

Amino-terminal deletions in the decorin core protein leads to the biosynthesis of proteoglycans with shorter glycosaminoglycan chains

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Received 9 March 1996

Abstract Analysis of the N-terminal sequence of decorin purified from connective tissues and comparison with the sequence deduced from the cDNA indicate that the nascent proteoglycan has a 14 amino acid residue N-terminal propeptide. Mammalian expression vectors encoding wild-type decorin and decorin with deletions in the propeptide were used to transform COS and CHO cells. Cells transformed with vectors encoding deletion variants of decorin synthesize proteoglycans with shorter galactosaminoglycan chains than cells transformed with wild-type decorin. This effect on the polysaccharide chain length may be due to a lower affinity between the core protein and the glycosyltransferases synthesizing the linkage region. Alternatively, the deletions may affect the intracellular transport of decorin. An antiserum prepared against the N-terminal propeptide immunoprecipitated decorin secreted by cultured cells, showing that decorin is exported with the N-terminal region intact.

Key words: Decorin; Glycosaminoglycan biosynthesis

1. Introduction

Decorin is a proteoglycan synthesized by cells in most types of connective tissues [1]. The amino acid sequence suggests that the 37 kDa core protein of decorin is composed of a central domain containing leucine-rich repeats flanked by globular N-terminal and C-terminal domains [2,3]. The central domain represents some 60% of decorin and is composed of ten approx. 20 amino acid residue long repeats. Similar repeats, with preferentially leucine residues in conserved positions, are present in a number of extracellular matrix proteins; biglycan [4], fibromodulin [5], lumican [6], chondroadherin [7] and PRELP [8]. The leucine-rich repeats also occur in cellular proteins, such as the RNase inhibitor. The 3-D structure of the RNase inhibitor shows that the leucine-rich repeats are arranged in a parallel α -helix/ β -sheet coil structure where each repeat represents one turn [9]. Leucine-rich repeat domains are involved in protein interactions and the inhibitor binds to RNase via the leucine-rich repeat domain [10]. Also, decorin binds to collagen type I via leucine-rich repeats [11,12].

The N-terminal domain contains four cysteine residues forming intrachain disulfide bonds and a Ser-Gly sequence substituted with the single glycosaminoglycan chain of decorin. The polysaccharide chain is composed of repeated uronic acid-galactosamine disaccharides. The length, sulfation

(4- and 6-sulfation of GalNAc) and uronic acid composition (GlcUA and IdUA) of the galactosaminoglycan chains vary depending on their tissue origin [13]. Amino acid sequence analysis of decorin isolated from tissues like rat uterus and human placenta shows the N-terminal rat sequence DEASGIIP [14] and the human sequence DEASGIGP [15]. These sequences conform with the proposed recognition sequence for glycosaminoglycan attachment [16]. Comparison of these N-terminal sequences with the sequences deduced from cDNA indicates that decorin is synthesized as a precursor with a 14 amino acid residue long propeptide (Fig. 1). Previous investigations have suggested that this propeptide is removed intracellularly, before secretion of decorin [17]. In contrast, it has been shown that biglycan, which is structurally related to decorin and also synthesized as a precursor, is secreted from cultured smooth muscle cells with the propeptide intact [18].

In the present study we have prepared cDNAs encoding rat decorins with deletions in the N-terminal propeptide. After transfection of the constructs in mammalian cells, we studied the role of the propeptide in the glycanation and secretion of decorin.

2. Materials and methods

2.1. Decorin deletion constructs and expression in mammalian cells

Eucaryotic expression vectors were constructed using PCR and rat decorin cDNA ligated in the *EcoRI* site of pBluescript KS II (Stratagene, La Jolla, CA) as a template. For PCR amplifications we used the T3 primer (ATTAACCTCACTAAAG) and RDec. D3 primer (GAGGCCTCGTCTCTCTAATCCTCTCTGCTCAAATGG), RDec. D4 primer (GAGGCCTCGTCTCTCTAAGCCCAAGAGACTTGCGC) or RDec. D5 primer (GAGGCCTCGTCTCTAAGTCAAATAATCCTCTCTG). Amplifications were performed for 30 cycles in a thermal cycler using Taq DNA polymerase (Boehringer Mannheim, Germany), 95°C for 30 s, 55°C for 40 s and 72°C for 120 s. The PCR products were subjected to nucleotide sequence analysis to confirm their correct sequences. The decorin constructs were cleaved with *HindIII*/*StuI* and ligated to the decorin cDNA/pKS II vector from which the *HindIII*/*StuI* fragment had been removed. Finally, the decorin cDNA constructs were moved from the pKS II vector to pcDNA I neo (Invitrogen, San Diego, USA) as *HindIII*/*BamHI* fragments. This eucaryotic expression vector contains the SV40 origin of replication and the expression of the decorin constructs are controlled by the cytomegalovirus promoter.

The decorin constructs were transiently expressed in COS cells as previously described [19]. The COS cells were transfected using electroporation (300 V, 500 μ F in a Bio-Rad gene pulser), grown for 48 h in Dulbecco's modified Eagle's medium (Gibco, Gaithersburg, MD, USA) supplemented with 10% fetal calf serum and then labeled in serum-free medium containing 0.1 mCi/ml [35 S]sulfate.

CHO cell lines expressing the decorin constructs were prepared as described [20]. The CHO DG 44 cells were cotransfected with decorin cDNA/pcDNA neo expression vector and pSV2 dhfr, using the calcium phosphate procedure. Clones were selected in nucleotide-free medium containing 10 nM methotrexate. CHO cell lines expressing

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the decorin constructs were labeled with [35 S]sulfate as described above for COS cells.

2.2. Analysis of decorin and pulse-chase labeling

Proteoglycans secreted into the culture medium were immunoprecipitated with a rabbit antiserum against purified rat decorin (generous gift from Dr. D. Heinegård, Department of Cell and Molecular Biology, Lund University) as described [21]. The immunoprecipitates were analyzed by polyacrylamide gel electrophoresis in SDS (SDS-PAGE) [22]. Proteoglycan samples were subjected to alkaline β -elimination by incubation in 0.5 M NaOH, 0.05 M NaBH₄ for 16 h. Size determinations of glycosaminoglycan chains were performed on a Sepharose CL 6B column (1×150 cm) eluted with 0.2 M NaCl and molecular weights were estimated according to Wasteson [23].

CHO cells were subjected to pulse-chase analysis. Cells were pulse-labeled for 5 min in sulfate-deficient EMEM medium (Nord Vac, Sweden) containing 1 mCi/ml [35 S]sulfate. After three washes with F-12 medium (Gibco, Gaithersburg, MD, USA), the cells were chased for 10–90 min in F-12 medium. Secreted proteoglycans were immunoprecipitated and analyzed by SDS-PAGE. The amounts of 35 S-proteoglycans in polyacrylamide gels were determined using a Bio Imaging analyzer (Fuji Photo Film Co., Japan).

The Iduronic acid content in glycosaminoglycans was estimated by Bio-Gel P6 (BioRad) gel chromatography following digestion with chondroitinase AC II (Seikagaku Kogyo Co., Japan) [13].

2.3. Preparation of antiserum

A rabbit antiserum against the N-terminal region of rat decorin was obtained after immunization with a decorin/glutathione thiotransferase fusion protein. The cDNA encoding decorin was prepared by PCR, using the T7 primer (GTAATACGACTCACTATAGGGC), the 5'-primer (GGGATCCCCATGAAGGCAACTCTCGTC) and decorin cDNA/pKS II vector as template. After amplification, as described above, the PCR product was cleaved with *Bam*HI and *Stu*I and ligated with *Bam*HI/*Sma*I cleaved pGEX-3X (Pharmacia, Sweden). The resulting plasmid, encoding glutathione thiotransferase fused to the first 32 amino acid residues of rat decorin (Met-1–Glu-32 in Fig. 1), was used to transform *E. coli* UT 5600. Fusion protein was prepared as described [24]. Bacteria carrying the prokaryotic expression vector were induced with IPTG and lysed by sonication. The bacterial extract was applied to glutathione-Sepharose 4B, washed extensively and bound decorin/glutathione thiotransferase fusion protein was eluted with glutathione. The purified fusion protein was mixed with Freund's complete adjuvant and used to immunize a rabbit as described [15].

3. Results and discussion

3.1. Expression and analysis of decorin deletion constructs

Rat decorin mRNA is translated in the ER to a 354 amino acid residue long protein. The primary translation products include a 16 residue signal peptide and a 14 residue long sequence in the N-terminus, which is absent from decorin assembled in the extracellular matrix [14,15]. To study the function of the N-terminal 14 residues in the biosynthesis of decorin, we made four rat decorin cDNA constructs (Fig. 1). In addition to the wild-type Rat. Dec, the constructs encoded deletion variants of rat decorin; RDec. D3 is deficient in five amino acid residues (Gly-24–Met-28), RDec. D4 lacks 12 residues (Gly-15–Met-28) and RDec. D5 is missing two residues (Phe-27–Met-28). The constructs were ligated to a eucaryotic

Rat. Dec.	M K A T L V L F L L A Q V S W A G P F E Q R G L F D F M L E D E A S G
RDec.D3	M K A T L V L F L L A Q V S W A G P F E Q R G - - - - - L E D E A S G
RDec.D4	M K A T L V L F L L A Q V S W A - - - - - - - - - - - L E D E A S G
RDec.D5	M K A T L V L F L L A Q V S W A G P F E Q R G L F D - - L E D E A S G

Fig. 1. N-terminal amino acid sequence of rat decorin and deletion constructs. The N-terminal sequence of wild-type decorin (Rat. Dec.) and deletion constructs: RDec. D3, RDec. D4 and RDec. D5. The signal peptide cleavage site is indicated by the large arrow. The small arrow indicates the hypothetical propeptide cleavage site.

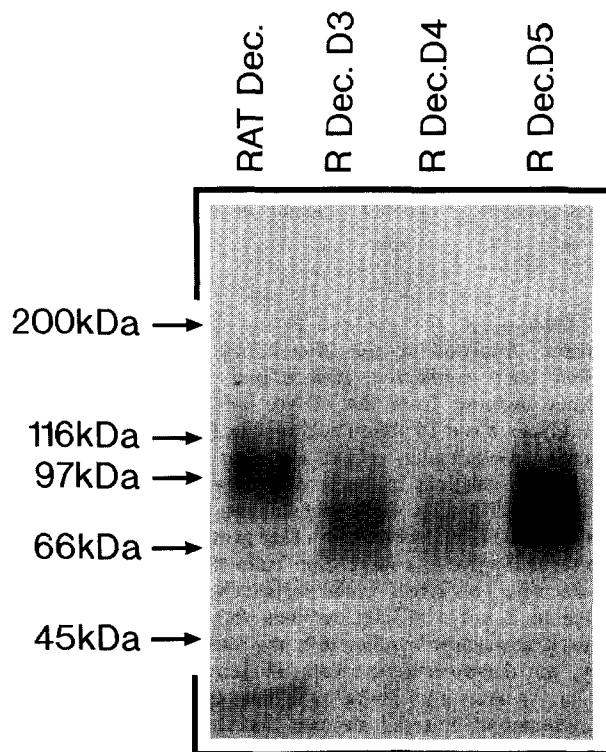


Fig. 2. SDS-PAGE of decorin synthesized by COS cells transformed with decorin and decorin deletion constructs. COS cells were transfected with the indicated proteoglycan cDNA constructs. The cells were metabolically labeled with [35 S]sulfate and proteoglycans secreted in the culture medium were immunoprecipitated with a decorin antiserum. The immunoprecipitates were analyzed on 7% SDS-PAGE.

expression vector and transiently expressed in COS cells. Proteoglycans secreted by transformed COS cells were metabolically labeled with [35 S]sulfate, immunoprecipitated and analyzed by SDS-PAGE (Fig. 2). The wild-type Rat. Dec. migrated with an apparent average M_r 100 kDa. The constructs RDec. D3 and RDec. D4 were similar in size, but smaller than the wild-type, with an approximate M_r 80 kDa. RDec. D5 migrated with an apparent M_r 90 kDa, intermediate in size compared to the wild-type and RDec. D3/RDec. D4 proteoglycans. These reductions in size on SDS-PAGE could not be due to the deletions in the core proteins, since that would result in size reductions of some 0.2–1.4 kDa. We therefore analyzed the size of the glycosaminoglycan chains attached to the core proteins. The 35 S-labeled glycosaminoglycan chains were released by alkaline β -elimination and subjected to Sepharose CL 6B gel chromatography (Fig. 3). The polysaccharides from the wild-type Rat. Dec had a K_{AV} 0.42 corresponding to M_r 32 kDa. The glycosaminoglycan chains from RDec. D3 and RDec. D4 were similar in size with a K_{AV} 0.49 corresponding to M_r 22 kDa. RDec. D5 proteoglycan contained 27 kDa glycosaminoglycan chains. The uronic acid compositions of the glycosaminoglycans from Rat. Dec, RDec. D3, RDec. D4 and RDec. D5 were similar (results not shown). The core proteins were substituted with chondroitin/dermatan sulfate chains containing 50–60% iduronic acid.

The constructs Rat. Dec and RDec. D3 were also used to transform CHO cells. The resulting CHO cell lines synthesized

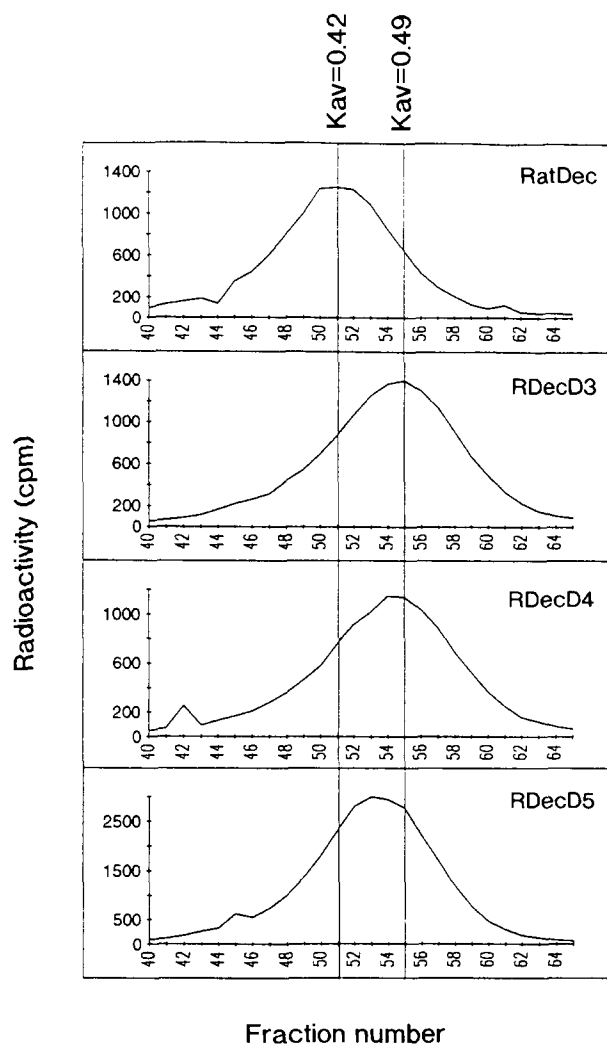


Fig. 3. Sepharose CL 6B gel chromatography of glycosaminoglycan chains from decorin and decorin deletion constructs. Immunoprecipitated [35 S]sulfate-labeled proteoglycans from transfected COS cells were subjected to alkaline β -elimination and chromatographed on a Sepharose CL 6B column eluted with 0.2 M NaCl.

proteoglycans with similar differences in size as observed when the constructs were transiently expressed in COS cells (Fig. 4). Decorin synthesized by the RDec. D3 CHO cells was substituted with approx. 30% shorter glycosaminoglycan chains than decorin glycosaminoglycan chains from the wild-type Rat. Dec CHO cells (results not shown).

The observed reduction in glycosaminoglycan chain length could be due to conformational changes in the core protein N-terminus, close to the Ser-Gly glycosaminoglycan substitution site. Possibly these changes affect the synthesis of the linkage region (-GlcUA-Gal-Gal-Xyl-) by lowering the affinity between the protein substrate and the glycosyltransferases. A slower rate of linkage region synthesis could possibly delay growth of the glycosaminoglycan chains and result in secretion of proteoglycans with shorter glycosaminoglycan chains. The subcellular location of the biosynthetic events leading to glycosylated decorin has not been studied in detail. The biosynthesis of aggrecan, the large chondroitin sulfate proteoglycan synthesized by chondrocytes, has been more extensively investigated [25]. Using subcellular fractionation of intracellu-

lar membranes [26] and in situ studies combining electron microscopic autoradiography and radiolabeling of semi-intact chondrocytes [27], it has been shown that the synthesis of aggrecan glycosaminoglycans takes place in a spatially well defined stepwise fashion. The synthesis begins with the addition of xylose to serine residues in the late ER, intermediate and possibly *cis*-Golgi compartments. The initial xylose substituted serine residue is elongated to form the linkage region -GlcUA-Gal-Gal-Xyl- in the *cis*-Golgi compartment, before the assembly of the glycosaminoglycan, which primarily takes place in the medial- and *trans*-Golgi. Since the synthesis of the linkage region and the polymerization of the glycosaminoglycan are temporally and spatially segregated, it is less likely that conformational changes affecting the early linkage region synthesis would lead to shorter glycosaminoglycan chains.

CHO cells, transformed with wild-type Rat. Dec. and RDec. D3, were subjected to pulse-chase labeling with [35 S]sulfate to compare the rate of proteoglycan secretion (Fig. 4). From the slopes in Fig. 4 we estimate that the

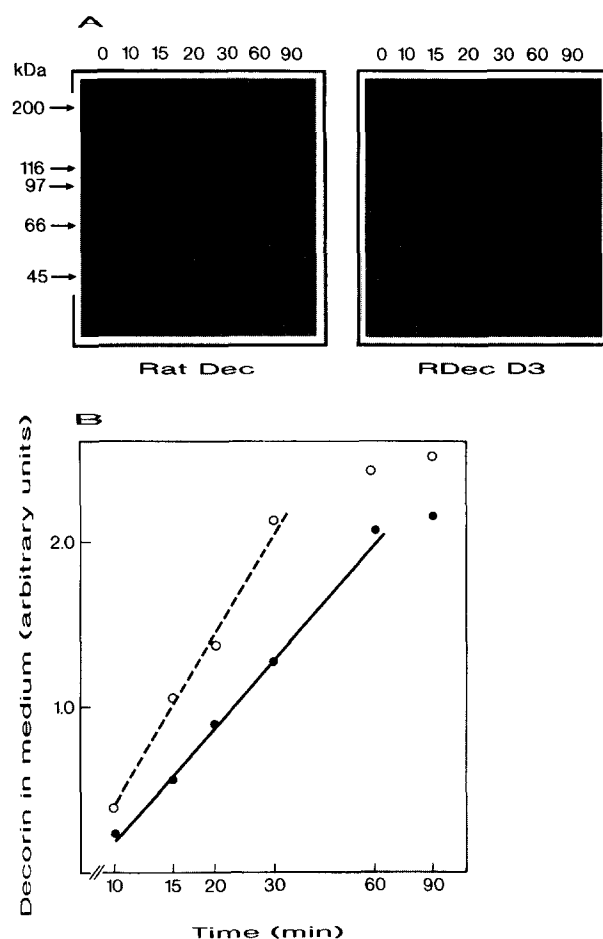


Fig. 4. Analysis of the rate of proteoglycan secretion from CHO cells transformed with decorin or RDec. D3. CHO cells transformed with wild-type Rat Dec. or RDec. D3 were subjected to pulse-chase analysis. The cells were pulse-labeled for 5 min with [35 S]sulfate and chased for 10–90 min in medium without radioactivity. Proteoglycans secreted into the culture medium were immunoprecipitated with a decorin antiserum and subjected to SDS-PAGE (A). The amounts of secreted Rat. Dec (●) and RDec. D3 (○) proteoglycans were determined using a phospho-imager and plotted versus log time of chase (B).

RDec. D3 CHO-cell line secreted proteoglycans at an approx. 30% faster rate than cells expressing the wild-type Rat. Dec. The time spent in the Golgi may determine the length of the glycosaminoglycan chain and possibly a faster transition through the Golgi could explain the observed reduction in glycosaminoglycan chain length. The vesicular transport of proteins along the secretory pathway is believed to occur by non-selective bulk flow [28]. However, studies of the transport of vesicular stomatitis virus G protein indicate that at least some proteins depend on a selective vesicular transport mechanism [29]. The correct transport of decorin between Golgi cisternae may depend on an intact N-terminal sequence.

3.2. N-terminal decorin antiserum

We also prepared a rabbit antiserum against the N-terminal 30 amino acid residues of rat decorin, including the signal peptide and 14 residues in the N-terminus. This antiserum precipitated decorin from [³⁵S]sulfate labeled culture media of rat skin fibroblasts and COS and CHO cells transformed with cDNA encoding rat decorin to the same extent as the antiserum prepared against purified rat decorin (results not shown). Apparently, the N-terminal 14 residues in decorin are not cleaved off inside the cell, but decorin is secreted with the N-terminal region intact. This suggests that the N-terminal 14 amino acid residues are proteolytically removed from decorin after deposition in the extra cellular matrix.

Acknowledgements: This work was supported by grants from the Swedish Medical Research Council, Anna-Greta Crafoords Stiftelse and the Medical Faculty, Lund University.

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