performing the assay at a final concentration of 1 mM SDS. However, this treatment had no effect on the absorbance (data not shown).

Precision and reproducibility of the assay

In order to measure the repeatability of the assay, samples from 3T3-F442A cells with low and high amounts of total protein were analyzed several times. At low (5.7 fmol) and high (42.8 fmol) levels of whole cell syndecan-4, the intra- and inter-assay coefficients of variation were 4.9% and 15.3%, and 3.3% and 9.7%, respectively (**Table 1**). These results show that the optimized sandwich ELISA is highly reproducible in the range of the standard curve. The sensitivity limit of the assay, defined as twice the background value, is less than 7 fmol syndecan-4 per well.

Deglycosylation conditions

As shown above, the optimal binding between the antigen and the antibody requires the removal of the GAG chains. A kinetic study (**Fig. 7**) shows that deglycosylation of a partially purified cell HSPG fraction from 3T3-F442A adipocytes with heparinase III and chondroitinase ABC yielded maximal absorbance value after 10 min incubation. When the same extract was incubated with heparinase III only, the maximal absorbance value was reached more slowly (Fig. 7). Finally, the incubation of the same cell HSPG fraction with only chondroitinase ABC did not result in recognition of the antigen by the antibody (data not shown).

Determination of syndecan-4 amount in various adult mouse tissues

Using this assay, we examined the distribution of syndecan-4 in various mouse tissues (**Fig. 8**). When expressed as ng per mg of tissue protein, the greatest abundance of syndecan-4 was found in the kidney, followed by the brain, testes, and

TABLE 1. Reproducibility of the ELISA for measurement of syndecan-4

Fraction	Protein/well	Intraassay CV (n = 5)	Interassay CV (n = 5)
	μg	%	%
Cellular whole lysate	27.1	4.9 (5.7 fmol)	15.3 (6.1 fmol)
	216.8	3.3 (42.8 fmol)	9.7 (46.0 fmol)
Cellular clarified lysate	21.2	3.5 (4.7 fmol)	10.0 (4.7 fmol)
	169.6	11.2 (35.2 fmol)	9.9 (40.5 fmol)
Cell HSPGs	5.0	2.5 (5.3 fmol)	5.8 (5.0 fmol)
	40.0	6.2 (29.1 fmol)	10.8 (26.7 fmol)

Inter-assay and intra-assay coefficients of variation (CV) are calculated from five replicates for each deglycosylated fraction from 3T3-F442A cells. The calculated amounts of syndecan-4 are indicated in parentheses.

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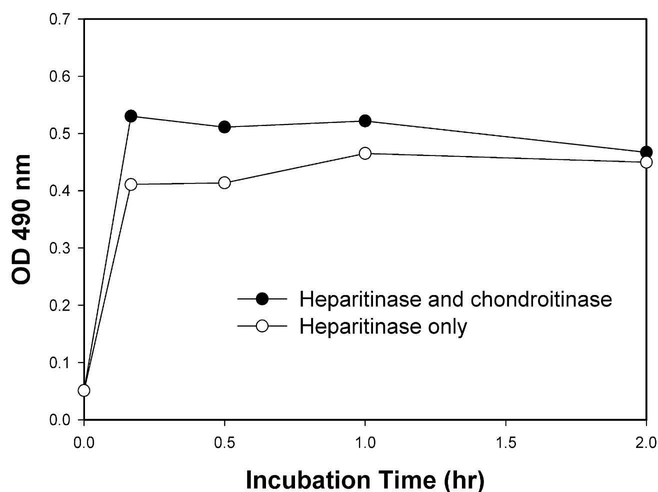


Fig. 7. Kinetic study of syndecan-4 deglycosylation as a function of incubation time with heparinase III and chondroitinase ABC (closed circle) or heparinase III only (open circle). Each point represents the average of two observations, which differed on average by less than 10%.

liver. Other tissues, such as the spleen, lung, stomach, and intestine, showed intermediate amounts. Syndecan-4 was diversely expressed in different adipose sites. For each of these tissues, western blotting analyses were carried out to verify that a single band at about 32,000 was detected with the anti-syndecan-4 antibody (data not shown).

DISCUSSION

We have developed a sandwich ELISA for mouse syndecan-4, using a polyclonal antibody directed against the extracellular domain of the transmembrane protein. This region of the protein, with the exception of the GAG attachment sites (1, 2), displays low sequence homology among the syndecans. In Western blot analyses of 3T3-F442A cells and of other tissues examined, the affinity purified syndecan-4 antibodies yielded a single band at a molecular weight of 32,000, similar to values previously reported in the literature. This apparent molecular weight is distinct from those reported previously for syndecan-1, syndecan-2, and syndecan-3; 80,000, 48,000 and 120,000, respectively (27). The aberrant electrophoretic mobility of syndecan-4 may be due to incomplete removal of GAG chains. However, immunoblotting performed with the recombinant 6-His-tagged syndecan-4 ED (free of GAG chains) also revealed an aberrant migration (Fig. 1). The anomalous migration of syndecan-4 core protein, even after deglycosylation of the GAG chains, was intensified when 6 M urea was added to acrylamide gels (data not shown).

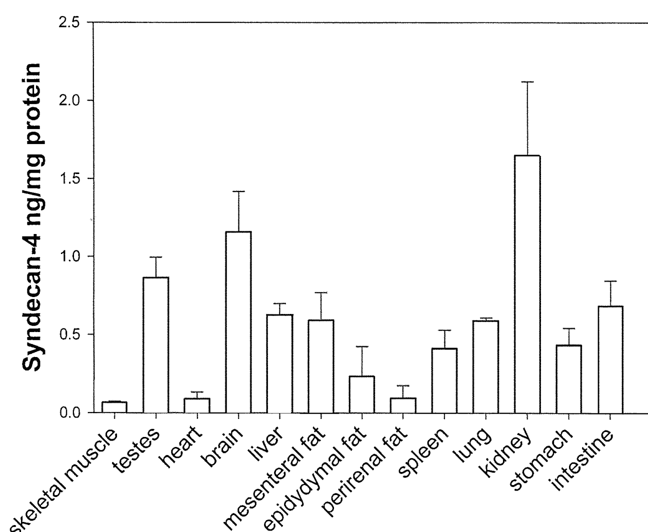
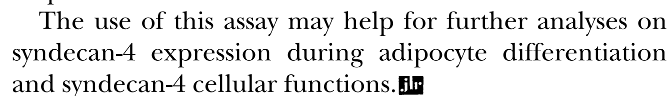


Fig. 8. Syndecan-4 content of various mouse tissues. Each point represents the mean ± SD of measurements made on three male mice (129 strain).

The optimized sandwich ELISA is highly reproducible in the range of the standard curve, and amounts from 7.1 to 71 fmol syndecan-4 per well can be detected. Western blotting (Fig. 1) and ELISA analyses (Fig. 6) show that syndecan-4 core proteins can be detected in the partially purified proteoglycan fraction from 3T3-F442A cells, as well as in the whole and clarified lysate. The data demonstrate that the affinity-purified antibodies can detect syndecan-4 epitopes after exposure to Triton X-100 and 4 M guanidine-HCl.

The enzymatic degradation of GAG chains using chondroitinase ABC (which splits N-acetylhexosamine β1-4 uronic acid linkage) and heparinase III (which splits N-sulphated hexosamine α1-4 uronic acid linkage) was found to be necessary for full expression of syndecan-4 epitopes (Figs. 6, 7). Considering that the potency of commercial sources of heparitinase may be variable and that manufacturers define units of heparitinase differently, it is essential that the optimum amounts of heparitinase and chondroitinase be determined for each batch of enzymes employed. Our results (Fig. 7) confirmed that GAG chains of mouse syndecan-4 from adipocyte cells contain mainly HS chains but also CS chains (3, 28).

Northern blot analyses have shown that syndecan-4 mRNA is abundant in the liver, kidney, and ovary, of intermediate amount in the brain and lungs, and low in the heart, skeletal muscles, skin, and small intestine (13, 14). Our results (Fig. 8) on syndecan-4 core protein expression in various mouse tissues are consistent with the literature and confirm that syndecan-4 is a widely expressed proteoglycan. The values given for various tissues should be considered semi-quantitative since the amounts of heparitinase and chondroitinase ABC were optimized only for adipose cells.



The use of this assay may help for further analyses on syndecan-4 expression during adipocyte differentiation and syndecan-4 cellular functions.

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