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Expression and Characterization of Minican, a Recombinant Syndecan-1 with Extensively Truncated Core Protein

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Syndecan-1 is an integral membrane heparan sulfate/chondroitin sulfate proteoglycan, involved in the control of cell growth and differentiation. The biological activities of syndecan-1 involve interactions with a variety of extracellular ligands, such as growth factors and matrix components, that are mainly mediated by the heparan sulfate moieties. The expression of syndecan-1 is downregulated in various malignant tumors, and low levels of expression appear to correlate with poor prognosis of some cancer types. On the other hand, the extracellular portion of syndecan-1 (ectodomain) has been demonstrated to inhibit the proliferation of various cancer cells in culture, suggesting that proteoglycan-like molecules should be studied further with regard to their antitumor activities. We have expressed, in CHO cells, a truncated syndecan-1 ectodomain ("minican") harboring domains for glycosaminoglycan attachment and antibody recognition. Analysis of recombinant minican indicates that it shares some of the biochemical and biological characteristics attributed to syndecan-1 ectodomain. Minican was thus substituted with heparan sulfate chains and bound to extracellular matrix proteins as well as fibroblast growth factors. Notably, minican inhibited the proliferation of S115 mouse mammary carcinoma cells and the effect seemed to involve inhibition of the Ras/Erk signaling pathway. Our data suggest that recombinant syndecan-1 with a minimal protein component is biologically active. This information may provide useful in further design of proteoglycan-like antitumor molecules. © 2002 Elsevier Science

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Syndecans are a family of four genetically distinct integral membrane proteoglycans substituted with heparan sulfate (HS) and chondroitin sulfate (CS) glycosaminoglycan chains (1, 2). The most well known member of this family, syndecan-1, has been proposed a role in biological processes such as cell differentiation, adhesion, and proliferation (3–5). These biological activities are thought to critically depend on HS-mediated interactions of syndecan-1 with a number of extracellular proteins, including components of the extracellular matrix (1, 2) and fibroblast growth factors (FGFs) (5–7).

Syndecan-1, originally cloned from mouse mammary epithelial cells (NMuMG) (8), has been implicated in the maintenance of normal cell growth and phenotype of epithelial cells (4, 9, 10). In carcinomas, the expression of syndecan-1 is generally downregulated and shows inverse correlation with the outcome of breast cancer as well as carcinomas of the head and neck region (11, 12). Moreover, in murine breast carcinoma cells (S115), which undergo malignant transformation when treated with testosterone, the transformation is associated with downregulation of syndecan-1 expression, whereas S115 cells that express syndecan-1 under a testosterone-sensitive promoter retain their syndecan-1 expression and untransformed phenotype in spite of testosterone treatment (13). The mechanisms by which syndecan-1 would counteract malignant transformation are not completely understood, but may involve inhibitory action on growth factor signals that promote tumor cell proliferation. In S115 cells, the testosterone-dependent transformation is thought to involve induction of FGF-8 expression (14),

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and in 3T3 fibroblasts, high levels of syndecan-1 expression have been shown to inhibit the cellular responses toward FGF-2 (15). Interestingly, soluble syndecan-1 ectodomain (i.e., the extracellular portion of the core protein containing the glycosaminoglycan moieties) inhibits growth of S115 cells and other tumor cells *in vitro*, whereas the corresponding effect is not achieved with syndecan-1 without HS chains (16) or free heparin (17). In addition, multiple HS chains of syndecan seem to be required to inhibit invasion of myeloma (ARH-77) cells into collagen gels (18). These findings suggest that a proteoglycan structure with multiple heparan sulfate chains clearly differs from syndecan-1 core protein or from free heparin/HS chains in its biological activity, and that these differences might be crucial for the antitumor effect of syndecan-1.

The purpose of this study was to investigate whether minican, expressed in Chinese hamster ovary (CHO) cells by cDNA transfection, would retain the biological activity characteristic of syndecan-1 ectodomain, in particular with regard to the ability to inhibit tumor cell proliferation. Potentially, factors such as the truncated core protein structure and "artificial" expression in transfected cells might influence the biological activity of the proteoglycan, for instance by altering its glycosaminoglycan substitution. Syndecan-1 ectodomain contains two clusters of glycosaminoglycan attachment sites, one toward the end of the N-terminus and the other at the C-terminal end of the ectodomain, close to the plasma membrane (1). Generally, the distal sites seem to favor assembly of HS, presumably due to the arrangement of amino acid sequences flanking the serine-glycine residues (17). Therefore, a 5-kDa segment of syndecan ectodomain containing the distal glycosaminoglycan attachment sites was selected as the "core protein" of minican.

Our results demonstrate that HS containing minican can be expressed CHO cells, a commonly used host for production of recombinant proteins. The minican thus produced shows biological activities comparable to syndecan-1 ectodomain in terms of protein binding and inhibition of S115 cell proliferation.

MATERIALS AND METHODS

Cell culture. S115, NMuMG and CHO cells were purchased from ATCC. Cells were cultured in plastic cell culture flasks or roller bottles. S115 cells were grown in Dulbecco's modified essential medium (DMEM, Sigma) supplemented with 5% fetal calf serum (FCS, Gibco), 1 mM L-glutamine, 2 mM sodium pyruvate, 10^{-9} M testosterone and penicillin and streptomycin. NMuMG cells were grown in the same medium without L-pyruvate and testosterone containing 10% FCS. CHO cells were grown in α-MEM (Gibco) containing 5% FCS and the same supplements as the medium used for NMuMG cells. The media of the transfected cells were supplemented with 250 μg/ml Geneticin (G418, Sigma). For metabolic labeling of the proteoglycans, the transfected cells were grown for three days in media supplemented with [^{3}H]GlcN (10 μCi/ml).

Cloning of minican. The cloning of the minican construct was done by PCR with mouse syndecan-1 cDNA as a template using a 5′-primer (AACCGGCAACTCGGATCCACGAAG) and a 3′-primer (CGTGGCTGTCAACAGTCACACGTC). The resultant PCR product (a 350 bp fragment corresponding to residues 196–448) was cleaved with *Pst*I at residue 292 and *Hin*cII at residue 448 yielding a 150-bp fragment. The fragment was subcloned into *Pst*I/*Stu*I-cleaved mouse syndecan-1 cDNA, resulting in a 1200-bp fragment. The sequences of the constructs were confirmed by dideoxy sequencing. The cloning of the syndecan-1 ectodomain has been described previously (15).

Transfections. Cells were transfected with 5 μg of vector or control DNA using the $CaPO_4$ method (19). Cells surviving selection with 750 μg/ml G 418 were harvested and clones were screened for expression of recombinant ectodomain or minican by Western analysis of conditioned media using the monoclonal antibody 281-2 against murine syndecan-1 (mAb 281-2) (8).

Northern analysis of minican transfectants. Confluent cells (10 cm plates) were washed with ice-cold PBS and the cells were lysed in 500 μl 4 M guanidinum isothiocyanate, pH 4.5, on ice. RNA was purified using phenol/chloroform extraction (20) after which samples (15 μg) of RNA were run on a reducing gel. The RNA was transferred onto a Hybond-N^+ nylon membrane (Amersham) in 20× SSC. The membrane was dried and the RNA was immobilized on the membrane with UV light. The membrane was prehybridized with a solution containing 1 M NaCl, 0.1% SDS, 10% dextran sulfate and denatured herring testis sperm DNA (0.2 mg/ml) for 3–4 h, and probed with $^{32}PO_4$-labeled PM-4 (21), a partial cDNA of mouse syndecan-1, overnight at +65°C. The membrane was washed with 1× SSC and 0.1× SSC containing 0.1% SDS at +65°C and exposed to Kodak X-Omat film at −70°C.

Western blots. Proteoglycans were purified from conditioned media by chromatography on a column of DEAE-Sephacel (Pharmacia) as described previously (21). Aliquots of the DEAE-purified proteoglycans were subjected to digestion with heparitinase I (EC 4.2.2.8, 10 mU/ml) and chondroitinase ABC lyase (EC 4.2.2.4, 250 mU/ml) (Seikagaku Corp. Tokyo, Japan) separately or in combination. The samples were combined with Laemmli's sample buffer, containing β-mercaptoethanol, heated to +95°C for 5 min and run on a 3–15% SDS–PAGE. The proteoglycans were transferred to a Hybond N^+ nylon membrane, fixed in 0.025% glutaraldehyde and the membranes were blocked using 5% instant fat-free milk powder in PBS containing 0.4% Tween 20. Immunoreactive material was detected with mAb 281-2 on Kodak X-Omat films using an ECL kit (Amersham).

Purification of recombinant proteoglycans for biological assays. Conditioned media were collected and applied to a column of DEAE-Sephacel. The column was extensively washed with 0.2 M NaCl, 0.05 M sodium acetate, pH 4.5 followed by elution of the bound material with 1 M NaCl, 0.05 M sodium acetate, pH 4.5. The proteoglycans were precipitated with 3 volumes of ethanol (+4°C overnight) and pelleted by centrifugation. The pellets were dissolved in 0.25 M Tris, 4 M guanidium hydrochloride. CsCl was added to reach the density of 1.4 g/ml and the solutions were centrifuged at 100,000*g* for 72 h at +18°C. The contents of the centrifuge tubes were fractionated and an aliquot of each fraction was subjected to protein measurement and slot-blot immunoassay with mAb 281-2. Appropriate fractions were pooled and diluted with 20 volumes of H_2O followed by another DEAE chromatography step and ethanol precipitation. Following centrifugation at 25,000*g* for 1 h, the formed pellet was dissolved in PBS and the solution was run through a CNBr immobilized mAb 281-2 Sepharose column. The column was washed with PBS and the 281-2 bound material was eluted with 50 mM triethylamine, pH 11.5. The pH of the eluates was adjusted to 7.0 with Tris–HCl. The eluates were pooled and subjected to DEAE chromatography and ethanol precipitation. The proteoglycans were dissolved in H_2O and sterilized by passage through a 0.22-μm filter. The glycosaminoglycan content of the purified proteoglycans was determined by mea-

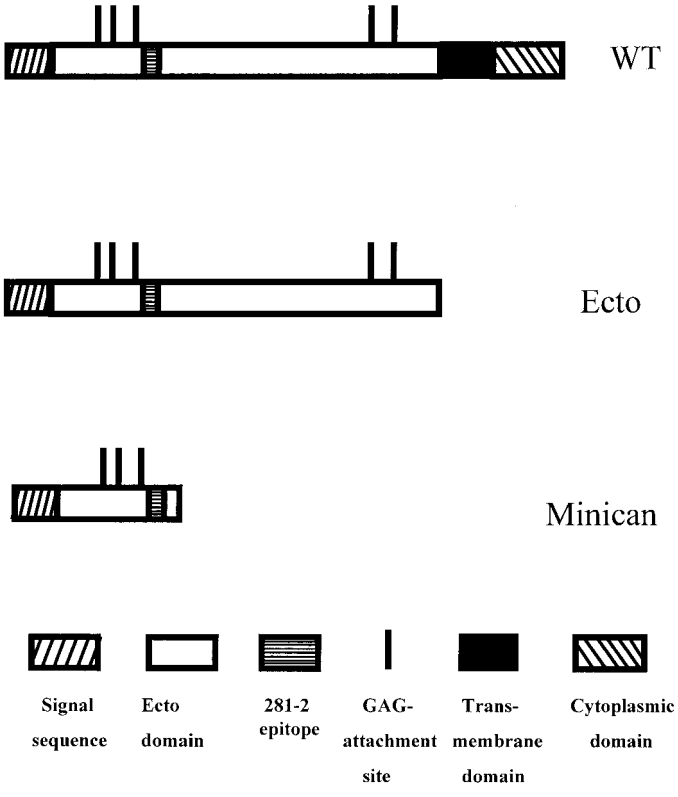

FIG. 1. Syndecan constructs. A schematic presentation of the domain structure of syndecan-1 and the ectodomain and minican constructs.

suring the uronic acid concentration using the phenyl-phenol method (22, 23).

Protein binding assay. In order to assess the binding of ectodomain and minican to FGFs and ECM proteins a nitrocellulose membrane assay with immobilized proteins was performed as described earlier (24). The membranes with immobilized proteins were incubated overnight with 2 μg/ml of immunaffinity purified proteoglycans at +4°C. The membranes were washed repeatedly with PBS, treated with 0.025% glutaraldehyde for 5 min, and washed with PBS. Bound proteoglycan was detected with mAb 281-2.

[^{3}H]Thymidine labeling of cells. S115 cells were plated on 24-well plates at a density of 100,000 cells/well and serum starved in DMEM containing 4% dextran-coated charcoal (DCC)-filtered FCS for three days after which the cells were kept in serum-free 50% HAM/50% DMEM for 24 h. The medium was changed to fresh HAM/DMEM and the cells were treated with proteoglycans/heparin (1 μg/ml) in the presence or absence of testosterone. Following a 24-h incubation, 1 μCi of [^{3}H]thymidine was added to each well for 2 h. The cells were washed three times with cold PBS, suspended into 100 μl 1 M NaOH and the suspension was transferred to 24-well plates for counting in a β-counter.

Analysis of ERK phosphorylation. S115 cells were serum starved and treated with testosterone and proteoglycans or heparin as described above. After a 10-h treatment, the cells were lysed into 100 μl 2× Laemmli buffer and the lysates were sonicated and run on a 10% SDS–PAGE. The proteins were transferred onto a nitrocellulose membrane (Schleier and Schuell). Phosphorylation of Erk 1/2 was detected with antibodies against phospho-Erk 1/2 (Sigma) whereas the sample loading was assessed by immunoblotting with anti-Erk2 antibody (Transduction Laboratories).

Disaccharide analysis. Proteoglycans were purified from unlabeled cells or cells metabolically labeled with [^{3}H]GlcN using DEAE chromatography and mAb 281-2 immunoaffinity chromatography as described above. The purified proteoglycans were subjected to treatment with nitrous acid at pH 1.5 (25). At this pH the reagent cleaves HS at N-sulfated GlcN residues. The unlabeled HS were labeled by reduction with NaB^3H_4. The cleavage products were chromatographed on a column of Sephadex G-15 (1 × 190 cm) in 0.2 M NH_4HCO_3 and fractions corresponding to disaccharides were pooled and desalted by centrifugal evaporation. The disaccharides were analyzed on a Partisil-10 SAX column eluted by a step gradient of KH_2PO_4. The disaccharide peaks were identified by comparing their elution positions with those of standard heparin disaccharides.

RESULTS

Expression of minican. To investigate the glycosylation and biological properties of recombinant syndecan ectodomain and minican, they were expressed in CHO and NMuMG cells by cDNA transfection. The ectodomain construct contained the entire extracellular part of syndecan-1 whereas the minican construct encompassed the N-terminal residues 1–68 including the distal glycosaminoglycan attachment site and the epitope for mAb 281-2 (Fig. 1). Northern analysis of wild type CHO cells or cells transfected with vector revealed two endogenous mRNA species (3.4 and 2.6 kb). The minican transfected cells revealed, in addition to the two endogenous mRNA species, a third band of 1.2 kb indicating expression of the minican construct (Fig. 2).

Minican is substituted with heparan sulfate. Proteoglycans were purified from conditioned media of cells transfected with the ectodomain or minican constructs by anion exchange chromatography, subjected to SDS–PAGE and assessed for reactivity with the mAb 281-2 by immunoblotting. Western analysis of

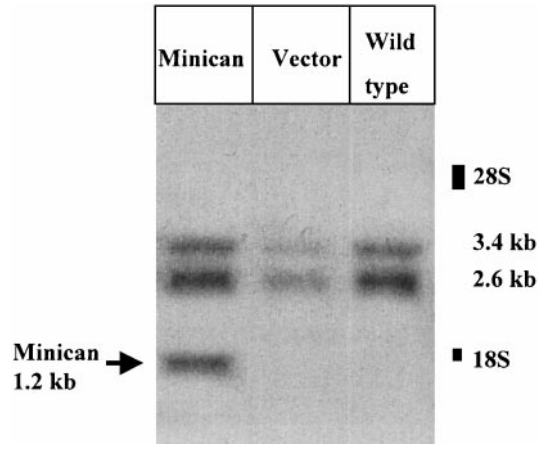

FIG. 2. Analysis of minican mRNA expression. RNA from wild-type or vector/minican transfected CHO cells were hybridized with mouse syndecan-1 cDNA probe. The 1.2-kb signal corresponds to the size of minican mRNA, whereas the 2.6- and 3.4-kb bands represent the two mRNA species of endogenous syndecan-1. The migration positions of 28S and 18S rRNA are indicated by vertical bars.

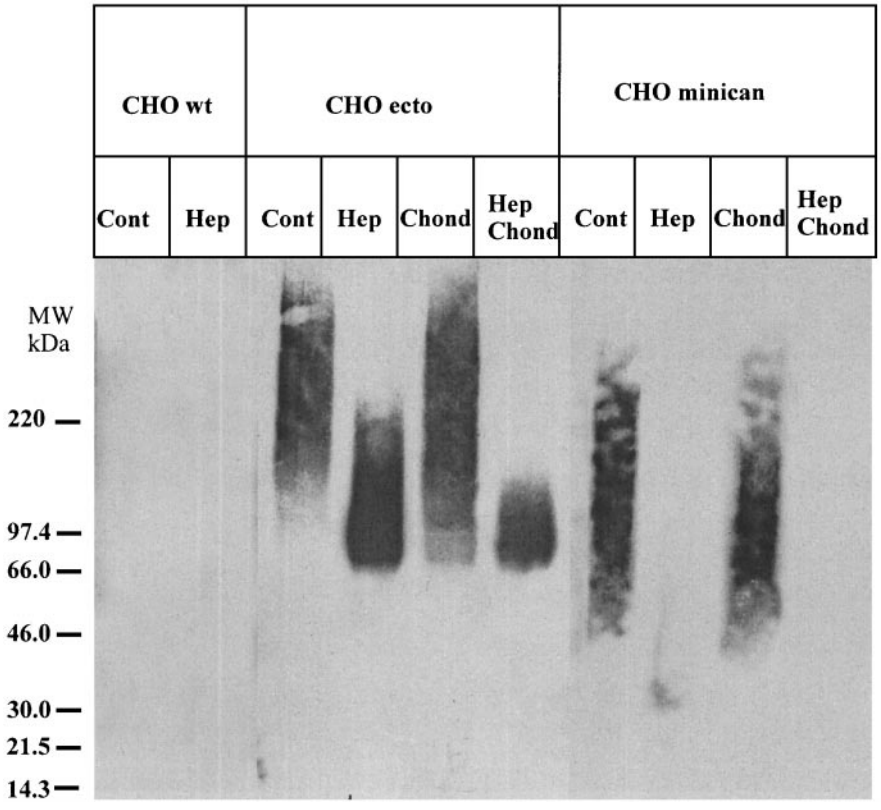

FIG. 3. SDS–PAGE analysis of minican and its glycosylation. Proteoglycans from the conditioned media of wild-type and minican transfected CHO cells treated with heparitinase (Hep) and/or chondroitinase ABC (Chond). The samples were run on 3–15% gradient SDS–PAGE followed by detection of minican by Western blotting with mAb 281-2.

wild type CHO cells did not reveal immunoreactivity because the mAb 281-2 does not recognize hamster syndecan-1 (Fig. 3, right lanes). By contrast, cells transfected with the minican construct were found to secrete mAb 281-2 reactive proteoglycan to their culture medium. (Fig. 3, left lanes). Minican appeared highly heterogeneous in size, with an average molecular weight of ~150 kDa. Treatment with chondroitinase had little or no effect on the electrophoretic mobility of minican, whereas heparitinase treatment abolished the minican smear almost completely. These results indicated that whereas the syndecan-1 ectodomain expressed in NMuMG cells is a hybrid proteoglycan containing both HS and CS (26, 27), minican contained almost exclusively HS.

Analysis of heparan sulfate O-sulfation. We next studied the O-sulfation of minican HS. Minican-transfected CHO cells were metabolically labeled with [^{3}H]GlcN. Proteoglycans were isolated and their HS moieties were subjected to deaminative cleavage with nitrous acid. The resultant disaccharides, representing HS sequences of consecutive N-sulfated disaccharide units, were recovered by gel chromatography and separated by strong anion exchange chromatography. The disaccharide species were identified by comparing their elution positions to those of heparin-derived disaccharide standards. The major sulfated disaccharide constituents of minican HS were the mono-O-sulfated IdoA(2-OSO_3)-GlcNSO_3 unit and the di-O-sulfated IdoA(2-OSO_3)-GlcNSO_3(6-OSO_3) unit (Fig. 4), which represented >60 and ~30% of the total O-sulfated disaccharide units, respectively. For comparison, we analyzed the O-sulfation pattern of HS from endogenous syndecan-1 of NMuMG cells. The results were largely similar to those obtained with minican HS, such that the major disaccharide species were the IdoA(2-OSO_3)-GlcNSO_3 and IdoA(2-OSO_3)-GlcNSO_3(6-OSO_3) units (Fig. 4).

Binding of minican to ECM proteins. Syndecan-1 is known to interact with components of the ECM and FGFs. To assess the binding of minican to such proteins we compared the binding properties of minican from CHO cells and syndecan-1 ectodomain from NMuMG cells. The protein binding properties of the latter syndecan-1 species have been characterized previously (28, 29). The extracellular proteins were immobilized on nitrocellulose filters, which were incubated in solution containing ectodomain or minican. The protein-bound proteoglycans were detected with mAb 281-2. Ectodomain and minican both showed

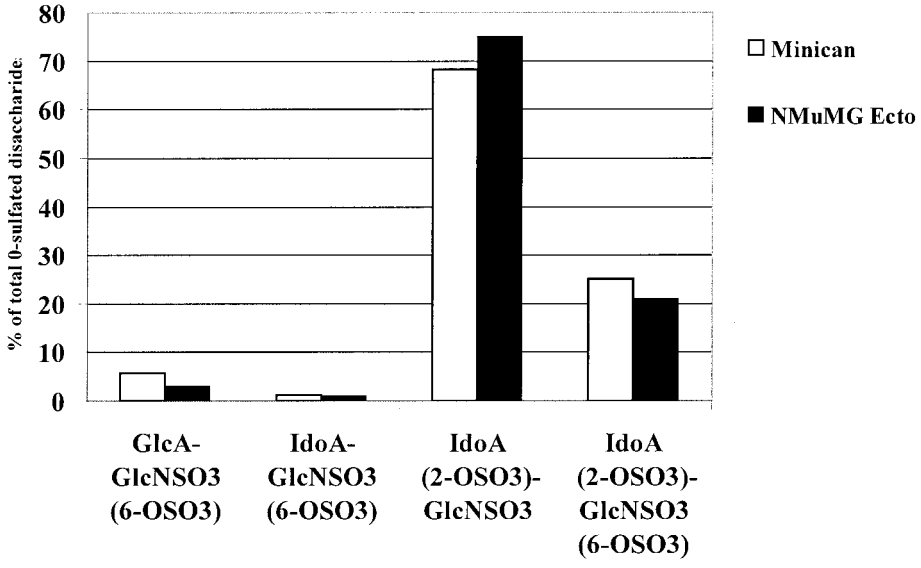

FIG. 4. Disaccharide composition of minican HS. HS from minican or syndecan-1 ectodomain from NMuMG cells (NMuMG ecto) was subjected to depolymerization by HNO_2 at pH 1.5. ^{3}H-labeled disaccharides from ectodomain or minican were recovered and subjected to strong anion exchange chromatography on a column of Partisil-10 SAX eluted with a step gradient of KH_2PO_4.

binding to collagen I, fibronectin and laminin but not to gelatin. Moreover, each of the tested proteoglycans bound FGF-2 and FGF-8b but failed to interact with FGF-1 (Fig. 5). These data suggest that syndecan-1 ectodomain and minican both have similar binding properties with regard to extracellular matrix components and FGFs.

Minican inhibits the testosterone-induced proliferation of S115-cells. Testosterone stimulation of S115 cells results in phenotypic transformation and highly enhanced proliferation, presumably due to testosterone-induced expression of FGF-8b (30). Previously, it has been shown that the testosterone-induced proliferation is only partially inhibited by exogenously added heparin or HS (17) whereas a much more efficient inhibition is achieved by treatment with syndecan-1 ectodomain (15). We thus applied immunopurified minican to the culture medium of testosterone-stimulated S115 cells and measured the incorporation of [^{3}H]thymidine into DNA. Testosterone treatment enhanced the incorporation ~20-fold compared to non-treated cells (Fig. 6). Minican inhibited the testosterone response by 80–90%, whereas heparin caused only ~25% inhibition of the [^{3}H]thymidine incorporation.

Given the findings that the testosterone-induced proliferation of S115 cells is mediated by FGFs acting in an auto-/paracrine fashion, we assessed the ability of minican to inhibit the phosphorylation of Erk. In testosterone stimulated cells, strong Erk 1/2 phosphorylation is seen 10 h after the start of the testosterone treatment, whereas untreated cells show no Erk phosphorylation (Fig. 7). The Erk phosphorylation in testosterone-treated cells was virtually abolished by

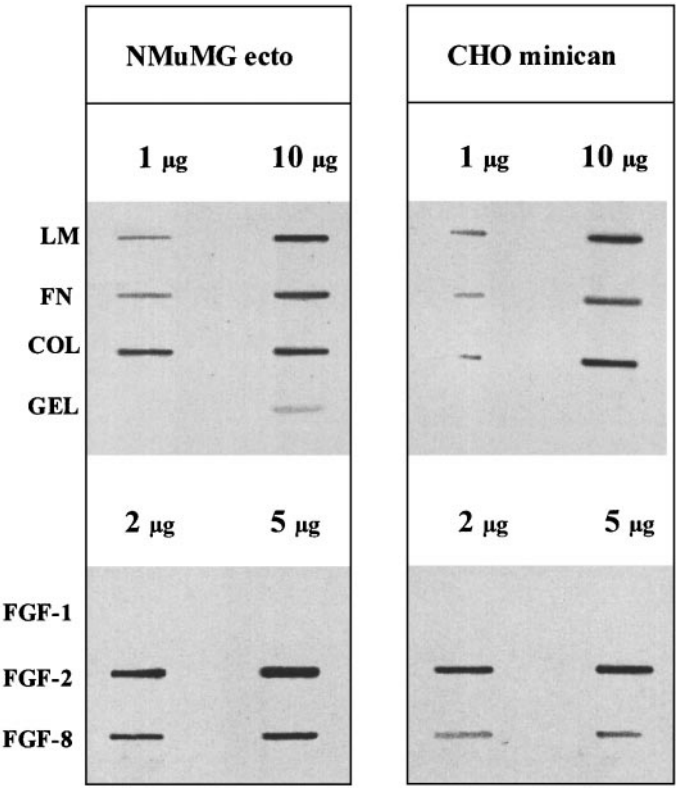

FIG. 5. Binding of syndecan-1 ectodomain and minican to proteins. Proteins were immobilized to nitrocellulose membrane using a slot blot apparatus. Unspecific binding sites were blocked with BSA and the membrane was incubated in PBS containing syndecan-1 ectodomain from NMuMG cells or minican from CHO cells. After washing the proteins were cross-linked with glutaraldehyde and the bound ectodomain/minican were detected by immunoblotting with mAb 281-2. LM, laminin; FN, fibronectin; COL, collagen I; GEL, gelatin.

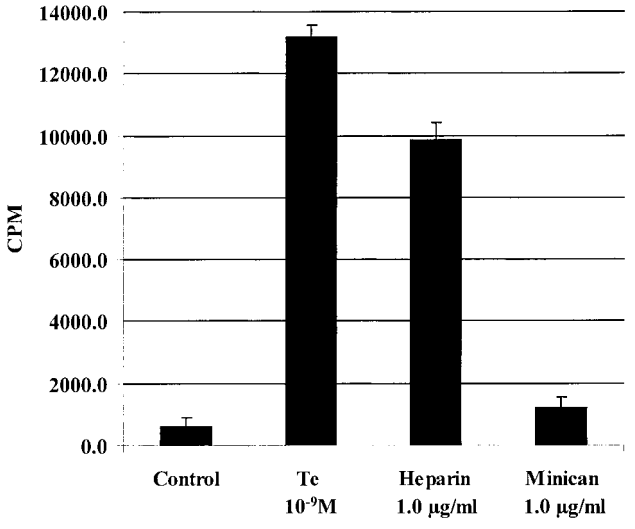

FIG. 6. Inhibition of testosterone-induced S115 cell proliferation by minican. Serum-starved S115 cells were stimulated with testosterone (Te) in the presence or absence of minican or heparin. Following a treatment of 24 h, the cells were assessed for [^{3}H]thymidine incorporation.

treatment with minican, whereas heparin had a somewhat weaker effect.

Together, these results indicate that minican can effectively inhibit the proliferation of transformed S115 cells, analogously to what has been previously demonstrated for the syndecan-1 ectodomain (16) and that inhibition of the Ras/ERK pathway is at least one of the mechanisms underlying such inhibition.

DISCUSSION

In this report we provide evidence that a novel proteoglycan construct ("minican"), containing a minor portion of the syndecan-1 core protein, shares many of the biochemical and biological activities attributed to the naturally occurring syndecan-1 ectodomain. Recombinant minican, expressed in CHO cells, was substituted with HS chains, bound to FGFs and matrix proteins, and inhibited the proliferation of S115 mammary carcinoma cells analogously to the endogenously expressed syndecan-1 ectodomain from NMuMG cells.

Exposure of S115 cells to testosterone induces expression of FGF-8 and perhaps also other species of the FGF family, and the transforming effect of testosterone can be inhibited by anti-FGF-antibodies (31). Moreover, we have recently demonstrated that the testosterone-induced transformation depends on HS, because treatment of S115 cells with sodium chlorate, an inhibitor of HS sulfation, renders the cells testosterone-unresponsive whereas the response is readily restored by addition of heparin (32). The antiproliferative activity of minican in the S115 cell model thus may be related to its ability to interfere with FGF signaling, because minican was able to interact with FGF-8 and FGF-2. In agreement with previous data, we found that minican inhibited the proliferation much more efficiently than heparin. A possible reason for this difference is that inhibition requires a multimeric arrangement of HS polymers, present in minican but not in heparin. In addition, minican HS is structurally different from heparin and may lack sequences that promote FGF signaling.

In addition to analyzing the antiproliferative effect of minican by the [^{3}H]thymidine incorporation assay, we also assessed its influence on the phosphorylation of ERK 1/2. ERK phosphorylation reflects the activation of the MAP kinase pathway as a result of signal transduction through FGF receptors (33). Whereas minican and syndecan-1 ectodomain virtually abolished the phosphorylation of ERK 1/2 in testosterone-treated S115 cells, also heparin treatment resulted in a marked downregulation of the phosphorylation. These findings suggest that in S115 cells, low level of ERK phosphorylation is still compatible with cell proliferation, because the latter parameter was only slightly decreased in heparin treated cells.

The glycosaminoglycan substitution of syndecan-1 appears to vary in a cell- and tissue specific manner. For instance, in NMuMG cells syndecan-1 contains a considerable proportion (~30%) of CS (26), whereas syndecan-1 from embryonic tooth mesenchyme carries almost exclusively HS chains (34). The glycosylation of syndecan-1 ectodomain expressed in CHO cells resembled the pattern seen in NMuMG cells. By contrast, minican seemed to contain only HS, in agreement with the findings that in intact syndecan-1 the HS chains are found at the distal glycosaminoglycan attachment sites whereas CS is incorporated into the proximal sites (27).

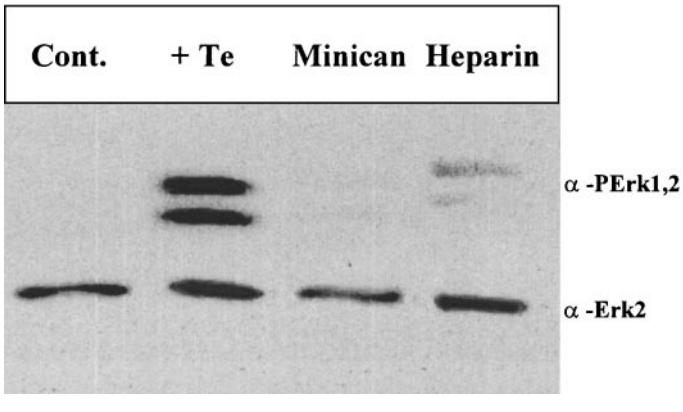

FIG. 7. Effect of minican on Erk phosphorylation. Serum-starved S115 cells were treated with testosterone in the presence of minican or heparin for 10 h. Cell lysates were analyzed for Erk 1/2 phosphorylation by immunoblotting with antibodies against phospho-Erk 1/2. The sample loading was assessed by immunoblotting with anti-Erk-antibodies.

In summary, the results of the current study demonstrate that recombinant proteoglycans with a minute core protein component can be successfully expressed in CHO cells. Such recombinant proteoglycans share many of the biological activities attributed to intact syndecan-1 ectodomain and may provide basis for the design of proteoglycan-like inhibitors of aberrant cell proliferation.

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