Chondroitin sulphate composition and structure in alternatively spliced CD44 fusion proteins

Michael PIEPKORN^{*1}, Peter HOVINGH[†], Kelly L. BENNETT[‡], Alejandro ARUFFO[‡] and Alfred LINKER^{†§}

^{*}Departments of Medicine (Dermatology) and Pathology, University of Washington School of Medicine, Seattle, WA, U.S.A., [†]Veterans Affairs Medical Center, Salt Lake City, UT, U.S.A., [‡]Bristol-Myers Squibb Pharmaceutical Research Institute, Seattle, WA, U.S.A., and [§]Departments of Pathology and Biochemistry, University of Utah School of Medicine, Salt Lake City, UT, U.S.A.

Previous studies have indicated that CD44 isoforms, spliced with variant exons, are heterogeneously glycanated with chondroitin sulphate and heparan sulphate chains. Because such alternative splicing may regulate divergent biological effects of the specific isoforms, we analysed the consequences of this process on the composition and structure of the chondroitin-sulphate chains. Recombinant chimaeras were engineered with and without exons V3–10 or V3,8–10 and expressed as Ig fusion proteins in COS cells. In addition, the chondroitin sulphates of wild-type isoforms were contrasted with those of isoforms mutated with serine-to-alanine codon substitutions at a putative Ser-Gly-Ser-Gly glycosaminoglycan acceptor site within exon V3. The chondroitin sulphates contained both 4- and 6-sulphated galactosamine residues, although there was a high content of non-sulphated galactosamine-containing repeat units. Splicing of exons V4–7, which contain

no Ser-Gly consensus motifs, resulted in increased glycanation with chondroitin-sulphate chains, as well as increased sulphation levels of the polymers. Comparison of wild-type and acceptor-site mutant isoforms showed that chondroitin-sulphate content declined by more than 60–80 % in the mutant, indicating that assembly of chondroitin-sulphate chains occurs there, and a general decrease in the sulphation level of the remaining chains was observed. Undersulphation of the recombinant chondroitin sulphates was shown by parallel analyses with native human keratinocyte CD44 molecules and is most probably an artifact of transient expression in COS cells. Our data indicate that combinatorial exon splicing exerts complex and distal effects on glycanation patterns and structure, which presumably modulate those functions that may be mediated through the chondroitin-sulphate moieties, such as motility and matrix invasion.

INTRODUCTION

Alternative exon splicing is a broadly conserved mechanism that serves to diversify the biological function of genes. This mechanism is exploited to a remarkable extent by the gene for CD44, an intrinsic membrane proteoglycan that exhibits marked heterogeneity of size and function [1]. The heterogeneity derives from the complex genomic structure of the single-copy gene, which is partitioned into 19 exons distributed over 50–60 kb of DNA; 12 of these are variable exons that can potentially be spliced into the membrane proximal ectodomain of the protein [2]. Cell-specific alternative splicing adds potential O- and N-linked polysaccharide assembly sites [3]. Such post-translational modifications by glycosylation provide additional means of structural heterogeneity and probably account in part for the functional versatility of the gene product [4,5]. The basic leukocyte form of CD44 (CD44H), with its variable glycanation by chondroitin sulphate, is expressed by multiple cell types, but epithelial cells such as keratinocytes additionally produce higher-molecular-mass CD44 isoforms. These include isoforms substituted with heparan-sulphate chains that are created by alternative exon splicing into the ectodomain [6,7]. Among the exons alternatively spliced by the epithelial cell-associated CD44 isoforms is the variable V3 domain, the splicing of which appears necessary for glycanation by heparan-sulphate chains and for the capacity to present heparin-binding cytokines for biologic function [4,8]. The specific functions of the covalent chondroitin-sulphate chains of CD44 are less clear, although evidence has emerged that CD44-related chondroitin sulphate has a role in melanoma-cell motility and invasive behaviour on collagenous matrices [9–11].

In these studies with human-recombinant-CD44 isoform-Ig fusion proteins produced in COS cells, our efforts were focused on characterizing the effects of alternative exon splicing on chondroitin-sulphate content and structure. This expression system allowed the production of sufficient material, which was readily isolated from culture supernatants, for biochemical analyses of the chains linked to alternatively spliced isoforms. Some of the effects of combinatorial exon splicing on the chondroitin-sulphate chains were thereby characterized, in doing so finding that exon splicing is coupled to effects on glycosaminoglycans assembled at distal sites within other exons. Moreover, the comparison of a V3-spliced CD44 isoform of wild-type sequence with a recombinant isoform mutated by site-directed mutagenic PCR primers at a putative glycosaminoglycan acceptor site within exon V3, allowed an analysis of its specific role in assembly of glycosaminoglycan chains. Finally, chondroitin-sulphate products of native CD44 molecules synthesized by cultured human keratinocytes, and of recombinant CD44V3–10, were directly compared in order to preliminarily evaluate ‘artificial’ effects of expression cloning on glycosaminoglycan structure.

MATERIALS AND METHODS

Materials

Pharmacia (Piscataway, NJ, U.S.A.) supplied Sepharose CL6B and Sephadex G50, G25 and G10. Delta chondroitin 4- and 6-sulphate sulphatases were obtained from Seikagaku Kogyo (Tokyo, Japan). Heparitinase I and heparinase (nomenclature of [12]) were purified from *Flavobacter heparinum* and were free

¹ To whom correspondence should be addressed at Box 356524, University of Washington, Seattle, WA 98195-6524, U.S.A.

Figure 1 Recombinant CD44 isoform-Ig fusion proteins

The Figure illustrates the isoforms that were generated for expression in the COS cell system. The complete exonic organization of the CD44 gene is depicted across the top, wherein the constitutive exons are represented by the solid, and the variable exons by the shaded, boxes (LP, leader peptide; TM, transmembrane domain). The target sequence of the V3 domain of the wild-type and mutant CD44V3-10 isoforms (bottom) includes positions 250 and 252 (numbered from the initiator methionine of the epican cDNA clone), where wild-type serine residues have been converted to alanine with mutagenic PCR primers.

of disaccharidase, oligosaccharidase, sulphatase and chondroitinase activities [12]. Glycosaminoglycan standards were bovine heparan sulphate [13] and shark-cartilage chondroitin 6-sulphate and human umbilical cord hyaluronic acid, both from Sigma (St. Louis, MO, U.S.A.). $\text{Na}_2^{35}\text{SO}_4$ (370 mCi/mM) and D-6-[^3H]-glucosamine hydrochloride (30 Ci/mM) were products of DuPont-New England Nuclear (Boston, MA, U.S.A.). Scintillation counting was carried out with a Packard Tri-Carb, model 1500, using Packard Opti Fluor. Testicular hyaluronidase, chondroitinase ABC, keratanase (from *Pseudomonas* sp.), Triton X-100, EDTA, *N*-ethylmaleimide, SDS, cetylpyridinium chloride and benzamidine were obtained from Sigma.

Fusion protein constructs

Construction of the vectors that were used to generate CD44-Ig fusion proteins has been described previously [4]. The following human CD44 isoforms were expressed in COS cells for this study and are illustrated in Figure 1. (1) CD44V8-10. (2) CD44V3-10 SGS wild-type (referring to the nominal Ser-Gly-Ser-Gly residues at positions 250-253 of the epican cDNA clone, as numbered from the initiator methionine [3]). (3) CD44V3, V8-10 SGS wild-type. (4) CD44V3-10 AGAG mutant, created in a mutagenic PCR protocol that converted the serine residues at positions 250 and 252 to alanine. For this purpose, the oligonucleotide, CGCCAGATCTACGTCTTCAAATAACATCTC-AGCAGGCTGGGAGCCAAATGAAGAAAATGAAGATGAAAGAGACAGACACCTAAGCTTTGCTGGAGCAGGCATTGATG, was used as a primer to change the two serines at the putative glycosaminoglycan attachment site in exon V3 to alanines in the mutant. The PCR products were generated from the wild-type constructs and then re-inserted into the same CD44-Ig vector at the engineered *Bgl*II/*Sph*I sites [4]. The fidelity of the PCR product was confirmed by direct nucleotide sequencing. (5) CD44V3, 8-10 AGAG mutant, created as above.

The glycosaminoglycans of the recombinant fusion proteoglycans were metabolically labelled for 48 h with [^{35}S]sulphate (50 $\mu\text{Ci/ml}$) and [^3H]glucosamine (20 $\mu\text{Ci/ml}$) in COS cell cul-

Figure 2 Sepharose CL6B chromatograms of glycosaminoglycan chains from CD44 isoforms

The chondroitin-sulphate chains of CD44V3, V8-10 were generated by sequential heparitinase I digestion of the recombinant proteoglycan, alkaline elimination of the resistant chains and elution from Sepharose CL6B (A). Similarly, recombinant CD44V3, V8-10 was digested with chondroitinase ABC, and the heparan-sulphate chains were cleaved from the core protein with alkaline borohydride and analysed on the column (B). The chondroitin-sulphate chains on CD44V8-10 were directly analysed on Sepharose CL6B following alkaline elimination and were pooled as indicated for detailed analyses (C). Radioactivity [^{35}S]sulphate ([^{35}S]-Sulfate, d.p.m. (DPM)) was quantified for each fraction eluted from the columns.

tures and isolated by standard protocols from the culture supernatants on Protein G–Sepharose [4]. The transfectant clones used in the labellings had been selected for equivalent levels of recombinant CD44 expression, as described [5]. In brief, binding assays employing anti-CD44 monoclonal antibodies against the 'constant' region were exploited in a flow cytometry protocol to identify those transfectants expressing approximately equal levels of recombinant product by their cytometry profiles. The only differences therefore were in splicing of the coding sequences for the alternative exons into the vector. Because the excess levels of radionuclides during labelling assured that the precursor pool was not rate limiting [14], structural variations in the glycosaminoglycans between isoforms should thus be due to exon splicing.

General analytical methods

Preparative size fractionation of products was on a 580 mm × 11 mm column of Sepharose CL6B eluted with 50 mM Tris, pH 7.5, containing 0.1 % (w/v) SDS, 0.35 M NaCl, 0.2 mM PMSF, 10 mM *N*-ethylmaleimide, 100 mM ϵ -aminocaproic acid, 10 mM EDTA and 5 mM benzamidine hydrochloride. Separation of degraded from non-degraded products following enzymic digestion was achieved with Sephadex G50 or Sephadex G25 columns (600 mm × 11 mm), eluted with 0.2 M NaCl in 10 % (v/v) ethanol. Preparations were desalted on Sephadex G10 (270 mm × 10 mm), eluted with distilled water.

Molecular mass estimates of the glycosaminoglycan chains from the CD44V3,V8–10 and CD44V8–10 fusion proteins were obtained by Sepharose CL6B chromatography, after removal of the core proteins. The estimates were interpolated from the linear plot of K_{av} (gel partition coefficient) versus log molecular mass, by reference to standards of known mass [13] and to published data [15]. For analysis of the chondroitin-sulphate chains on the CD44V3,V8–10 and CD44V8–10 isoforms, the product was digested with heparitinase (heparinase 3 [12]), eluted from Sepharose CL6B, cleaved with alkaline borohydride and eluted again from Sepharose CL6B (refer to Figure 2).

A combination of specific enzymic degradation, with heparitinase and chondroitinase ABC, and pH 1.5 nitrous acid deaminative depolymerization was used to identify glycosaminoglycan classes [12,13,16–18]. Relative degradation, as determined by Sephadex G-50 chromatography, was used to assign the proportional content of chondroitin sulphates among the other glycosaminoglycans.

Disaccharide analysis of glycosaminoglycans derived from CD44V3,V8–10 and CD44V8–10 isoforms

Detailed compositional structure of the chondroitin sulphates was analysed by HPLC. Samples of radionuclide-labelled glycosaminoglycans, which were recovered from the CD44V3,V8–10 and CD44V8–10 isoforms after alkaline elimination, were combined with carrier chondroitin sulphate (from shark cartilage; Sigma), digested with chondroitinase ABC, eluted from Sephadex G25 columns and desalted on Sephadex G10. The disaccharides were identified by analysis before and following digestion with delta chondroitin 4- or 6-sulphate sulphatase. The digests were resolved by HPLC (Waters Associates) on a Whatman Particil 10 SAX (strong anion-exchange) column [19], which was eluted with a linear 45 min gradient of water to a final concentration of 0.5 M potassium phosphate buffer, pH 6.6, with a 5 min interlude of isocratic elution with 70 % (v/v) water, using a flow rate of 1.4 ml/min. During the peak elutions, fractions of two drops were collected and concurrently analysed for absorbance at

230 nm on a Gilford spectrophotometer and for radioactivity by liquid-scintillation counting. Peak fractions were pooled, freeze-dried and taken up in standard volumes for final quantification of radioactivity.

Analysis of glycosaminoglycans of wild-type and linkage-region mutant CD44V3–10 and CD44V3,8–10 fusion proteins

Wild-type and mutant CD44V3–V10 and CD44V3,8–10 recombinant fusion proteins were metabolically labelled and isolated as before. The labelled material was treated with alkaline borohydride overnight at 4 °C, neutralized with acetic acid and combined with 1.3 mg of chondroitin sulphate and 1.4 mg of heparan sulphate standards as carriers. Some samples were isolated on a larger scale by elution with a linear salt gradient from Whatman DE Cellulose 52 columns (5 cm × 1 cm). The isolates were clarified by centrifugation, and the glycosaminoglycans, all of which were beta-eliminated, were precipitated with ethanol. Each sample was treated with chondroitinase ABC and size-fractionated by elution from Sephadex G50 columns (600 mm × 11 mm) with 0.2 M NaCl in 10 % ethanol (Figure 3). The disaccharide eluates present within the Sephadex G50 included volume fractions were desalted by elution from Sephadex G10 (270 mm × 10 mm) with distilled water, and then analysed by HPLC using strong anion-exchange fractionation on Spherisorb S5 SAX (25 mm × 4.6 mm) columns (Phase Separations, Norwalk, CT, U.S.A.), eluted with a 0.05–0.50 M potassium phosphate convex gradient, pH 6.0, controlled with the Waters Automated Gradient Controller.

Isolation and analysis of CD44 chondroitin sulphate from cultured human neonatal keratinocytes

To provide comparisons of the recombinant isoforms with native CD44 molecules, human neonatal foreskin keratinocytes were cultured in serum-free KGM (Clonetics, San Diego, CA, U.S.A.) and metabolically labelled, as for the recombinant products (above), with [³⁵S]sulphate and [³H]glucosamine, in parallel with COS cells expressing the CD44V3–10 wild-type construct. Detergent extracts of the keratinocytes were immunoprecipitated with anti-CD44, recognizing an epitope common to all CD44 isoforms (Pgp-1; PharMingen, San Diego, CA, U.S.A.), and the glycosaminoglycans of the precipitate, along with those of the recombinant CD44V3–10 isoform, were isolated by β -elimination and analysed by gel filtration, anion-exchange chromatography and selective enzymic digestion, as before.

RESULTS

The general strategy was to first compare chondroitin-sulphate content and structure between a pair of CD44 constructs differing in splicing of the V3 exon: CD44V3,V8–10 versus CD44V8–10, as depicted in Figure 1. Previous studies had shown that only V3-spliced CD44 isoforms are substituted with heparan-sulphate chains and are capable of presenting heparin-binding cytokines for biological function [4,8]; however, it is plausible that V3 splicing also adds assembly sites for chondroitin-sulphate chains. Secondly, we compared the glycosaminoglycans of CD44V3–10 and CD44V3,8–10 isoforms of wild-type sequences with mutant isoforms in which codons for alanine residues were substituted for serine codons at the Ser-Gly-Ser-Gly tetrapeptide within the V3 coding region. We accordingly tested the hypotheses that alternative exon splicing and site-directed mutation at a candidate glycosylation site gives rise to variations in the composition of the glycosaminoglycan chains. Finally, analytical comparisons were obtained between the recombinant isoforms and native

Figure 3 Preparative Sephadex G50 elutions of the glycosaminoglycans from wild-type and mutant CD44V3–10 isoforms

The alkaline-eliminated glycosaminoglycans of the wild-type (A) and (B), and mutant (C) and (D), proteins were digested with chondroitinase ABC (ABCCase) (A) and (C), and the undegraded void volume material treated with heparitinase I and subsequently with nitrous acid (B) and (D), and then eluted from columns of Sephadex G50. The fractions that were pooled for analyses of the different product categories are indicated in the panels. ‘Glycoprotein’ refers to CD44 molecules substituted with neuraminic acid-containing O-linked glycans, whereas ‘oligosaccharides’ under the heparan- and chondroitin-sulphate headings denotes the enzyme-generated products of those glycosaminoglycan classes. Radioactivity ($10^{-3} \times [^3\text{H}]\text{glucosamine}$ ([^3H]-Glucosamine), d.p.m. (DPM)) was quantified for each fraction eluted from the columns.

CD44 molecules synthesized by cultured human keratinocytes, in order to evaluate possible differences resulting from the expression system on the content and structure of the covalent glycosaminoglycan chains.

Preliminary characterization of CD44V3,V8–10-, CD44V8–10- and CD44V3–10-derived glycosaminoglycans

Following their separation from the CD44V3,V8–10 and CD44V8–10 core proteins by alkaline elimination and borohydride reduction, the glycosaminoglycan chains were eluted from Sepharose CL6B to obtain estimates of the relative masses by reference to the standards (Figure 2). The chondroitin-sulphate chains of CD44V3,V8–10, isolated following enzymic removal of the heparan sulphates, eluted as shown in Figure 2(A); for comparison, elution of CD44V3,V8–10 glycosaminoglycans digested with chondroitinase ABC (leaving behind the heparan sulphates) is depicted in Figure 2(B). The chondroitin sulphates of the CD44V8–10 isoform, which lacked heparan-sulphate chains in this study (confirming previous data), were similarly analysed, and fractions for further analysis were pooled

Table 1 Relative size of CD44 isoform glycosaminoglycans

The partition coefficients (K_{av}) of glycosaminoglycan classes after alkaline elimination and reduction are given for the indicated sources. The range of values corresponds to a molecular mass of approx. 15–25 kDa.

Product	K_{av}
CD44V8–10	
Chondroitin sulphate	0.21
CD44V3,V8–10	
Chondroitin sulphate	0.27
Heparan sulphate	0.27

as indicated (Figure 2C; fraction D represents inorganic radio-nuclide). The relative partition coefficients (K_{av}) of the respective chondroitin-sulphate chains, with those of the heparan sulphates from the CD44V3,V8–10 isoform for comparison, are listed in Table 1. The average molecular mass distributions of the chondroitin sulphates from the two isoforms were comparable,

Table 2 Sulphation levels of chondroitin and heparan sulphates from CD44 isoforms

The overall levels of sulphation are given for the indicated glycosaminoglycans, expressed as ratios of the radionuclide labels determined for each product category.

Product	^{35}S Sulphate/ ^3H glucosamine ratio
CD44V8–10	
Chondroitin sulphate*	0.10
Chondroitin sulphate†	0.02
Chondroitin sulphate‡	0.02
CD44V3,V8–10	
Chondroitin sulphate	0.11
Heparan sulphate	0.26
Standard	
Monosulphated disaccharide (av.)	1.38

* Fraction A from Figure 2(C).
† Fraction B from Figure 2(C).
‡ Fraction C from Figure 2(C).

although qualitatively the corresponding elution patterns illustrated in Figure 2(A) and 2(C) were distinctive.

The overall degree of sulphation for the chondroitin-sulphate chains was assessed by calculation of ^{35}S sulphate/ ^3H glucosamine labelling ratios, in comparison with the reference monosulphated disaccharide standard and with the heparan sulphates of the V3-spliced isoform (Table 2). These data indicated that the chains of the recombinant products were quite undersulphated. Specifically, the overall ^{35}S sulphate/ ^3H glucosamine ratios were between 0.02 and 0.11, whereas that for the specific monosulphated disaccharide standard was 1.38. We were not able to determine if there was microheterogeneity of sulphate content across the molecular-mass distributions. A subsequent analysis of CD44V3–10 SGSG wild-type and CD44V3–10 AGAG mutant isoforms confirmed that the chondroitin sulphate polymers of the recombinant products were consistently undersulphated relative to standard chondroitin sulphates. In this analysis, the ^{35}S sulphate/ ^3H glucosamine labelling ratios were 0.15 and 0.19 for the CD44V3–V10 mutant and wild-type isoforms respectively, which, although somewhat higher than the earlier values, remained substantially below those of the reference standards.

Because earlier observations had indicated that the isoforms were substituted with substantial amounts of neuraminic acid-containing O-linked glycans, which would complicate the chromatographic separations, we employed the strategy of salt-gradient elution of the β -eliminated glycosaminoglycan chains from Whatman DE-cellulose anion-exchange columns, to better resolve these glycans from the chondroitin-sulphate chains.

Analysis of glycosaminoglycan content of wild-type and mutant isoforms of the CD44V3–10 and CD44V3,8–10 fusion proteins

Evidence that alternative exon splicing and site-directed mutation alter the content and structure of the glycosaminoglycan moieties was sought by parallel analyses of two pairs of alternatively spliced, wild-type and mutant constructs. The heparan and chondroitin sulphates and non-glycosaminoglycan polysaccharides isolated from the CD44V3,8–10 and CD44V3–10 wild-type and mutant fusion proteins by alkaline elimination were preparatively analysed on Sephadex G50 columns after sequential chondroitinase ABC digestion and either nitrous acid or hepari-

Table 3 Quantitative yields of chondroitin sulphates from wild-type and mutant CD44V3–10 and CD44V3,8–10 isoforms

The metabolically labelled products of the COS cell lines, which were transfected with constructs for the wild-type (SGSG) or mutant (AGAG) CD44V3–10 and CD44V3,8–10 isoforms, were assigned to the chondroitin sulphate category by differential enzymic digestion and chromatographic resolution.

Construct	$10^{-3} \times$ Chondroitin sulphate yield, d.p.m. (^{35}S sulphate/ ^3H glucosamine)
CD44V3–10	
Wild-type	369/102
Mutant	32/22
CD44V3,8–10	
Wild-type	134/92
Mutant	117/41

Table 4 Proportional yields and sulphation levels of chondroitin sulphates from wild-type and mutant CD44V3–10 and CD44V3,8–10 isoforms

The metabolically labelled enzyme-generated products of the wild-type (wt) (SGSG) or mutant (mt) (AGAG) CD44V3–10 and CD44V3,8–10 isoforms were analytically assigned to the indicated categories. The 'oligosaccharides' refer to O-linked glycans resistant to chondroitinase and heparitinase. For the ^{35}S / ^3H column, note that the standard disaccharide ratio is 3.54.

(a)			
Product	Product yield (%)		
	By ^3H	By ^{35}S	$^{35}\text{S}/^3\text{H}$
CD44V3–10 wt			
Chondroitin sulphate	20.7	45.0	3.60
Oligosaccharides	59.5	25.8	0.72
CD44V3–10 mt			
Chondroitin sulphate	2.7	19.4	1.47
Oligosaccharides	71.8	52.5	0.15
CD44V3,8–10 wt			
Chondroitin sulphate	12.1	36.9	2.56
Oligosaccharides	56.3	30.4	0.45
CD44V3,8–10 mt			
Chondroitin sulphate	4.5	16.6	1.45
Oligosaccharides	65.8	62.7	0.38
(b)			
Product	Product yield (total, d.p.m.)		
	By ^3H	By ^{35}S	
CD44V3–10 wt	(470)	(817)	
CD44V3–10 mt	(794)	(159)	
CD44V3,8–10 wt	(309)	(280)	
CD44V3,8–10 mt	(1827)	(761)	

tinase I degradation (illustrated for CD44V3–10 products in Figure 3).

The yields of the chondroitin sulphates isolated from the constructs are listed in Table 3. In general, there were relatively fewer chondroitin-sulphate chains associated with wild-type CD44V3,8–10 than with wild-type CD44V3–10. Moreover, the chondroitin sulphates of wild-type CD44V3,8–10 were less sulphated than were those derived from wild-type CD44V3–10 (refer to Table 4). The mutants with site-directed amino acid

Table 5 Analysis of chondroitin sulphate disaccharides from alternatively spliced CD44 isoforms

The disaccharide composition of the chondroitin sulphates from the CD44V3,V8–10 and CD44V8–10 isoforms was analysed by HPLC after chondroitinase ABC digestion. See Figure 2(C) for origins of fractions A, B and C: disacch, disaccharide.

Product	Yield (%) [³⁵ S]Sulphate	[³ H]Glucosamine	[³⁵ S]/[³ H] ratio
CD44V8–10, fraction A			
Delta non-sulphate disacch	0	77	0
Delta 6-sulphate disacch	31	10	1.3
Delta 4-sulphate disacch	55	13	1.7
Inorganic sulphate	14	—	—
Delta 4,6-sulphate disacch	0	0	—
CD44V8–10, fraction B			
Delta non-sulphate disacch	0	67	0
Delta 6-sulphate disacch	19	14	1.3
Delta 4-sulphate disacch	45	19	1.5
Inorganic sulphate	25	—	—
Delta 4,6-sulphate disacch	0	0	0
CD44V8–10, fraction C			
Delta non-sulphate disacch	0	61	0
Delta 6-sulphate disacch	24	15	1.1
Delta 4-sulphate disacch	55	24	1.5
Inorganic sulphate	21	—	—
Delta 4,6-sulphate disacch	0	0	0
CD44V3,V8–10			
Delta non-sulphate disacch	0	84	0
Delta 6-sulphate disacch	8	10	1.6
Delta 4-sulphate disacch	3	6	1.0
Inorganic sulphate	89	—	—
Delta 4,6-sulphate disacch	0	0	0

substitutions at the putative V3 glycosaminoglycan acceptor site contained much less chondroitin sulphate than did the wild-type product: by the ³H-label, there were decreases of 78 % and 55 % in the chondroitin-sulphate content of the CD44V3–10 and CD44V3,8–10 mutant isoforms respectively (Table 3). In a separate confirmatory experiment, glycosylation by chondroitin sulphates decreased by 85 % in the mutant CD44V3–10 construct.

A more detailed analysis of the proportional yields of the chondroitin sulphates and their sulphation levels from the wild-type and mutant constructs is given in Table 4. In comparisons of the paired wild-type and mutant isoforms, both the yields by [³H]glucosamine incorporation and degree of sulphation of the glycosaminoglycan chains declined in the two mutant constructs. The sulphation level of those chondroitin-sulphate chains remaining in the mutants declined by more than 60 % on average, as measured by [³⁵S]/[³H] labelling ratios of the enzymic degradation products (Table 4). As shown in Table 4 and Figures 3(B) and 3(D), a substantial amount of glucosamine-labelled material resistant to chondroitinase ABC and heparitinase was present in the isolates. CD44 is heavily substituted with O-linked acetyl-galactosamine and neuraminic acid-containing oligosaccharides [5,8]. These are the most likely source of the resistant glycans. However, because sulphate was present, albeit at low levels, we treated one of the fractions with keratanase, followed by chromatography on Sephadex G50. This analysis revealed that some degradation did occur, although only a small yield of disaccharide was obtained. Whereas this may indicate the presence of keratan sulphate, keratanases as a group appear not to be entirely specific for keratan sulphate, but can degrade some galactose- and hexosamine-containing oligosaccharides [20,21]. It is significant

Table 6 Analysis of chondroitin sulphate disaccharides from wild-type and mutant CD44V3,8–10 and CD44V3–10 isoforms

The disaccharide composition of the chondroitin sulphates was characterized by HPLC after chondroitinase ABC digestion. Percentage yield is given from [³H] (d.p.m.); abbreviation: disacch, disaccharide.

Construct and product	Yield (%)
CD44V3,V8–10 wt	
Delta non-sulphate disacch	14.6
Delta 6-sulphate disacch	32.4
Delta 4-sulphate disacch	53.0
CD44V3,V8–10 mt	
Delta non-sulphate disacch	29.3
Delta 6-sulphate disacch	54.3
Delta 4-sulphate disacch	16.4
CD44V3–10 wt	
Delta non-sulphate disacch	12.1
Delta 6-sulphate disacch	51.4
Delta 4-sulphate disacch	36.4
CD44V3–10 mt	
Delta non-sulphate disacch	30.3
Delta 6-sulphate disacch	35.4
Delta 4-sulphate disacch	34.3

that the content of these resistant glycans was similar for the two wild-type isoforms, whereas mutation of a putative glycosaminoglycan assembly site induced an approx. 2-fold increase in this material (Table 4).

Analysis of chondroitin sulphate disaccharide composition

In order to assess the effects of variant exon V3 splicing on chondroitin sulphate structure, the chondroitinase ABC-generated disaccharide profiles of the wild-type CD44V3,V8–10 and CD44V8–10 chondroitin sulphates were characterized by HPLC, using delta chondroitin 4- and 6-sulphate sulphatases to analytically resolve the corresponding disaccharide repeat units. The column was calibrated by resolution of the chondroitinase ABC digests from shark-cartilage chondroitin 6-sulphate, which yielded three major disaccharide peaks: delta chondroitin 6-sulphate, delta chondroitin 4-sulphate and delta chondroitin 4,6-disulphate. Digestion of the standard with either the chondroitin 4- or the chondroitin 6-sulphate sulphatase attenuated the corresponding disaccharide peak and generated non-sulphated disaccharides, as expected. The chondroitinase ABC-generated disaccharides from the CD44V3,V8–10 and CD44V8–10 chondroitin sulphates were then analysed (Table 5). For both CD44 isoforms, the major products generated by the chondroitinase were non-sulphated disaccharide units. Among the sulphated disaccharides, slightly more delta 4-sulphated products (57–61 % by the [³H]glucosamine label, compared with 6-sulphated moieties) were obtained from CD44V8–10, whereas the reverse pattern was evident for the CD44V3,V8–10 isoform (62 % delta chondroitin 6-sulphate); however, measured amounts of label in the latter products were low, thereby limiting any conclusions from these comparisons. No disulphated disaccharides were identified in any of the fractions. This is in agreement with the data shown in Table 2.

In separate analyses comparing wild-type and mutant constructs, the chondroitin sulphates of paired CD44V3,8–10 and CD44V3–10 wild-type and mutant isoforms were digested in

Table 7 Chondroitin sulphate content of keratinocyte and recombinant CD44 proteoglycans in anion-exchange fractions

Proportional (%) distribution of ^{35}S -labelled carbohydrate product is tabulated for the pooled eluates from an anion-exchange column. The products have been partitioned between the four fractions, and the percentages of each fraction accounted for by chondroitin sulphate are listed. The anion-exchange fractions eluted within the indicated ranges of NaCl molarity.

Product	Anion-exchange fraction ([NaCl]) ...	Distribution of product (%)			
		0.03–0.11	0.12–0.19	0.20–0.24	0.25–0.38
Keratinocyte CD44					
Labelled carbohydrate		2	9	21	68
Chondroitin sulphate		0	0	0	12
Recombinant CD44V3–10					
Labelled carbohydrate		10	59	26	6
Chondroitin sulphate		2	8	4*	

* Combined fractions eluting from 0.2 to 0.38 M NaCl.

* Combined fractions eluting from 0.2 to 0.38 M NaCl.

parallel with chondroitinase ABC and the derived disaccharides characterized by HPLC (Table 6). The chondroitin-sulphate disaccharides from the CD44V3–10 mutant contained slightly more, and those from the CD44V3,8–10 mutant and CD44V3–10 wild-type much more, 6- than 4-sulphated galactosamine residues; in contrast, the material from CD44V3,8–10 wild-type chondroitin sulphates had more 4- than 6-sulphated disaccharide units. Yields of non-sulphated disaccharides were inexplicably low compared with the previous analyses. These data nevertheless suggest that both combinatorial splicing of the variant exons and mutation at a glycosaminoglycan assembly site influence the patterns of post-translational sulphation of the chondroitin sulphate galactosamine moieties.

Parallel analysis of recombinant CD44V3–10 and human keratinocyte CD44 proteoglycans

The glycosaminoglycans of recombinant CD44V3–10 and cultured human keratinocyte CD44 were cleaved from the core proteins by alkaline elimination, separated on Sephadex G-50, chromatographed on Whatman DE-52 and treated sequentially with chondroitinase ABC and nitrous acid. Proportional content of chondroitin sulphate was assigned in each case by elution from Sephadex G-50 (Table 7). All of the chondroitin sulphates from keratinocyte CD44 eluted from the anion-exchange matrix between 0.25 and 0.38 M NaCl, whereas most of the respective recombinant CD44V3–10 chondroitin sulphates eluted much earlier, between 0.12 and 0.19 M NaCl. Thus the chondroitin sulphates of COS-cell-produced CD44V3–10 are substantially undersulphated relative to the native proteoglycan isoforms.

DISCUSSION

Information on the glycosaminoglycans of alternatively spliced proteoglycans is presently limited. We exploited an Ig-fusion protein vector for expression of CD44 isoforms [4,8], to better define the effects of alternative exon splicing on the chondroitin-sulphate content and structure of this proteoglycan. Heterogeneity in the chondroitin sulphates and related N- and O-linked carbohydrate moieties may regulate, for example, the binding

affinity of the specific isoforms for hyaluronan [5,22–24] and other matrix molecules, and hence their biological properties.

The current analyses of the chondroitin sulphates attached to human recombinant CD44 isoform–Ig fusion proteins indicated that, although both 4- and 6-sulphated galactosamine residues were identified, the chains were generally quite undersulphated relative to reference standards and native keratinocyte CD44 proteoglycan, and there was a high yield of non-sulphated galactosamine-containing units after chondroitinase treatment. Nevertheless, glycosaminoglycan content and structure were shown in these studies to be substantially altered by combinatorial splicing of the variable exonic domains. The major findings were obtained from the direct comparisons of the wild-type and mutant CD44V3–10 and CD44V3,8–10 isoforms (Tables 3 and 4). These analyses demonstrated that splicing of exons V4–7, which contain no consensus motifs for glycosaminoglycan assembly, was associated with increased glycanation of the isoform by chondroitin-sulphate chains. Further effects of this alternative exon splicing were observed in the microheterogeneity of glycosaminoglycan structure, as reflected by a general increase in the sulphation level of the chondroitin-sulphate chains and specifically by an increased content of galactosamine 6-sulphate. Thus splicing of exons V4–7 may allow greater utilization of Ser-Gly assembly sites in the proteoglycan and possibly more optimal substrate configuration for the action of sulphotransferases. Whereas the range of biological roles of exons V4–7 remains to be elucidated [5], a function, in the transduction of signals that initiate cell proliferation, has been hypothesized [25]. With respect to exon V3, mutations at the Ser-Gly-Ser-Gly glycosaminoglycan assembly locus within that domain correlated with substantially decreased chondroitin-sulphate glycanation, and a decrease in sulphation level of those chains that remained, in both CD44 constructs; concurrently, there was an increase in the O-linked glycan content of the isoforms. Taken together, these observations indicate that variant exon splicing, including those without potential glycosaminoglycan assembly motifs, and molecular modifications at such assembly sites, exert distal effects on the overall level of carbohydrate substitution and specifically on the content and structure of the glycosaminoglycan moieties at other loci within the proteoglycan. Related conclusions have been drawn by other investigators, as it has been shown in a recombinant system expressing chimaeric syndecan-1 that utilization of potential heparan-sulphate assembly sites is not independently determined, but rather is coupled to the occupancy of other, distal, Ser-Gly dipeptides [26].

The observed differences in the ratios of 4- and 6-sulphate between the galactosamine-containing repeat units of the wild-type and mutant CD44V3–10 and CD44V3,8–10 chondroitin sulphates (Tables 5 and 6) are potentially of interest. The biological significance of the occurrence of the two different structures, however, is not well understood at present. Differences in the tissue distribution, the arrangement of negative charges, and physical properties in solution phase, as well as in the solid state [27,28], indicate a potentially specific biological function for each type of chondroitin-sulphate polymer, rather than merely random processing.

The generally low sulphate content of the recombinant glycosaminoglycans was shown by the parallel analyses of recombinant and native CD44 chondroitin sulphates to apparently be an 'artifactual' effect of the COS expression system. The low sulphate density of the heparan-sulphate chains (results not shown) could explain previous observations of failure of recombinant V3-domain-containing CD44 fusion proteins to ligate one of the heparin-binding cytokines [4]. In any case, the undersulphation of the recombinant CD44 fusion proteins adds certain

constraints on the use of the COS-cell expression system to study the structure and function of proteoglycans.

Previous studies had indicated that no heparan-sulphate chains are substituted on the CD44 core protein without alternative splicing of the domain encoded by exon V3 [4,8]. The direct comparison in the current studies between the glycosaminoglycans of the CD44V8–10 and CD44V3,V8–10 isoforms confirmed that only the latter product had heparan-sulphate chains, suggesting that the Ser-Gly-Ser-Gly site within V3 is the major heparan-sulphate assembly site. It was therefore unexpected that the mutant CD44V3–10 and CD44V3,8–10 isoforms, created by substituting alanine for serine codons at the target tetrapeptide coding region, also displayed a markedly reduced content of chondroitin sulphate glycosaminoglycans, which declined by 60–80% compared with the corresponding wild-type isoforms. Thus either the Ser-Gly-Ser-Gly tetrapeptide may be a versatile attachment site for heparan- or chondroitin-sulphate chains or else alterations at that locus have distal effects at other potential glycosaminoglycan assembly sites within the core protein of CD44, as suggested above.

It is clear from the available data that combinatorial splicing of the variable exons into the ectodomain of the basic CD44H isoform has complex effects on both the composition and structure of the covalent chondroitin-sulphate chains, as well as on the N- and O-linked oligosaccharides and heparan-sulphate chains. The effects of variant exon splicing on the glycanation patterns that were observed in these studies presumably modulate those functions of the isoforms that are mediated via the polysaccharide moieties. Such functions could be reflected in the previous observation that variant exon splicing reduces the hyaluronic acid binding properties of the CD44 proteoglycan [5]. Among the other potential functions of the chondroitin sulphates that may be specifically modulated by this mechanism is their emerging role in mediating motility and invasive behaviour in provisional matrices, such as during neoplastic transformation or in wound repair [9–11]. Further studies focusing on the effects of variant exon splicing in these processes are thus warranted.

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REFERENCES

- 1 Jackson, D. G., Buckley, J. and Bell, J. I. (1992) *J. Biol. Chem.* **267**, 4732–4739
- 2 Screation, G. R., Bell, M. V., Jackson, D. G., Cornelis, F. B., Gerth, U. and Bell, J. I. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 12160–12164
- 3 Kugelman, L. C., Ganguly, S., Haggerty, J. G., Weissman, S. M. and Milstone, L. M. (1992) *J. Invest. Dermatol.* **99**, 381–385
- 4 Bennett, K. L., Jackson, D. G., Simon, J. C., Tanczos, E., Peach, R., Modrell, B., Stamenkovic, I., Plowman, G. and Arrufo, A. (1995) *J. Cell Biol.* **128**, 687–698
- 5 Bennett, K. L., Modrell, B., Greenfield, B., Bartolazzi, A., Stamenkovic, I., Peach, R., Jackson, D. G., Spring, F. and Aruffo, A. (1995) *J. Cell Biol.* **131**, 1623–1633
- 6 Brown, T. A., Bouchard, T., St. John, T., Wayner, E. and Carter, W. G. (1991) *J. Cell Biol.* **113**, 207–221
- 7 Cooper, D. L., Dougherty, G., Harn, H.-J., Jackson, S., Baptist, E. W., Byers, J., Datta, A., Phillips, G. and Isola, N. R. (1992) *Biochem. Biophys. Res. Commun.* **182**, 569–578
- 8 Jackson, D. G., Bell, J. I., Dickinson, R., Timans, J., Shields, J. and Whittle, N. (1995) *J. Cell Biol.* **128**, 673–685
- 9 Faassen, A. E., Mooradian, D. L., Tranquillo, R. T., Dickenson, R. B., Letourneau, P. C., Oegema, T. R. and McCarthy, J. B. (1991) *J. Cell Sci.* **105**, 501
- 10 Knutson, J. R., Iida, J., Fields, G. B. and McCarthy, J. B. (1996) *Mol. Biol. Cell* **7**, 383–396
- 11 Henke, C. A., Roongta, U., Mickelson, D. J., Knutson, J. R. and McCarthy, J. B. (1996) *J. Clin. Invest.* **97**, 2541–2552
- 12 Linker, A. and Hovingh, P. (1972) *Methods Enzymol.* **28**, 902–911
- 13 Linker, A. and Hovingh, P. (1973) *Carbohydr. Res.* **29**, 41–62
- 14 Silbert, C. K., Humphries, D. E., Palmer, M. E. and Silbert, J. E. (1991) *Arch. Biochem. Biophys.* **285**, 137–141
- 15 Gordon, P. B., Zanger, D. R. and Hatcher, V. B. (1986) *Carbohydr. Res.* **151**, 121–134
- 16 Linker, A. and Hovingh, P. (1975) *Biochim. Biophys. Acta* **385**, 324–333
- 17 Linker, A. (1979) *Biochem. J.* **183**, 711–720
- 18 Piepkorn, M., Hovingh, P. and Linker, A. (1988) *J. Cell. Physiol.* **135**, 189–199
- 19 Linhardt, R. J., Wang, H., Lagareth, D. and Bae, J. (1992) *J. Biol. Chem.* **267**, 2380–2387
- 20 Kitamikado, M., Makoto, I. and Li, Y.-T. (1981) *J. Biol. Chem.* **256**, 3906–3909
- 21 Aeed, P. A., Guido, D. M., Mathews, W. R. and Elhammer, A. P. (1992) *Biochemistry* **31**, 6951–6961
- 22 Katoh, S., Zheng, Z., Oritani, K., Shimozato, T. and Kincade, P. W. (1995) *J. Exp. Med.* **182**, 419–429
- 23 Lesley, J., English, N., Perschl, A., Gregoroff, J. and Hyman, R. (1995) *J. Exp. Med.* **182**, 431–437
- 24 Takahashi, K., Stamenkovic, I., Cutler, M., Dasgupta, A. and Tanabe, K. K. (1996) *J. Biol. Chem.* **271**, 9490–9496
- 25 Zöller, M. (1995) *J. Mol. Med.* **73**, 425–438
- 26 Zhang, L., David, G. and Esko, J. D. (1995) *J. Biol. Chem.* **270**, 27127–27135
- 27 Scott, J. E., Chen, Y. and Brass, A. (1992) *Eur. J. Biochem.* **209**, 675–680
- 28 Scott, J. E., Heatley, F. and Wood, B. (1995) *Biochemistry* **34**, 15467–15474