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Deglycosylation of Chondroitin Sulfate Proteoglycan and Derived Peptides†

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ABSTRACT: In order to define the domain structure of proteoglycans as well as identify primary amino acid sequences specific for attachment of the various carbohydrate substituents, reliable techniques for deglycosylating proteoglycans are required. In this study, deglycosylation of cartilage chondroitin sulfate proteoglycan (CSPG) with minimal core protein cleavage was accomplished by digestion with chondroitinase ABC and keratanase, followed by treatment with anhydrous HF in pyridine. Nearly complete deglycosylation of secreted proteoglycan was verified within 45 min of HF treatment by loss of incorporated [^{3}H]glucosamine label from the proteoglycan as a function of time of treatment, as well as by direct analysis of carbohydrate content and xylosyltransferase acceptor activity of unlabeled core protein preparations. The deglycosylated CSPG preparations were homogeneous and of high molecular weight (approximately 370 000). Comparison of the intact deglycosylated core protein preparations with newly synthesized unprocessed precursors (apparent M_r ~360 000) suggested that extensive proteolytic cleavage of the core protein did not occur during normal intracellular processing. Furthermore, peptide patterns generated after clostripain digestion of core protein precursor and of deglycosylated secreted proteoglycan were comparable. With the use of the clostripain digestion procedure, peptides were produced from unlabeled proteoglycan, and two predominant peptides from the most highly glycosylated regions (the chondroitin sulfate rich regions of the proteoglycan) were isolated, characterized, and deglycosylated. These peptides were found to follow similar kinetics of deglycosylation and to acquire xylose acceptor activity comparable to the intact core protein.

Chondroitin sulfate proteoglycan (CSPG)[1] consists of a core protein to which various types of carbohydrate chains are attached. Studies using cultured chondrocytes from embryonic chick sterna (Habib et al., 1984; Campbell & Schwartz, 1988) or from the rat chondrosarcoma (Kimura et al., 1981; Fellini et al., 1984) have contributed significantly to our understanding of the events involved in the assembly of proteoglycans. Extensive posttranslational processing of the core protein involves addition of N-linked oligosaccharides and xylose in the RER (Habib et al., 1984; Hoffman et al., 1984), followed by addition of the remainder of the glycosaminoglycan chains and O-linked oligosaccharides (Thonar et al., 1983) and processing of the N-linked oligosaccharides in the Golgi apparatus. All of these steps lead to the formation of a very large and polydisperse proteoglycan [M_r ~(1-4) × 10^6], which is approximately 90% carbohydrate by weight (Hassell et al., 1986). The polydispersity observed in proteoglycans synthesized by cultured rat chondrosarcoma chondrocytes can be primarily accounted for by variations in the number or size of the attached carbohydrate chains (Fellini et al., 1981). However, several studies using proteoglycans synthesized in vivo indicate that heterogeneity in the length of the core protein may also contribute to this polydispersity (Rosenberg et al., 1976; Thyberg et al., 1975; Heinegard, 1977; Buckwalter & Rosenberg, 1982). Newly synthesized core protein, immunoprecipitated from the RER of both rat chondrosarcoma and embryonic chick sternal cultured chondrocytes, has yielded a molecular weight value of 370 000 by SDS-polyacrylamide electrophoresis (Habib et al., 1984; Kimura et al., 1981). Also cell-free translation of mRNA from either system (Vertel et al., 1984) yielded immunospecific core protein which was homogeneous and also of rather large molecular weight (M_r ~340 000). These results can be contrasted with the few available estimates for the size of the core protein in the completed proteoglycan. Chondroitinase and keratanase (when necessary) digestion of proteoglycan from rat chondrosarcoma or chick epiphyses resulted in preparations of core protein which consisted of 60% protein by weight and migrated on SDS-PAGE with an apparent molecular weight of 400 000 (Kimura et al., 1981; Kimata et al., 1982; Oike et al., 1982). This would give an estimate for the molecular weight of the

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[1] Abbreviations: CSPG, chondroitin sulfate proteoglycan; RER, rough endoplasmic reticulum; Endo H, endo-β-*N*-acetylglucosaminidase H; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PITC, phenyl isothiocyanate; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetate; PMSF, phenylmethanesulfonyl fluoride; NEM, *N*-ethylmaleimide; HBSS, Hank's balanced salt solution; Tris, tris(hydroxymethyl)aminomethane; GuHCl, guanidine hydrochloride.

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core protein of approximately 240 000, if the assumption is made that the protein and carbohydrate entities contribute equally to the migration of the molecule in the gel. In early experiments, anhydrous HF treatment of hyaluronidase-digested rat chondrosarcoma proteoglycan was shown to remove most (>95%) of the residual carbohydrate, yielding a core protein with an apparent molecular weight of 210 000 on SDS–PAGE (Schwartz et al., 1985). The reduction in molecular weight of the core protein after HF treatment of the completed proteoglycan suggested that significant proteolytic cleavage may have occurred either intracellularly, in the extracellular matrix, or during sample preparation. These findings, in conjunction with the observed heterogeneity of the core protein in many systems, have left wanting a reliable estimate for the size of the core protein in completed extracellular proteoglycan, and whether intracellular proteolytic processing of core protein occurs.

Furthermore, there is increasing evidence that core protein is composed of domains with structural and functional specificity (Heinegard & Alexsson, 1977; Oike et al., 1982; Pêrin et al., 1984; Bonnet et al., 1983; Stevens & Hascall, 1986; Haynesworth et al., 1987). These studies have used clostripain, trypsin, or CNBr to produce peptide fragments, yet in no single study have fragments representing all of the major domains been produced, isolated, or characterized. In particular, peptides originating from the carbohydrate-substituted regions have not been purified and primary sequence determined before and after deglycosylation in order to provide direct evidence for the requirements for initiation of chondroitin sulfate, keratan sulfate, or *O*-linked oligosaccharides. Finally, no study of proteoglycans has directly compared predicted protein sequences from cDNA clones encoding portions of core protein with peptides isolated and directly sequenced from those same regions.

In order to accomplish some of these objectives, we have attempted to provide a comprehensive analysis of core protein using a combination of techniques from protein chemistry and molecular biology. In this paper, we report procedures for deglycosylating CSPG accompanied by minimal damage to the core protein. Application of these procedures to both in vitro and in vivo systems suggests that extensive proteolytic cleavage does not occur intracellularly and that the core protein is therefore secreted in a homogeneous, high molecular weight form. Furthermore, these methods have been used to deglycosylate two major peptides from the chondroitin sulfate rich domain of core protein, yielding for the first time reliable estimates for the protein portions of such peptides in the absence of carbohydrate. Generation and deglycosylation of these derived fragments are the first steps toward further studies of the sequence, organization, and properties of the core protein molecule.

Materials and Methods

Materials. L-[^{3}H]Serine (24 Ci/mmol) was purchased from Amersham; UDP-[^{14}C]xylose (267 mCi/mmol) and D-[6-^{3}H]glucosamine (33 Ci/mmol) were from New England Nuclear. Other materials were obtained from the following sources: F12 media and gentamycin from Gibco; UDP-xylose, pepstatin, phenylmethanesulfonyl fluoride, benzamide hydrochloride, 6-aminocaproic acid, *N*-ethylmaleimide, cycloheximide, anisole, and clostripain from Sigma; rabbit anti-rat IgG, endo-β-*N*-acetylglucosaminidase H, chondroitinase ABC, and keratanase from ICN Immunobiologicals (formerly Miles); guanidine hydrochloride (sequanal grade), PITC, and amino acid standards from Pierce; CsCl (special biochemical grade) from Gallard Schlesinger; fucose, xylose, glucose, galactose, mannose, glucuronolactone, *N*-acetylgalactosamine, *N*-acetylglucosamine, *N*-acetylneuraminic acid, and 5% SP2401 on 100–120 Supelcoport prepacked gas chromatography columns from Supelco; Sepharose CL-6B and Sephadex G-100 from Pharmacia; 70% HF in pyridine from Columbia Organic; ion retardation resin AG11A8 from Bio-Rad; and testicular hyaluronidase from Leo Helsingborg Laboratories, Sweden.

Cell Culture. Cultures of chick sternal chondrocytes were established from 14-day embryos according to previously described procedures (Habib et al., 1984; Cahn et al., 1967) with a few modifications, including the use of Falcon Primaria culture plates.

Labeling of Chick Sternal Chondrocytes. Harvested chick sternal chondrocytes prepared as described were suspended in calcium and magnesium-free HBSS at (6–9) $\times$ 10^7 cells/mL and incubated with 3–400 μCi/mL [^{3}H]serine or 200 μCi of [^{3}H]glucosamine or [^{35}S]methionine in unsupplemented F12 media at 37 °C for various times. Cells and media were processed as previously described (Habib et al., 1984; Campbell & Schwartz, 1988).

Immunoprecipitation. Labeled chondrocytes and media were prepared for immunoprecipitation and as described (Habib et al., 1984; Campbell & Schwartz, 1988). To immunoprecipitate core protein, an aliquot of labeled material was diluted to 200 μL with lysis buffer, and 20 μL of the monoclonal antibody S103L, which has been shown to precipitate chick core protein specifically, was added (Habib et al., 1984). After 2 h of incubation at 25 °C, 5 μL of normal rat serum and 200 μL of rabbit anti-rat IgG (Miles) were added and incubated 2 more h at 4 °C. The immunoprecipitates were centrifuged and washed once in lysis buffer, a second time in lysis buffer containing 0.1% SDS, and a third time in 10 mM Tris-HCl, pH 7.4, containing 0.1% Nonidet P-40.

Preparation of Proteoglycan Monomer from Embryonic Chick Epiphyses or Rat Chondrosarcoma. Extraction and purification of chondroitin sulfate proteoglycan monomer from the epiphyses of the tibias and femurs of 14-day chick embryos or from Swarm rat chondrosarcoma were essentially as described (Schwartz et al., 1985; Hascall & Kimura, 1982).

Enzyme Digestions. [^{3}H]Serine-labeled core protein immunoprecipitates were solubilized and digested with endoglycosidase H to remove mannose-enriched N-linked oligosaccharides (Habib et al., 1984; Campbell & Schwartz, 1988). After digestion, the samples were precipitated with 10% trichloroacetic acid and analyzed by SDS–polyacrylamide gel electrophoresis. Proteoglycan immunoprecipitated from the media of labeled cells was incubated with 0.2 unit of chondroitinase ABC and 0.2 unit of keratanase in 100 μL of Tris-HCl buffer, pH 7.4, containing 5 mM EDTA, 1 mM PMSF, 10 mM NEM, and 0.36 mM pepstatin at 37 °C for 4 h. The presence of these protease inhibitors has been shown by Oike et al. (1980) to prevent degradation of the core protein during the enzymatic treatment. The digested immunoprecipitates were then either directly run on SDS–polyacrylamide gels or lyophilized in preparation for HF treatment.

Proteoglycan isolated from chick epiphyses was dialyzed into 100 mM Tris-HCl buffer, pH 7.4, containing the mixture of proteases listed above, and 2.5 units of chondroitinase ABC and 0.5 unit of keratanase were added per 10 mL of sample. Digestion was allowed to continue for 4 h at 37 °C after which the digested material was run on a column (1.5 $\times$ 50 cm) of CL6B eluted in 50 mM sodium acetate, pH 6.5, with 150 mM NaCl, to separate the resultant core protein preparation from

enzyme and free carbohydrate. The void volume was pooled, dialyzed vs H_2O, and again either analyzed directly by SDS–PAGE or lyophilized prior to HF treatment. For comparison with previous studies, proteoglycan isolated from chick epiphyses was also digested with hyaluronidase. Monomer was dialyzed into 50 mM sodium acetate buffer, pH 5.0, containing 150 mM NaCl, 10 mM EDTA, 100 mM 6-aminocaproic acid, and 5 mM benzamide hydrochloride and digested with 30 μg/mL testicular hyaluronidase for 4 h at 37 °C (Keiser & Hatcher, 1979). The digest was then run on a column of CL6B and handled as described above.

Peptides Generated by Clostripain Digestion. Immunoprecipitated pulse-labeled core protein (precursor) or deglycosylated pulse–chase-labeled proteoglycan was isolated by SDS–polyacrylamide gel electrophoresis and digested with clostripain directly in the gel. The technique used was a modification of the one-dimentional peptide mapping methods described by Cleveland (1983). Briefly, samples were run on a 5% SDS–polyacrylamide gel, and 8-mm-wide slices of gel containing the material of interest were excised, washed for 1 h in 25% MeOH, and lyophilized. Gel slices were then rehydrated in a 1.5-mL microfuge tube with 40 μL of 200 mM sodium acetate and 200 mM Tris buffer, pH 7.7, containing 5 mM $CaCl_2$, 1.25 mM DTT, 1 mM PMSF, and 5 units/mL clostripain (40–60 units/mg). Rehydrated slices were incubated at 37 °C for 1 h followed by the addition of 40 μL of 2× concentrated sample buffer. After being heated at 100 °C for 8 min, the slices were placed into the wells of a 5–18% gradient gel, electrophoresed, and analyzed by autoradiography.

The method for treatment of unlabeled chondroitinase-digested CSPG with clostripain was modified slightly. Proteoglycan was dissolved to a concentration of 4 mg/mL in 100 mM Tris and 100 mM sodium acetate, pH 7.6, containing 50 mM $CaCl_2$, 2.5 mM DTT, and 1 mM PMSF. Clostripain was preincubated in the same buffer for 2–3 h at 23 °C and added to a concentration of 5 units/mL, and the mixture was incubated for 16 h at 37 °C. The peptides were purified on DEAE-cellulose, preequilibrated in 50 mM Tris-HCl, pH 8.0, containing 0.2% CHAPS. Samples (containing 10 mg of clostripain-digested proteoglycan, or less) were loaded onto columns (2 × 5 cm), washed with 30 mL of preequilibration buffer, and eluted stepwise with the same buffer containing 0.25 M NaCl, 0.4 M NaCl, and finally with 1.0 M NaCl. Samples were then run on a Toya Soda 3000 or 4000 column (7.5 × 600 mm, with a 7.5 × 75 mm precolumn) and eluted with 0.15 M ammonium acetate, pH 7.0. The optical density of the effluent was measured at 230 nm, and fractions within each peak were pooled, concentrated by vacuum centrifugation, recycled, and reconcentrated before analysis.

Deglycosylation of CSPG by HF in Pyridine. Lyophilized enzymatically treated CSPG was dried in a polyethylene container at 40 °C for 24 h under vacuum prior to chemical deglycosylation. The dried core protein preparation was treated with HF in pyridine with anisole as a scavenger of released carbohydrate entities [0.1 mL of anisole (mL of HF in pyridine)$^{-1}$ (10 mg of core protein)$^{-1}$] (Olson et al., 1985). Reaction was carried out with gentle stirring at 25 °C, and then the vessel was rapidly evacuated in a desiccator at 4 °C for 20 min. Samples to be analyzed by SDS–PAGE were further evacuated for 40 min in a Speed Vac concentrator (Savant) prior to chloroform/methanol precipitation (Wessel & Flugge, 1984).

In order to collect enough deglycosylated proteoglycan for analyses of amino acid and carbohydrate content as well as xylosyltransferase acceptor activity, an alternate procedure for removing the HF and cleaved carbohydrate was employed. In this procedure, the vessel was evacuated for an additional hour at 4 °C, and then the sample was run on an ion retardation column (0.7 × 30 cm) of AG11A8 (Bio-Rad) eluted with H_2O. The void volume was pooled and run on a column (1.5 × 25 cm) of Sephadex G-100 eluted in 50 mM sodium acetate, pH 6.5, with 150 mM NaCl. The void volume, which contained all of the eluted protein, was pooled and dialyzed vs H_2O.

Analytical Procedures. SDS–polyacrylamide slab gel electrophoresis was carried out by the method of Laemmli (1980), using 3.75% resolving gels and 3.0% stacking gels as previously described (Habib et al., 1984; Campbell & Schwartz, 1988). Samples for amino acid analysis were hydrolyzed in distilled 6 N HCl containing 0.1% phenol under vacuum at 110 °C for 24 h, coupled to PITC, and run on an octadecyl column (4.5 × 250 mm, IBM), using a Varian 5000 liquid chromatography instrument as previously described (Heinrikson & Meredith, 1984). Carbohydrates were quantitated as the trifluoroacetate derivatives of the *O*-methyl glycosides using inositol as an internal standard (Zanetta et al., 1972). The derivatized samples were analyzed by gas-liquid chromatography (Hewlett Packard 5840 chromatograph and 5840A data processor) on an SP-2401 column (6 ft × $^1/_8$ in. i.d.). The column temperature was programmed from 100 to 200 °C at a rate of 1 °C/min. Assays for xylosyltransferase acceptor activity were performed as described previously (Schwartz & Rodén, 1974; Olson et al., 1985).

Results

Limit Deglycosylation of CSPG. Improved procedures for nearly complete deglycosylation of chondroitin sulfate proteoglycan (CSPG) of cartilage consist of enzymatic digestion with chondroitinase and keratanase followed by treatment with anhydrous HF in pyridine. On the basis of the previous work of Oike et al. (1980, 1982), chondroitinase and keratanase digestion was used to remove the majority of carbohydrate substituents from CSPG, leaving only the oligosaccharides and the linkage region carbohydrates still attached. Figure 1 shows the results obtained when these procedures were used to deglycosylate [^{3}H]serine-labeled CSPG secreted by cultured chick sternal chondrocytes. As expected, chondroitinase and keratanase digestion (lanes A and J) yielded a homogeneous core protein preparation of apparent M_r ~400 000. HF treatment was then used to remove the remaining carbohydrate residues, which still account for a significant fraction (40% by weight) of the enzymatically digested core protein preparation, using new methods for rapidly removing the core protein after HF treatment to help minimize degradation of the protein core (see Materials and Methods). A steady decrease in the apparent molecular weight of the core protein preparation was observed (Figure 1, lanes D–G), while the core protein preparation remained homogeneous and of rather high molecular weight (M_r ~370 000 after 2 h of HF treatment). These experiments were repeated by using either [^{35}S]-methionine- or [^{3}H]leucine-labeled core protein, and identical results were obtained in each case, reducing the possibility that any prominent degradation products might have been overlooked due to lack of serine content (data not shown).

While greater than 85% of the total radioactivity of the samples was recovered after 2 h of HF treatment and subsequent chloroform/methanol precipitation, the yield of intact core protein decreased more significantly with time of HF treatment (Figure 1, lanes D–G). Quantitation of the recovery of intact core protein by slicing and counting the appropriate

Table I: Recovery and Content of Core Protein Preparations after Deglycosylation of CSPG

% recovery of [^{3}H]Ser-labeled intact core protein[a]			
chondroitinase + keratanase digested	0.5 h HF	1 H HF	2 h HF
99	59	41	22

	amino acid content (residues/1000)[b]			
amino acid	chondroitinase + keratanase digested	0.5 h HF	1 h HF	2 h HF
Asx	56.9	55.2	51.8	53.1
Glx	142.1	135.6	138.4	142.0
Ser	135.6	132.0	141.2	135.6
Gly	123.1	138.3	141.2	125.8
Tyr	73.5	66.0	63.5	73.3
Ala	88.0	95.8	99.7	87.7
Arg	35.5	34.4	32.5	34.6
Pro	62.9	59.7	54.4	62.3
Tyr	34.7	37.1	29.8	38.1
Val	57.7	54.2	51.8	57.1
Met[c]	19.4	19.0	19.6	18.5
Ile	46.8	43.4	42.7	48.5
Leu	61.4	56.1	59.6	61.7
Phe	44.0	47.0	49.2	37.5
Lys	11.3	12.1	13.2	10.5

[a] Recovery of intact core protein after deglycosylation of CSPG. Cultured embryonic chick sternal chondrocytes were labeled for 2 h with [^{3}H]serine and then chased for 24 h. Media were collected, immunoprecipitated for the core protein of CSPG, and deglycosylated by using chondroitinase and keratanase followed by HF treatment. Samples were electrophoresed on 3.7% gels (similar to those shown in Figure 1) which were sliced and counted to quantitate the recovery of intact core protein ([^{3}H]serine). [b] CSPG extracted and purified from chick epiphyses was deglycosylated, and the resultant core protein preparations were hydrolyzed, coupled to PITC, and analyzed as described under Materials and Methods. [c] Parallel samples run on a Beckman analyzer with coupling to ninhydrin uniformly yielded analyses which were very similar for every residue except methionine. The alternate method gave estimates of methionine content of approximately 7.9 residues/1000.

Table II: Removal of Carbohydrate by HF Treatment

	μg of carbohydrate[a]/mg of protein[b]			
carbohydrate	chondroitinase + keratanase digested	0.5 h HF	1 h HF	2 h HF
Xyl	62.6	27.4	25.0	7.0
Gal	236.0	86.4	70.6	34.6
GlcUA	61.8	24.0	nd[c]	nd
Man	17.4	15.7	20.6	12.2
GalNAc	152.0	81.0	63.6	33.8
GlcNAc	76.2	43.8	33.6	17.8
NeuNAc	77.6	6.60	7.20	1.84
total (% remaining)	684 (100)	285 (42)	221 (32)	107 (15)

	xylosyltransferase acceptor act. (cpm μg^{-1} h^{-1})[d]		
chondroitinase + keratanase digested	0.5 h HF	1 h HF	2 h HF
8.3	251	359	392

[a] CSPG extracted and purified from chick epiphyses was deglycosylated and analyzed for carbohydrate content as described under Materials and Methods. [b] Quantitative amino acid analysis was used to estimate the protein content of the samples. [c] Not detected. [d] Xylosyltransferase acceptor activity of deglycosylated CSPG. CSPG extracted and purified form chick epiphyses was deglycosylated, and the resultant core protein preparations were tested in the xylosyltransferase assay for acceptor activity as described under Materials and Methods.

region of the gel suggested that the core protein degraded with first-order kinetics and a half-life of approximately 51 min (see Table I). Random cleavage of peptide bonds in the HF may account for the loss of intact core protein in this experiment since no distinct breakdown products were detected in any of the samples. Even a very low rate of random peptide bond cleavage in this harsh chemical treatment could significantly reduce the yield of intact core protein considering the very large size of this molecule. The loss of core protein in radiolabeled experiments may be due to the small amount of material used, as much better recovery of intact core protein was observed when unlabeled epiphyseal CSPG or derived peptides were deglycosylated (as discussed below).

To confirm the effectiveness of the HF treatment, [^{3}H]-glucosamine-labeled CSPG was deglycosylated, and the amount of carbohydrate removed at each step was quantitated by slicing and counting the region of the gel containing the intact core protein (data not shown); [^{35}S]methionine-labeled CSPG was used as a control for loss of intact core protein during the procedure. The enzymatic digestion proved successful at removing the majority of the carbohydrate (93%) as expected, and HF then rapidly removed most of the rest of the carbohydrate residues. After only 45 min of HF treatment, 99% of the [^{3}H]glucosamine had been removed from the remaining intact core protein.

Unlabeled CSPG from chick epiphyses was also deglycosylated by these procedures, and sufficient amounts were accumulated to permit analysis of carbohydrate content, xylosyltransferase acceptor activity, and amino acid content. As shown in Figure 1 (equal amounts of material were deglycosylated in lanes N–R), loss of intact core protein during deglycosylation was apparently less of a problem in these preparative experiments than in the previous analytical ones although by 4 h of HF treatment (lane R) there was significant loss. Perhaps the greater mass of proteoglycan used in these experiments facilitated more complete recovery of the protein core. Carbohydrate analysis indicated that prior to HF treatment, the core protein preparation from chick epiphyses contained approximately 40% carbohydrate, or 684 μg of carbohydrate/mg of protein (Table II). HF treatment for 2 h removed 85% of this residual carbohydrate, which consists primarily of linkage region sugars and the oligosaccharides remaining after enzymatic digestion. Thus, the proteoglycan was converted by enzymatic digestion and HF treatment from a molecule consisting of >90% carbohydrate to one of approximately 10% carbohydrate. The dramatic decrease in xylose content is particularly notable. Approximately 90% of the xylose residues were removed after 2 h of HF treatment, indicating that effective removal of the chondroitin sulfate linkage region was occurring. This observation was verified by testing the core protein preparations at various times of HF treatment for xylosyltransferase acceptor activity (Table II). As expected, acceptor activity was very low after only enzymatic digestion (0-min time point), since the linkage region was intact; however, acceptor activity rapidly increased and reached a plateau during the course of HF treatment. This hyperbolic pattern is consistent with quick removal of the majority of the xylose residues, followed by a steady approach toward limit deglycosylation, similar to the pattern observed for glucosamine loss. In contrast to these results, very little change in amino acid content was detected after HF treatment (Table I), confirming the effectiveness of the HF treatment in removing the remaining carbohydrate (especially chondroitin sulfate residues) while leaving intact the protein core.

Comparison of Deglycosylated CSPG and Precursor Core Protein. Having established the efficacy of the deglycosylation procedures, the resultant core protein preparation was compared to the intracellular precursor form. When precursor core protein immunoprecipitated from [^{3}H]serine pulse-labeled embryonic chick sternal chondrocytes was analyzed by SDS–

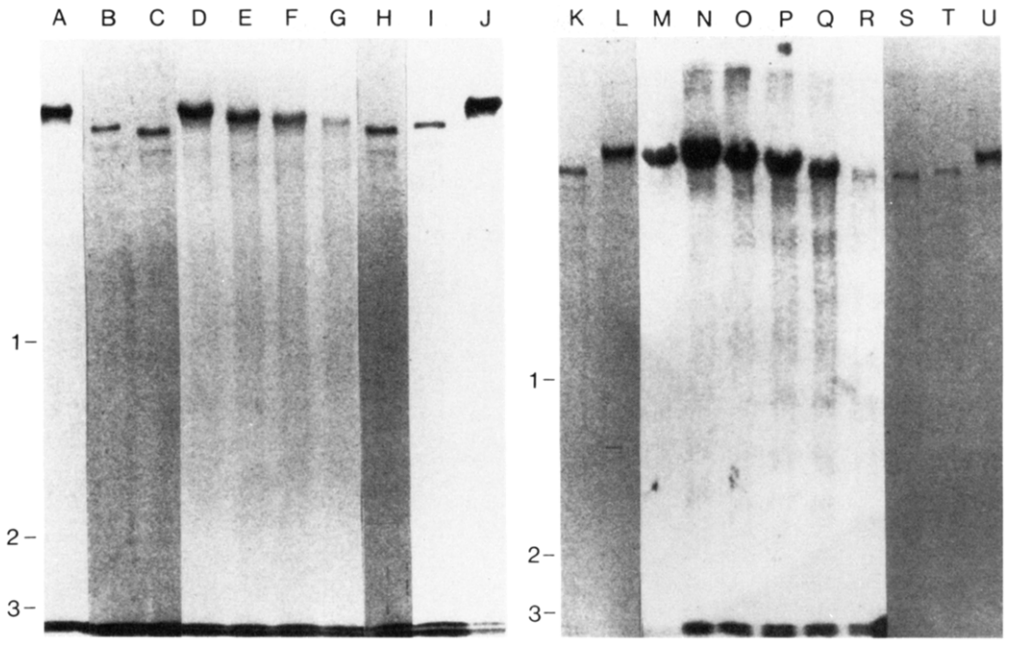

FIGURE 1: SDS-polyacrylamide gel electrophoresis of embryonic chick CSPG at various stages of deglycosylation. The samples in lanes A–L and S–U are derived from [^{3}H]serine-labeled CSPG and precursor core protein synthesized by chick sternal chondrocytes in culture. In contrast, the unlabeled samples in lanes M–R are derived from CSPG synthesized in vivo by embryonic chick epiphyses. Lanes A–J, [^{3}H]serine-labeled CSPG secreted by chick sternal chondrocytes in culture was isolated by immunoprecipitation, digested with chondroitinase and keratanase, and then treated with HF in pyridine as described under Materials and Methods. Also shown for comparison is precursor core protein immunoprecipitated from pulse-labeled chondrocytes. Samples were electrophoresed on a 3.75% SDS-polyacrylamide gel and put up for autoradiography for 24 or 48 h. Controls omitting primary or secondary antibodies have shown that material of M_r <240 000 represents nonspecific immunoprecipitate (1). Lanes A and J, chondroitinase- and keratanase-digested CSPG. Lanes D–G, chondroitinase- and keratanase-digested CSPG treated with HF for 15, 30, 60, and 120 min, respectively. Lanes B and I, precursor core protein. Lanes C and H, precursor core protein digested with endoglycosidase H. Lanes B, C, and H were taken from the 48-h exposure and combined with the other lanes which were taken from the 24-h exposure. Molecular weight standards indicated in the margin are as follows: (1) myosin (200 000); (2) β-galactosidase (116 000); and (3) phosphorylase B (92 000). Lanes K–U, unlabeled CSPG purified from embryonic chick epiphyses at various stages of deglycosylation. Also run on the gel (3.70% SDS–PAGE) for comparison is precursor core protein immunoprecipitated from [^{3}H]serine pulse-labeled chick sternal chondrocytes. Likewise, chondroitinase- and keratanase-digested CSPG from [^{3}H]serine pulse–chase-labeled sternal chondrocytes was also analyzed. After electrophoresis, the gel was stained with Coomassie brilliant blue R and then dried and put up for autoradiography. A picture of the dried Coomassie-stained gel (lanes M–R) was lined up with the autoradiograph to allow direct comparison of the relative migration rates of the various samples to be made. Lanes K and S, precursor core protein from pulse-labeled sternal chondrocytes digested with endoglycosidase H. Lanes L and U, chondroitinase- and keratanase-digested CSPG from pulse–chase-labeled sternal chondrocytes. Lane T, precursor core protein from pulse-labeled sternal chondrocytes. Lane M, chondroitinase- and keratanase-digested CSPG from chick epiphyses. Lanes N–R, chondroitinase- and keratanase-digested CSPG from chick epiphyses treated with HF for 15, 30, 60, 120, and 240 min, respectively. The molecular weight standards indicated in the margin are the same as those used for the first gel.

PAGE (Figure 1, lanes B and I), the newly synthesized, N-glycosylated core protein was shown to migrate with an apparent molecular weight of 370 000, as previously reported (Habib et al., 1984; Campbell & Schwartz, 1988). Endoglycosidase H digestion of this material yielded a precursor core protein, theoretically devoid of any carbohydrate, which migrated with an apparent molecular weight of 360 000 (Figure 1, lanes C and H). Realizing the limitations of molecular weight estimations in these gel systems, the fact that the core protein from deglycosylated CSPG (Figure 1, lane G) migrated at apparoximately the same rate as the unprocessed endo H digested precursor strongly suggested a similarity in size and/or composition. Since neigher core protein at this point should contain significant amounts of carbohydrate to influence migration rate, these results would indicate that significant intracellular proteolytic cleavage is not occurring in this in vitro system during modification of core protein to CSPG. If such cleavage was occurring, the deglycosylated core protein would migrate more rapidly than the precursor.

Unlabeled CSPG synthesized by embryonic chick epiphyses was also isolated and deglycosylated to determine whether significant intracellular proteolytic cleavage occurs in vivo. As before, HF treatment (Figure 1, lanes N–R) yielded a homogeneous, high molecular weight core protein preparation. Also included in Figure 1 for comparison is unprocessed core protein from chick sternal chondrocytes (lane S). Again, these two core protein preparations exhibit very similar migration rates in the gel system, suggesting that extensive proteolytic cleavage of the core protein does not occur in vivo in embryonic chick epiphyses. This is significant since this sample consists of molecules that have resided in the tissue during the life of the day-14 embryos.

Our early attempts to deglycosylate CSPG from rat chondrosarcoma by hyaluronidase digestion followed by HF treatment and purification by gel filtration yielded a core protein preparation which migrated with an apparent molecular weight of approximately 210 000 on SDS–PAGE (Schwartz et al., 1985). As shown previously (Kimura et al., 1981), the precursor core protein from these cells (like the chick cells) appears to be of rather high molecular weight (370 000), suggesting that proteolytic cleavage occurred intracellularly, in the extracellular matrix, or during preparation. Given these contrasting results, a comparison of hyaluronidase and the combined chondroitinase and keratanase digestion was carried out followed by HF treatment of chick CSPG. As previously reported for rat chondrosarcoma CSPG (Schwartz et al., 1985), a prominent band at M_r ~220 000 was observed in the chick preparations treated with hyaluronidase, which was then reduced slightly to approximately 210 000 after HF treatment (data not shown). Thus, degradation during preparation (in the hyaluronidase digestions) evidently ac-

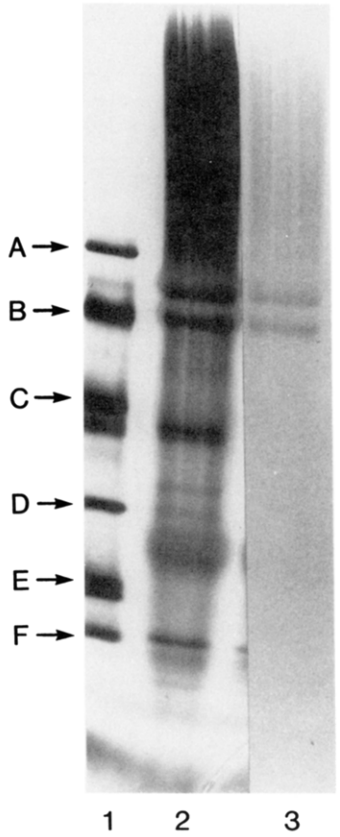

FIGURE 2: Clostripain digest of chondrotinase-treated unlabeled CSPG. Chondroitinase-treated unlabeled CSPG (D_1D_1) was treated with clostripain for 16 h at 37 °C, electrophoresed on a 5–18% polyacrylamide gel, and stained first with Alcian blue (lane 3) and then with ammoniacal silver (lane 2). Lane 1 contains molecular weight markers: (A) phosphorylase B (92.5K); (B) BSA (66.2K); (C) ovalbumin (45K); (D) carbonic anhydrase (31K); (E) soybean trypsin inhibitor (21.5K); (F) lysozyme (14K).

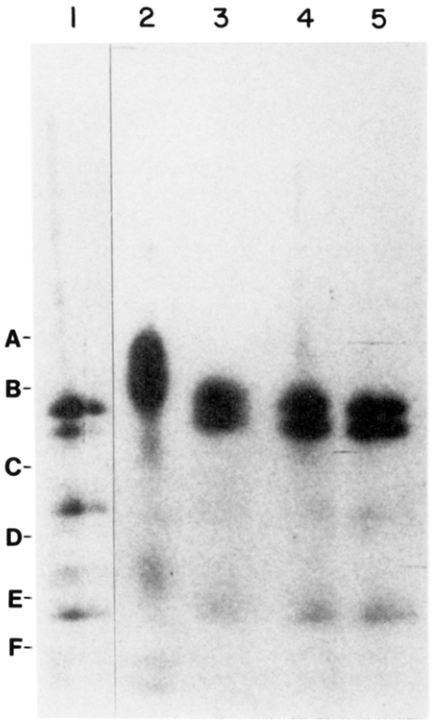

FIGURE 3: Peptide patterns generated by clostripain digestion of precursor core protein and deglycosylated CSPG. Immunoprecipitated [^{3}H]serine pulse-labeled core protein (precursor) or deglycosylated pulse–chase-labeled CSPG was isolated by SDS-polyacrylamide gel electrophoresis and digested with clostripain directly in the gel (see Materials and Methods). Digested samples were then electrophoresed on a 5–18% gradient gel which was put up for autoradiography. Lane 1, endoglycosidase H digested pulse-labeled core protein (precursor) immunoprecipitated from chick sternal chondrocytes. Lanes 2–5, CSPG immunoprecipitated from the media of pulse–chase-labeled chondrocytes which have been digested with chondroitinase and keratanase followed by HF treatment for 0, 30, 60, and 90 min, respectively. Molecular weight standards indicated in the margin are as indicated for Figure 2.

counts for the extensive proteolytic cleavage observed in the earlier studies of rat chondrosarcoma proteoglycan and of the current studies on chick CSPG.

Deglycosylation of Peptides from Chondroitin Sulfate Rich Domains. The deglycosylation procedure has also been extended to peptide fragments derived from proteoglycans. As part of a comprehensive study to investigate the domain structure of chick CSPG in detail, peptides from all regions of the core protein were generated by clostripain digestion and subsequently identified by differential chondroitinase and keratanase susceptibility and carbohydrate analysis (in preparation). An example of the peptide pattern obtained from clostripain treatment of chondroitinase-digested unlabeled chick CSPG, stained with Alcian blue and ammoniacal silver [a special staining procedure developed in order to detect the otherwise poorly detectable proteoglycan fragments; see Krueger and Schwartz (1987)], is shown in Figure 2. Three predominant peptides of apparent molecular masses 86, 75, and 27 kDa, several smaller peptides, and a heterogeneous smear from ~90 kDa to the top of the gel are routinely observed.

We have focused for further deglycosylation and characterization studies on the two largest distinct peptides that arise after chondroitinase treatment, i.e., the 86- and 75-kDa species, designated CS-A and CS-B. It should be mentioned that no change in migration of these peptides was observed after subsequent keratanase digestion, indicating that they contain primarily chondroitin sulfate chains. Furthermore, carbohydrate analysis revealed that they consist predominantly of the residues xylose, galactose, glucuronic acid, and *N*-acetylgalactosamine in molar proportions (0.79:2.09:1.26:0.86) characteristic of the known CS linkage pentasaccharide (1:2:1:1). These peptides were then further deglycosylated with HF/pyridine. Unfortunately, after removal of the linkage region sugars, the peptides no longer stained sufficiently, even with the Alcian blue/ammoniacal procedure. Therefore, Figure 3 shows peptide patterns generated after clostripain treatment of [^{3}H]serine-labeled CSPG previously digested with chondroitinase and keratanase (lane 2). The 86- and 75-kDa peptides (CS-A and CS-B) were heavily labeled with serine and were now the predominant species, although all the peptides detected by the Alcian blue/ammoniacal silver staining were revealed by longer exposure. Also, peptide patterns derived from [^{35}S]methionine- or [^{3}H]leucine-labeled proteoglycan (not shown) were identical qualitatively, reducing the possibility that prominent peptides were overlooked. The major peptides were still present after HF treatment for up to 90 min, yielding final molecular weight values of 58K and 50K for CS-A and CS-B, respectively (Figure 3, lanes 2–5). CS-A and CS-B followed similar kinetics of deglycosylation to that observed for CSPG (Figure 1), reaching limit deglycosylation after 1.5 h of HF treatment with quantitatively no loss of material, further verifying the effectiveness of this procedure. Interestingly, the peptide patterns of precursor core protein and HF-deglycosylated CSPG (Figure 3, lanes 1 and 5) were strikingly similar, again suggesting that significant intracellular processing of core protein by proteolysis does not

occur during biogenesis of CSPG.

Carbohydrate analysis of purified CS-A and CS-B before and after 90 min of HF treatment revealed that the linkage region sugars xylose, galactose, *N*-acetylgalactosamine, and glucuronic acid were reduced as follows: from 110 to 15, 290 to 60, 175 to 40, and 120 to 45 μg of carbohydrate/mg of protein, respectively. These values are similar overall to those presented in Table II for the intact CSPG for these four residues, characteristic of chondroitin sulfate chains. In contrast, *N*-acetylglucosamine, mannose, and fucose, more characteristic of keratan sulfate or N-linked oligosaccharides, were less prevalent or not present at all on these peptides, even before HF treatment (i.e., 36, 5, and 0 μg of carbohydrate/mg of protein, respectively). Xylosyltransferase acceptor activity of the peptides was in the same range as the intact core protein; increasing from 4 cpm/μg of protein before to 245 cpm/μg of protein after HF deglycosylation. The excellent yield of the deglycosylated peptides in these experiments suggests that the deglycosylation procedures should be particularly useful for the further investigation and characterization of the glycosylated domains of proteoglycans.

Discussion

Chondroitin sulfate proteoglycan is an extremely complex and polydisperse macromolecule. Variation in the number or size of carbohydrate chains is the main source of this polydispersity (Fellini et al., 1981); however, in many systems, heterogeneity of core protein length also appears to be an important factor (Rosenberg et al., 1976; Heinegard, 1977; Buckwalter & Rosenberg, 1982). Unfortunately, little fundamental information is available about the structure of the core protein primarily because it is so heavily glycosylated and thus has been inaccessible for study. We have recently developed procedures which allow nearly complete deglycosylation of the proteoglycan with minimal core protein degradation. Application of these techniques to proteoglycans synthesized by embryonic chick chondrocytes yielded core protein preparations which were homogeneous and of high molecular weight (approximately 370 000), suggesting that significant intracellular proteolytic cleavage does not occur during production of the 2.5×10^6 Da CSPG. These findings raise the possibility that the heterogeneity observed in several previous studies may have resulted from degradation either in the extracellular matrix or during preparation (as observed artifactually with the use of hyaluronidase). Proteoglycans in which heterogeneity of core protein length has been observed share in common the fact that they originate from adult tissue types, bovine nasal septum, or humoral articular cartilage, in particular. Perhaps with time after secretion, the core protein is proteolytically degraded while residing in the extracellular matrix. However the CSPG accumulating in embryonic chick epiphyses up to day 14 appears to have a homogeneous and high molecular weight core protein.

Our results suggesting that extensive intracellular proteolytic processing of the core protein does not normally occur must be qualified given the inherent limitations of the gel system at resolving high molecular weight proteins as well as the fact that complete deglycosylation is difficult both to achieve and to establish. With these considerations in mind, the possibility remains that a small but undetectable percentage of the core protein might be removed during intracellular processing. In fact, some core proteins from simpler proteoglycans appear to be proteolytically processed in a manner similar to other secretory proteins, i.e., by the removal of a propeptide (Bourdon et al., 1986). The removal of a propeptide from the core protein of cartilage CSPG, while potentially of physiological importance, would be very difficult to detect, considering the large size of the precursor. Nevertheless, the demonstration that the core protein is secreted in an apparently homogeneous, high molecular weight form is significant given previous data suggesting otherwise (Kimura, 1981; Schwartz et al., 1985).

From this and our other work, we have also generated a series of major peptides from chick CSPG by clostripain digestion, identified the domains from which they originate, and characterized them with respect to several structural and functional properties (unpublished results). Two of the peptides (CS-A and CS-B) that appear to contain the largest amount of carbohydrate, even after chondroitinase and keratanase treatment, have been further deglycosylated by the HF procedure. These peptides follow the same pattern of deglycosylation as the intact proteoglycan, with a limit digestion by 90 min of treatment. Carbohydrate removal was as efficient, although the sugar composition was slightly different as expected for peptides primarily substituted with chondroitin sulfate chains. The deglycosylation procedure has allowed for the first time available estimates of the molecular size of the protein portion of peptide fragments derived from highly glycosylated molecules like proteoglycans. The significant reduction in molecular weight (86K to 58K and 75K to 50K for CS-A and CS-B, respectively), as well as the substantial loss of carbohydrate, should emphasize the severe limitations of all previous studies in drawing conclusions on the nature and properties of only partially (enzymatically) deglycosylated core protein peptides. The ability to effectively deglycosylate proteoglycans, or fragments derived from them, promises to play an important role in the further study of the sequence, organization, and properties of core protein molecules.

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Interaction of an Organophosphate with a Peripheral Site on Acetylcholinesterase†

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ABSTRACT: *O*-Ethyl *S*-[2-(diisopropylamino)ethyl] methylphosphonothioate (MPT) is an active site directed inhibitor of acetylcholinesterase (AChE). Inhibition of the *Electrophorus electricus* (G4) enzyme follows classical second-order kinetics. However, inhibition of total mouse skeletal muscle AChE and inhibition of the individual molecular forms from muscle, including the monomeric species, do not proceed as simple irreversible bimolecular reactions. Similarly, complex inhibition kinetics are observed for the purified enzyme from *Torpedo californica*. AChE can be cross-linked with glutaraldehyde into a semisolid matrix. Under these conditions the abnormal concentration dependence for MPT inhibition is accentuated, and a range of MPT concentrations can be found where inhibition of polymerized AChE is far less than that observed at lower concentrations. Inhibition in certain concentration ranges is partially reversible after removal of all unbound ligand. Thus, there are two different modes of organophosphorus inhibition by MPT: the classical irreversible phosphorylation of the active site and a reversible interaction at a site peripheral to the active center. Propidium, a well-studed peripheral site ligand, can prevent the later interaction. Hence, the second site of MPT interaction with AChE may overlap or be linked to the peripheral anionic site of AChE characterized by the binding of propidium and other peripheral site inhibitors.

Acetylcholinesterase (AChE;[1] acetylcholine hydrolase; EC 3.1.1.7) is a widely distributed enzyme found in neural and nonneural tissues (Hall, 1973; Rieger & Vigny, 1976; Ott & Brodbeck, 1978; Massoulié & Bon, 1982). The enzyme exists in several molecular forms. In skeletal muscle, AChE is present as soluble and membrane-bound globular forms and as dimensionally asymmetric forms. The asymmetric forms are found not only in the motor end plate (Hall, 1973; Vigny et al., 1976), where they are primarily localized in the synaptic basal lamina, but also in intracellular loci (Dreyfus et al., 1983). In the electric organ of *Torpedo* both classes of molecular species have been found, whereas in *Electrophorus* only the asymmetric species have been identified. The catalytic properties of all the molecular forms of AChE appear identical in kinetic parameters or susceptibility to inhibition. The AChE active center is known to include a nucleophilic subsite that

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[1] Abbreviations: AChE, acetylcholinesterase; MPT, *O*-ethyl *S*-[2-(diisopropylamino)ethyl] methyl phosphonothioate; BuChE, butyrylcholinesterase; DFP, diisopropyl fluorophosphate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

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