Ghelis, C., & Yon, J. (1982) *Protein Folding*, p 562, Academic, New York.
Gratton, E., Alcala, R., Marriott, G., & Prendergast, F. (1986) in *Progress and Challenges in Natural and Synthetic Polymer Research* (Kwabata, C., & Bishop, A., Eds.) Ohmshu, Tokyo.
Hartmann, H., Parak, F., Steigmann, W., Petsko, G. A., Ponzi, R. D., & Frauenfelder, H. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 4967–4971.
Irace, G., Bismuto, E., Savy, F., & Colonna, G. (1986) *Arch. Biochem. Biosphys. 244*, 459–464.
James, D., & Ware, W. (1985) *Chem. Phys. Lett. 120*, 455–459.
Kuriyan, J., Wilz, S., Karplus, M., & Petsko, G. A. (1986) *J. Mol. Biol. 192*, 133.
Lakowicz, J. R., Cherek, H., & Balter, A. (1981) *J. Biochem. Biophys. Methods 15*, 131–146.
Pace, C. N. (1975) *CRC Crit. Rev. Biochem. 3*, 1–43.
Ragone, R., Colonna, G., Bismuto, E., & Irace, G. (1987) *Biochemistry 26*, 2130–2134.
Szabo, G. A., & Rayner, D. M. (1980) *J. Am. Chem. Soc. 102*, 554–563.
Tanford, C. (1968) *Adv. Protein Chem. 23*, 121–282.
Teale, F. W. J. (1959) *Biochim. Biophys. Acta 35*, 543.
Wetlaufer, D. B. (1962) *Adv. Protein Chem. 17*, 303–390.

Structural Characterization of Heparan Sulfate Proteoglycan Subclasses Isolated from Bovine Aortic Endothelial Cell Cultures†

Michael G. Kinsella* and Thomas N. Wight

Department of Pathology SM-30, School of Medicine, University of Washington, Seattle, Washington 98195

Received May 28, 1987; Revised Manuscript Received September 25, 1987

ABSTRACT: Labeled heparan sulfate proteoglycans (HSPG) were isolated from wounded and confluent cultures of bovine aortic endothelial cells by nondegradative extraction with 4 M guanidine hydrochloride and detergent. HSPG were separated from more highly charged chondroitin or dermatan sulfate proteoglycans by ion-exchange chromatography, and subclasses of different hydrodynamic size were isolated by gel filtration. Three major subclasses of HSPG were characterized structurally with respect to the presence and relative size of protein core, the presence and amount of nonsulfated oligosaccharide, and size and structure of heparan sulfate (HS) chains. The largest (600–800-kDa) HSPG subclass (I), isolated from cell layers and media of confluent cultures, bears 38-kDa HS chains on an apparently heterogeneous class of relatively large glycoprotein cores. HSPG II (150–200 kDa), isolated from cell layer or media, has 22-kDa HS chains and smaller core glycoproteins (<50 kDa). HSPG III, the subclass of smallest hydrodynamic size, has 13-kDa HS chains and a glycopeptide core of less than 15 kDa. All subclasses bear varying proportions of non-sulfated oligosaccharides of similar sizes. Comparisons of HS chain structure indicated that the different subclasses have similar proportions (49–55%) of *N*-sulfate, with both *O*-sulfate and highly N-sulfated blocks of disaccharide distributed similarly along HS chains. In addition, HS chains from subclasses II and III contain sequences that are insensitive to periodate oxidation or heparitinase digestion, suggesting that they contain increased proportions of iduronate. HSPG isolated from wounded cultures were structurally similar to those isolated from confluent cultures, except the lowest M_r subclass (HSPG III) has longer HS chains (22 kDa) and little or no associated protein. Like the 22-kDa HS II chains isolated from confluent cultures, media HS III chains isolated from wounded cultures contained a heparitinase-insensitive portion, similar in size to the heparitinase-resistant portion of the 13-kDa HS III chain from confluent cultures.

Vascular endothelial cells synthesize a variety of connective tissue macromolecules including both heparan sulfate proteoglycans (HSPG)[1] and chondroitin or dermatan sulfate proteoglycans (Kramer et al., 1982; Oohira et al., 1983). Although the functions of proteoglycans in endothelial cell biology are incompletely understood, several studies suggest a role for HSPGs in the binding of anticoagulant factors (Marcum et al., 1986) and lipoprotein lipase (Shimda et al., 1981; Cheng et al., 1981) to the endothelial cell surface and as the putative source of "heparin-like" molecules that may be involved in growth control (Castellot et al., 1981). In addition, HSPGs are incorporated into the structure of basement membranes, where they may influence vascular permeability (Kanwar & Farquhar, 1979). More generally, HSPG is associated with the cell surface of many types of cells, either integrated into the plasma membrane or bound to a receptor (Kjellēn et al., 1980; Norling et al., 1981; Rapraeger & Bernfield, 1983). Cell surface HSPG associated with other matrical molecules such as fibronectin (Hayman et al., 1982;

† This work was supported by a grant-in-aid from the American Heart Association and NIH Grant HL 18645 (T.N.W.).

* Author to whom correspondence should be addressed.

[1] Abbreviations: BAEC, bovine aortic endothelial cells; HSPG, heparan sulfate proteoglycan; HS, heparan sulfate; Tris, tris(hydroxymethyl)aminomethane; Gdn-HCl, guanidine hydrochloride; glcN, glucosamine; glcUA, glucuronic acid; idoUA, iduronic acid; EDTA, ethylenediaminetetraacetic acid; DEAE, diethylaminoethyl; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; GAG, glycosaminoglycans; CS, chondroitin sulfate; DSPG, dermatan sulfate proteoglycan; PMSF, phenylmethanesulfonyl fluoride.

0006-2960/88/0427-2136$01.50/0 © 1988 American Chemical Society

Woods et al., 1984) and collagen (Hedman et al., 1982; Koda et al., 1985) may play a role in cell adhesion and spreading (Laterra et al., 1983).

Cell culture studies have revealed at least three size classes of HSPG in endothelial cell cultures, but it is not clear whether a relationship exists between any of these classes (Oohira et al., 1983; Buonassissi & Colburn, 1983; Marcum et al., 1986; Keller et al., 1987). Recent work has demonstrated an increased proportion of lower molecular weight HSPG subclasses present in endothelial cell cultures either undergoing "sprouting" (Oohira et al., 1983) or in which migration has been stimulated by wounding (Kinsella & Wight, 1986). These results suggest that the synthesis and/or turnover of HSPG is altered under conditions of increased proliferation and migration. Clearly, a comparison of the structure of HSPG subclasses may provide insight into whether some of the subclasses are related by metabolic processing or turnover mechanisms.

Materials and Methods

Materials. Guanidine hydrochloride (grade I), Tris base, EDTA, papain (crude powder), dithiothreitol, iodoacetamide, L-cysteine, pepstatin, soybean trypsin inhibitor, *N*-ethylmaleimide, and phenylmethanesulfonyl fluoride were all purchased from Sigma Chemical Co., St. Louis, MO; 6-aminohexanoic acid and benzamidine were from Eastman Kodak Co., Rochester, NY; chondroitin ABC lyase and heparitinase (EC 4.2.2.8; *Flavobacterium heparinum*) were from Miles Laboratories, Elkhart, IN (now ICN Biochemicals, Irvine, CA); sodium borohydride was from Wilshire Chemical Co., Gardena, CA; Spectrapor dialysis tubing was from Spectrum Medical Industries, Inc., Los Angeles, CA; Aquacide I was from Calbiochem, San Diego, CA; Sephadex G-50, DEAE-Sephacel and Sepharose CL-2B, CL-4B, and CL-6B were from Pharmacia Fine Chemicals, Piscataway, NJ; Bio-Gel P-10 was from Bio-Rad Laboratories, Richmond, CA; $Na_2[^{35}S]O_4$ (carrier free), D-[6-^{3}H]glucosamine hydrochloride (31 Ci/mM) and Triton X-100 were from New England Nuclear (NEN), Boston, MA; L-[G-^{3}H]serine (10 Ci/mM) and [^{35}S]methionine (1198 Ci/mM) were from ICN Radiochemicals, Irvine, CA; and all cell culture supplies were from Grand Island Biological Co., Grand Island, NY. All other chemicals were reagent grade.

Cell Culture Radiolabeling and Proteoglycan Extraction. Cultures of BAEC, isolated from cow thoracic aortas and maintained as previously described (Kinsella & Wight, 1986), were single labeled with 50 μCi/mL [^{35}S]sulfate or double labeled with [^{35}S]sulfate and 20 μCi/mL D-[6-^{3}H(N)]glcN-HCl or 25 μCi/mL [^{3}H]serine and 25 μCi/mL [^{35}S]sulfate for either 24 or 48 h by replacement with fresh medium containing 10% heat-inactivated fetal bovine serum. Each preparation included 1–10 × 100 mm dishes, each containing approximately 1×10^7 cells. Cultures were fed every 4 days and were labeled 7–10 days after reaching confluence (approximately 14 days in culture). Wounded cultures were prepared from confluent cultures at 10–12 days in culture by multiscratch wounding (Kinsella & Wight, 1986) and were labeled with 50 μCi/mL [^{35}S] sulfate between 24 and 72 h after wounding.

To extract proteoglycans from labeled cultures, media were removed and solid Gdn-HCl was added to a concentration of approximately 4 M. The cell layers were extracted for 15 min with ice-cold 4 M Gdn-HCl, pH 5.8, containing 2% Triton X-100, 2.5 mM EDTA, 0.1 M 6-aminohexanoic acid, 5 mM benzamidine, 5 mM phenylmethanesulfonyl fluoride, and 10 mM *N*-ethylmaleimide and then harvested by scraping. Pooled media and cell layer samples were stored separately at −70 °C until they were processed further.

Isolation of HSPG Subclasses. Media and cell layer extracts were equilibrated in 8 M urea with 2 mM EDTA, 0.3% Triton X-100, 0.25 M NaCl, and 50 mM Tris-HCl, pH 7.5, by chromatography on Sephadex G-50F and immediately applied to DEAE-Sephacel (5–10 mL) in the same buffer. Unbound radioactivity was removed from the column by washing with 5 volumes of urea buffer. Bound radioactivity was eluted from the column with a linear gradient of 0.25–0.70 M NaCl in urea buffer (total volume of 200 mL). Fractions containing the two major salt-eluted labeled peaks were pooled separately. The first of the labeled peaks, which contained all of the HSPG (Kinsella & Wight, 1986), was concentrated by applying the diluted sample to an 0.6–0.8-mL DEAE-Sephacel minicolumn and eluting bound radioactivity with sequential washes of 4 M Gdn-HCl containing 0.5% Triton X-100 (Yanagishita & Hascall, 1984a). Recovery of applied radioactivity exceeded 90%. Concentrated HSPG samples were then chromatographed in 4 M Gdn-HCl with 0.5% Triton X-100 on a preparative Sepharose CL-4B column 1.5 × 125 cm). Subclasses of HSPG, defined on the basis of Sepharose CL-4B elution position, were pooled. Pooled HSPG subclasses were placed in Spectrapor III low molecular weight cutoff dialysis tubing and concentrated by contact desiccation before dialysis against distilled water. Aliquots were prepared for analysis by precipitation with ethanol (4 volumes of 1.3% potassium acetate in 95% ethanol, 2 h at −20 °C). Recoveries of labeled material by ethanol precipitation were greater than 85%. [^{35}S]Methionine-labeled HSPG subclasses were run on 4–10% gradient SDS–PAGE (Laemmli, 1970) and enhanced for autoradiography with Enlightening (NEN, Boston, MA), before exposure at −70 °C of Kodak XAR-2 film.

Analytical Gel Filtration Chromatography. Gel filtration chromatography of untreated HSPG subclasses and heparitinase-treated core protein preparations was carried out on Sepharose CL-2B, CL-4B, and CL-6B (all columns 0.7 × 100–120 cm) in 0.1 M Tris-HCl buffered 4 M Gdn-HCl with 0.5% Triton X-100, pH 7.0. Estimation of the apparent molecular weight of [^{3}H]serine-labeled core preparation (after heparitinase treatment) was done from a plot of log MW versus K_{av} on Sepharose CL-6B of protein standards (Pharmacia Fine Chemicals, Piscataway, NJ), iodinated with [^{125}I]NaI by using an insolubilized lactoperoxidase-catalyzed reaction (Iodogen, Pierce Chemical Co., Rockford, IL). Additional standards, including type III procollagen (100 kDa) and procollagen [α1(III)$_3$] trimer (291 kDa), metabolically labeled with [^{3}H]proline, were provided by Dr. J. Bonadio, Department of Pathology, University of Washington. Chromatography of protein standards was carried out in 4 M Gdn-HCl with 0.5% Triton X-100. Glycosaminoglycans prepared from parent HSPG subclasses by alkaline β-elimination or papain digestion (see below) were chromatographed on Sepharose CL-6B (0.7 × 105 cm) in 0.1 M Tris-HCl and 0.2 M NaCl, pH 7.0. Estimates of HS chain size were made by comparison of Sepharose CL-6B elution K_{av}, determined experimentally, with a previously published standard curve of log MW versus K_{av} on Sepharose CL-6B for chondroitin sulfate chains of various known molecular weight (Wasteson, 1971). Secondary GAG standards of known molecular weight, including chondroitin sulfate chains from macaque aortic smooth muscle cell aggregating CSPG (43 kDa; Chang et al., 1983) and aA1 chondrosarcoma CSPG (12.5 kDa; Stevens & Hascall, 1981) were also chromatographed on the same column in order to standardize and validate our M_r estimates.

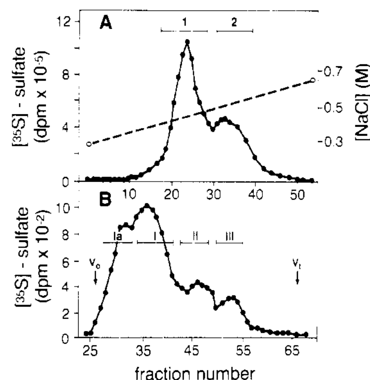

FIGURE 1: Preparative ion-exchange and gel filtration chromatograph of Gdn-HCl extracts of confluent cell layers. [^{35}S]Sulfate-labeled cell extracts were equilibrated in 8 M urea buffer with 0.25 M NaCl and detergent, applied to DEAE-Sephacel, and fractionated as described under Materials and Methods (A). Two peaks (1, 2) eluting during the salt gradient from DEAE-Sephacel were pooled as shown by the bars. Pool 1 from DEAE-Sephacel, which contains HSPGs, was then chromatographed on Sepharose CL-4B in 4 M Gdn-HCl and detergent, and four subfractions (Ia, I, II, and III) were pooled as indicated by the bars (B). [^{35}S]Sulfate label (●); NaCl concentration (- - -) (A).

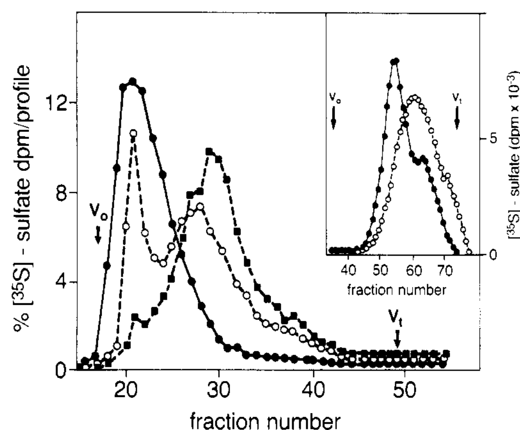

FIGURE 2: Gel filtration chromatograph of HSPG Ia before and after reduction and alkylation. HSPG subclass Ia was prepared from media or cell layer extracts and chromatographed on Sepharose CL-4B in 4 M Gdn-HCl with detergent either before or after reduction and alkylation of disulfide bonds as described under Materials and Methods. Unreduced aliquots of HSPG Ia from cell layer (●) or media eluted at the void volume, while varying proportions of reduced-cell HSPG Ia from cell layer (○) or media (■) were included on the column. (Inset) Elution of HSPG Ia (●) and I (○) from Sepharose CL-2B (K_{av} of 0.39 and 0.55, respectively).

Nitrous acid digests and periodate/alkali-treated samples (see below) were run on Bio-Gel P-10 (0.7 × 100 cm) columns in 0.5 M ammonium bicarbonate. Void and total volumes of gel filtration columns were determined by the elution position of [^{3}H]DNA and [^{35}S]sulfate, respectively.

Enzymatic and Chemical Procedures. Reduction of disulfide bonds was carried out in 4 M Gdn-HCl with 0.5% Triton X-100, 2 mM EDTA, 5 mM dithiothreitol, and 0.1 M Tris-HCl, pH 8.5, for 5 h at 40 °C. Reduced disulfide bonds were alkylated with iodoacetamide (3.5 mM) by addition to the reduced HSPG subclasses, followed by incubation at 4 °C overnight in the dark (Hassell et al., 1985).

Heparan sulfate chains were released from core proteins of isolated HSPG subclasses by papain digestion or alkaline borohydride treatment (Wight & Hascall, 1983). For enzymatic degradation, samples were digested with 30 μg/mL papain in 0.1 M sodium acetate buffer with 5 mM EDTA and 5 mM cysteine, pH 7.0, for 4 h at 65 °C. Alkaline β-elimination was carried out for 24 h at 45 °C with 1 M sodium borohydride in 0.05 M NaOH and terminated by neutralizing treated samples with glacial acetic acid. Papain-released glycosaminoglycan chains were identified as HS containing by sensitivity to deaminative cleavage with nitrous acid at pH 1.5 (Lindahl et al., 1973). Prior to further analysis, HS chains released by alkali from HSPG subclasses were treated with 0.03 unit/mL chondroitin ABC lyase in enriched Tris buffer, pH 7.8 (Saito et al., 1968), for 3 h at 37 °C to remove any contaminating chondroitin or dermatan sulfate chains. Heparan sulfate chains were separated by Bio-Gel P-10 chromatography from digestion products and nonsulfated oligosaccharides and recovered by ethanol precipitation. Heparitinase digestion of HSPG monomers or isolated HS chains was carried out at 37 °C for 4 h with 10 mIU/mL of enzyme in 50 mM Tris-HCl, pH 7.2, with 5 mM calcium acetate, 5 mM PMSF, 5 mM benzamidine, 100 mM 6-aminohexanoic acid, and 100 μg/mL each of pepstatin and soy bean trypsin inhibitor [modified from Hassell et al. (1985)]. To assess the extent and distribution of N-sulfated residues in HS chains, deaminative cleavage was carried out according to the procedure of Shively and Conrad (1976). Periodate oxidation of glcUA residues and subsequent cleavage by alkaline elimination of the oxidized products were carried out according to the protocol of Fransson et al. (1980).

RESULTS

Isolation of HSPG Subclasses from Confluent Cultures. Media and cell layer extracts from radiolabeled cultures were chromatographed on DEAE-Sephacel to remove the bulk of the contaminating protein and to separate HSPG from the more highly charged DSPG (Figure 1A). Profiles of both cell layer and media extracts reveal two major [^{35}S]sulfate-labeled peaks (1,2) that eluted during a 0.25–0.7 M NaCl gradient. Peak 1 represented 75–80% of the label incorporated into cell layer associated macromolecules and 65–75% of the corresponding media radioactivity extracted from confluent cultures, while peak 2 contained the bulk of the remaining incorporated [^{35}S]sulfate. In representative preparations, peak 1 eluted from DEAE-Sephacel between 0.35 and 0.45 M sodium chloride, while peak 2 eluted between 0.45 and 0.55 M sodium chloride. In pooled samples of peak 1, greater than 90% of the [^{35}S]sulfate radioactivity was susceptible to nitrous acid deaminative cleavage as judged by Sephadex G-50 chromatography (not shown). When portions of pooled peak 1 samples from either media or cell layer extracts were chromatographed on Sepharose CL-4B in 4 M Gdn-HCl buffer with 0.5% Triton X-100, four peaks (Ia, I, II, III) were eluted within the included volume of the column at, respectively, K_{av} 0.11, 0.25, 0.45, and 0.63 (Figure 1B). In media and cell layer extracts from confluent cultures, HSPG subclasses Ia and I predominated, typically representing approximately 70% of the DEAE peak 1 associated [^{35}S]sulfate radiolabel. Gel filtration chromatography without detergent resulted in poor recovery of applied radioactivity (<25%), largely at the expense of the larger M_r peaks. Preparative Sepharose CL-4B columns were used to provide material from each of the subclasses for the analyses described below.

Analytical Sepharose CL-4B chromatography only partially resolved HSPG subclasses Ia and I from media or cell layer. When the leading shoulder of the major peak (Ia) was pooled separately from the remainder of the peak (I) and both were rechromatographed on Sepharose CL-2B, the two HSPG subclasses eluted with K_{av} of 0.39 and 0.55, respectively (Figure 2, insert). After reduction and alkylation, however, most media-derived HSPG Ia was found to elute primarily with HSPG I (K_{av} = 0.25) on Sepharose CL-4B (Figure 2). In

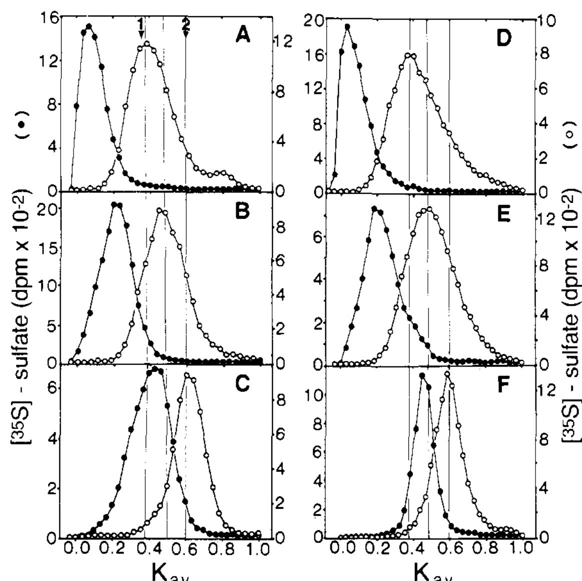

FIGURE 3: Gel filtration of cell layer and media derived subclasses of [^{35}S]sulfate-labeled HSPG on Sepharose CL-6B before and after treatment with papain. HSPG subclasses I (A, D), II (B, E), and III (C, F) were isolated from cell layer (A–C) or media (D–F) extracts and chromatographed either before (●) or after treatment with papain (○). Untreated samples were chromatographed in 4 M Gdn-HCl buffer with detergent, while treated samples were chromatographed in 0.1 M Tris-HCl buffer, pH 7.0, with 0.2 M NaCl. Vertical reference lines mark K_{av} = 0.38, 0.48, and 0.59, from left to right. (Arrow heads) Reference standard GAG elution positions: 1, 43 kDa; 2, 12.5 kDa.

contrast to media-derived HSPG Ia, approximately 40% of cell layer HSPG Ia was unaffected by reduction and alkylation and continued to elute near the Sepharose CL-4B void volume after treatment. Reduction of HSPG II did not affect its elution position from Sepharose CL-4B (not shown). These results suggest that media-derived HSPG Ia and most cell layer HSPG Ia represent disulfide-bonded aggregates of HSPG I, formed either during culture or during dissociative extraction with Gdn-HCl.

Characterization of the Polysaccharide Components of HSPG Subclasses of Confluent Cultures. The size of the component GAG chains and the presence of protein core was assessed for each of the isolated subclasses by chromatography on Sepharose CL-6B before and after papain digestion (Figure 3) or alkaline elimination (not shown). All HSPG subclasses were found to shift elution position after digestion, indicating that all were proteoglycans. Three size classes of papain-released GAG chains were found. HSPG I (Figure 3A,D) which eluted from Sepharose CL-6B at K_{av} of 0.10, whether derived from media or cell layer extracts, released GAG chains of K_{av} = 0.38 (M_r ~38 kDa). Analysis of HSPG Ia, from both media and cell layer, yielded results identical with those presented for HSPG I (not shown). HSPG II, however, which eluted from Sepharose CL-6B at K_{av} ~0.20 (Figure 3B,E), released GAG chains of K_{av} = 0.48 (M_r ~22 kDa). Digestion of HSPG III (K_{av} = 0.48) released smaller GAG chains, which eluted from Sepharose CL-6B at K_{av} = 0.59–0.61, M_r ~13 kDa (Figure 3C,F). Heparan sulfate chains released by alkali treatment from the different HSPG subclasses eluted from Sepharose CL-6B with K_{av}s indistinguishable from those heparan sulfate chains released by papain treatment (not shown).

HS chains, labeled with [^{3}H]glcN and [^{35}S]sulfate, were prepared by alkaline borohydride treatment of the various HSPG subclasses. After chromatography on Bio-Gel P-10 (see Figure 6), HS chains were isolated from the column void volume by ethanol precipitation and characterized by quantitative deaminative cleavage with nitrous acid at low pH

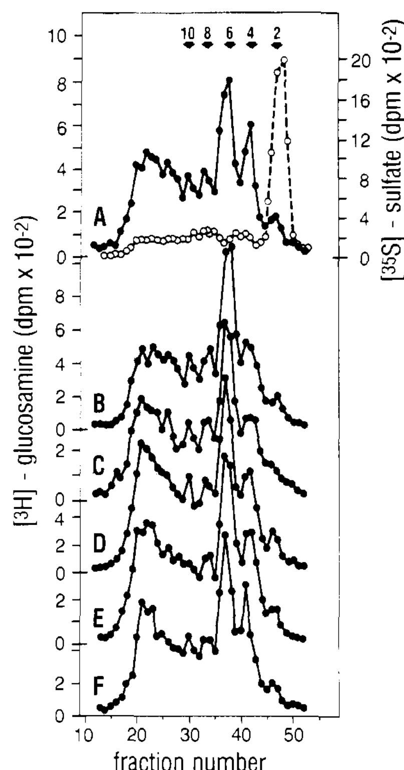

FIGURE 4: Bio-Gel P-10 gel filtration chromatograph of double-labeled HS chains, prepared by β-elimination from parent HSPG subclasses, after quantitative deaminative cleavage with nitrous acid as described under Materials and Methods. Profiles of cell layer (A–C) and media (D–F) derived, nitrous acid generated [^{3}H]oligosaccharide from HS I (A, D), II (B, E), and III (C, F) are shown. A representative [^{35}S]sulfate profile is shown in (A) for nitrous acid degraded cell layer HS I. Arrows denote the elution positions of di- through decasaccharide (2–10) [^{3}H]Glucosamine (●); [^{35}S]sulfate (○).

(Shively & Conrad, 1976). Nitrous acid degradation products were separated by rechromatography on Bio-Gel P-10 to assess the relative extent and distribution of N-sulfation among the different populations of HS chains (Figure 4). Nitrous acid treatment results in the liberation of free sulfate from glcNSO$_3$ along the HS chain, with the cleavage of the adjacent glycosidic bonds and the subsequent release of a series of [^{3}H]-oligosaccharides bearing *O*-sulfate-substituted residues. Hence, the proportion of N-/O-sulfation can be calculated (Carlstedt et al., 1983) from the relative amounts of *O*-[^{35}S]sulfate eluting with oligosaccharide peaks in the included volume from the Bio-Gel P-10 column and *N*-[^{35}S]sulfate eluting as free sulfate at the column total volume (sulfate label distribution, which did not appear to differ among the different HS chain subclasses, is shown only in Figure 4A). In addition, the relative frequency with which *N*-sulfate-substituted residues are distributed along the HS chain can be determined by a comparison of the relative proportions of the various N-desulfated oligosaccharides generated by nitrous acid degradation. Oligosaccharides are separated on Bio-Gel P-10 into five included ^{3}H-labeled peaks, corresponding to di- through decasaccharide, and a peak eluting near the void volume that represents larger oligosaccharide fragments. Comparisons of the HS chains prepared from the various HSPG subclasses indicate that all contain similar proportions of *N*-sulfate substitution (49–55%). In addition, deaminative cleavage of isolated HS chain subclasses suggests that they differ neither in the distributions of N-sulfation, since all have similar profiles of ^{3}H-labeled oligosaccharide (Figure 4), nor O-sulfation, since no differences were noted in the proportions of [^{35}S]sulfate coeluting with ^{3}H-labeled oligosaccharides after degradation (not shown).

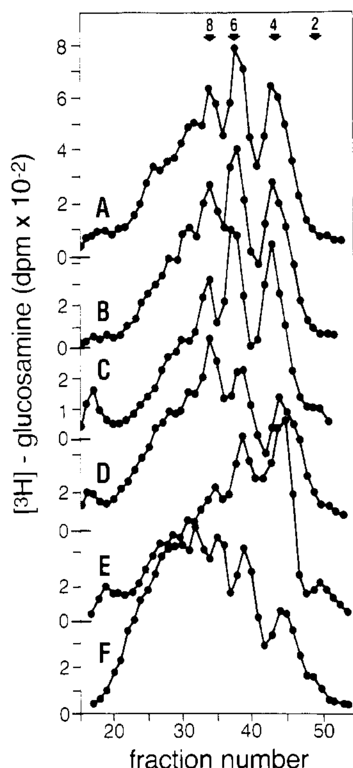

FIGURE 5: Bio-Gel P-10 gel filtration chromatograph of double-labeled HS chains, prepared by β-elimination from parent HSPG subclasses, after periodate oxidation and alkali cleavage as described under Materials and Methods. Profiles of cell layer (A–C) and media (D–F) derived [3H]oligosaccharide from HS I (A, D), II (B, E), and III (C, F) are shown. Arrows denote the elution positions of di- through octasaccharide (2–8).

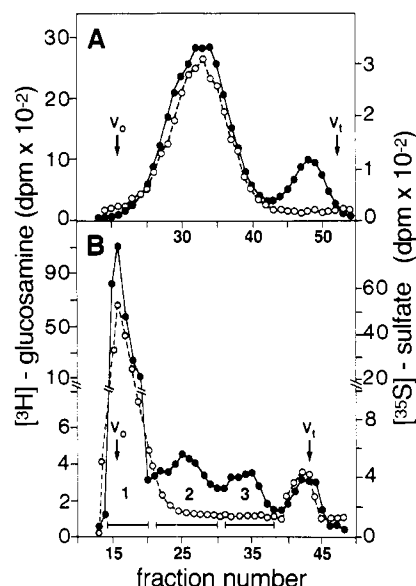

FIGURE 6: (A) Gel filtration profiles of cell layer HSPG I, double labeled with [35S]sulfate (○) and [3H]glcN (●), after removal of core protein. HSPG I was digested with papain as described under Materials and Methods and chromatographed on Sepharose CL-6B. Two peaks eluted from the column, one double labeled, representing HS chains, and another, at a more included position (open arrow), representing nonsulfated oligosaccharide. (B) HSPG I chains eluted from Bio-Gel P-10 as three [3H]glcN-labeled peaks after β-elimination from the core protein as described under Materials and Methods. HS chains eluted at the column void volume (pool 1), while two peaks representing nonsulfated oligosaccharide eluted at more included positions (pools 2 and 3). Only the void volume peak and the total volume peak, which contains chondroitin ABC lyase sensitive radioactivity (see Materials and Methods), contained [35S]sulfate. HS chain and oligosaccharide pools are marked with horizontal bars (1–3).

HSPG subclasses were also characterized by examination on Bio-Gel P-10 of the oligosaccharide fragments obtained from isolated HS chains by periodate oxidation followed by alkaline eliminative cleavage of the oxidized residues (Figure 5). This procedure oxidizes and cleaves the HS chain adjacent to glcUA when present in extended runs (i.e., in regions of the chain that have undergone relatively little epimerization to idoUA) and not adjacent to residues bearing glcNSO3 (Fransson et al., 1980). These results indicate that HS chains derived from all cell layer HSPG (Figure 5A–C) and media HSPG I and II (Figure 5D,E) are cleaved in a similar manner, with the majority of degradation products eluting as tetra-, hexa-, or octasaccharide. In contrast, media HS III (Figure 5F) was less sensitive to periodate oxidation, with the majority of cleavage products eluting larger than hexasaccharide. These results suggest that media HS III chains may be idoUA rich, since nitrous acid cleavage (Figure 4) indicates that media HS III does not bear increased proportions of $glcNSO_3$.

HSPGs were also found to bear nonsulfated oligosaccharides when HSPG subclasses, double labeled with [35S]sulfate and [3H]glcN, were papain digested and chromatographed on Sepharose CL-6B. Figure 6 depicts representative profiles of double-labeled HSPG I from cell layer, either after papain digestion on Sepharose CL-6B (Figure 6A) or after β-elimination on Bio-Gel P-10 (Figure 6B). Similar digestions of other subclasses gave similar profiles (not shown). [35S]-Methionine-labeled preparations of HSPG I showed no apparent contamination with other glycoproteins when submitted to SDS–PAGE (not shown), suggesting that alkali-released nonsulfated oligosaccharides are derived from the HSPG subclasses. After digestion of each HSPG subclass with papain, radiolabel eluted from the Sepharose column in two peaks: (1) a double-labeled peak that eluted at differing K_{av}s

Table I: Relative Percentages of HS Chains and Nonsulfated Oligosaccharides in HSPG Subclasses

HSPG	HS chain[a,b]	oligosacc 1[a,c]	oligosacc 2[a,d]
cell			
I	85	9	6
II	95	3	3
III	96	2	1
media			
I	85	8	4
II	81	10	9
III	80	10	10

[a] Percentages of [3H]glcN radioactivity as calculated from Bio-Gel P-10 profiles of β-eliminated HSPG subclasses. [b] HS chains are represented as pool 1 in the representative Bio-Gel P-10 profile (Figure 6b). [c] Oligosaccharide 1 was calculated from pool 2 as represented in Figure 6b. [d] Oligosaccharide 2 was calculated from pool 3 as shown in Figure 6b.

as previously described for HS chains isolated from the different HSPG subclasses (see Figure 3) and (2) a more included (K_{av} ~0.87, approximately 2.5–3 kDa) second peak, labeled only with [3H]glcN, which represents nonsulfated N- or O-linked oligosaccharide. Upon Bio-Gel P-10 chromatography, oligosaccharides labeled only with [3H]glcN and released from chondroitin ABC lyase digested HSPG subclasses by alkaline borohydride treatment eluted in two included peaks, while free, double-labeled HS chains eluted at the void volume (Figure 6B). Relative proportions of ^{3}H-labeled HS chain and [3H]oligosaccharide in the different HSPG subclasses were found to vary (Table I). In cell layer HSPGs, the proportion of ^{3}H radioactivity associated with oligosaccharide decreased with decreasing hydrodynamic size of the HSPG subclasses, a correlation not found among media HSPG subclasses. This observation may suggest either that basic structural differences

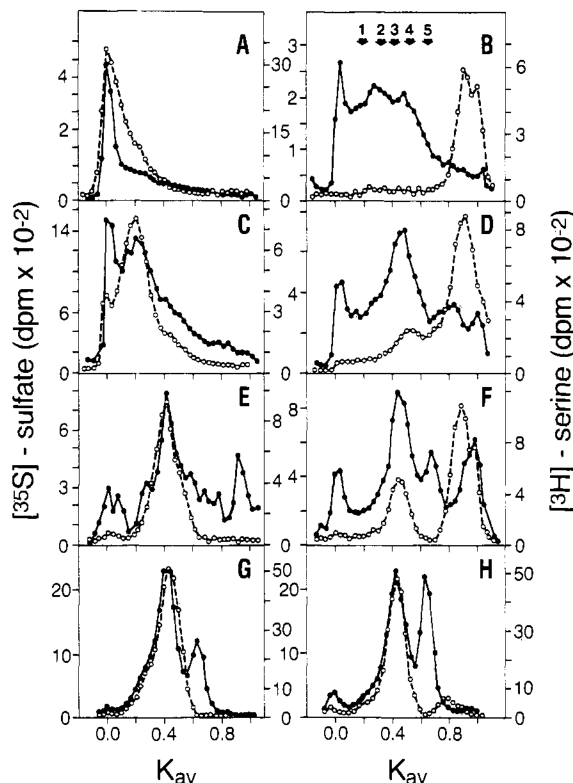

FIGURE 7: Gel filtration chromatograph of HSPG subclasses, double labeled with [^{3}H]serine (●) and [^{35}S]sulfate (○), before and after heparitinase digestion. HSPG subclasses were prepared from cell layer and media extracts as previously described, and aliquots were treated with buffer alone as a control (A, C, E, G) or digested with heparitinase (B, D, F, H) and chromatographed on a calibrated Sepharose CL-6B column in 4 M Gdn-HCl with detergent as described under Materials and Methods. Profiles for cell layer HSPG I (A, B), II (C, D), III (E, F) and media HSPG III (G, H) are shown. Arrows marked 1–5 denote elution positions of standard proteins: 1, collagen [α1(III)$_3$] trimer (291 kDa); 2, α1(III) procollagen (100 kDa); 3, bovine serum albumen (67 kDa); 4, chymotrypsinogen A (25 kDa); 5, ribonuclease A (13.7 kDa).

exist among the different isolated subclasses or that HSPG parent monomers are degraded differently in the media and cell layer compartments (see Discussion).

HSPG Subclass Core Protein Analysis. Isolated subclasses of HSPG, labeled with both [^{3}H]serine and [^{35}S]sulfate, were chromatographed in 4 M Gdn-HCl with 0.5% Triton X-100 on Sepharose CL-6B columns calibrated with radiolabeled protein standards before and after heparitinase digestion to provide estimates of relative average core protein hydrodynamic size (Figure 7). Under these chromatographic conditions, a plot of the elution position of labeled marker proteins versus log MW was linear (not shown). Heparitinase digestion of cell layer HSPG I caused the ^{3}H-label to shift from the void volume into a broad peak with a K_{av} of 0.22 (ranging from approximately 100 to 300 kDa), while [^{35}S]sulfate, which initially coeluted with the ^{3}H-label, eluted near the total volume of the column (Figure 7A,B). The width of the peak of heparitinase-treated core, which may be caused either by the presence of multiple heterogeneous cores (Lories et al., 1987) or the inclusion of contaminating HSPG II core (Figure 7D), precludes an accurate estimation of the M_r of HSPG I core(s). Cell layer HSPG II shifted from a major double-labeled peak (K_{av} = 0.25) before digestion to a major [^{3}H]serine-labeled peak at K_{av} = 0.43 after treatment (Figure 7C,D). Note that about 20% of the sulfate label continues to coelute with the major ^{3}H-labeled peak after digestion of HSPG II (Figure 7D), indicating partial sensitivity to the enzyme. The small amount of ^{3}H-label that eluted in the void volume of the column after digestion of HSPG subclasses I and II also voided on Sepharose CL-4B (not shown), an elution position that was unchanged after disulfide bond reduction. The identity of this peak is unknown. Subclasses I and II derived from media gave similar results (not shown).

HSPG III from cell layer or media eluted from Sepharose CL-6B as one double-labeled peak (K_{av} = 0.38, cell layer; 0.41–0.43, media) prior to heparitinase treatment (Figure 7E,G). The identity of other peaks labeled only with [^{3}H]-serine is unknown. After digestion with heparitinase, only a portion of the ^{35}S radioactivity shifted to an elution position near the column total volume, indicating partial sensitivity to the enzyme (Figure 7 F,H). Cell layer HSPG III, in which (similar to cell layer HSPG II) the majority of the sulfate label was heparitinase sensitive, shifted slightly (from K_{av} = 0.38 to 0.41) after digestion, with a corresponding increase in the amount of a ^{3}H-labeled peak that eluted at K_{av} = 0.63 (Figure 7F). While the majority of media HSPG III did not shift elution position after digestion, there was an increased amount of a ^{3}H-labeled peak that also eluted at K_{av} = 0.63 (Figure 7H). From these observations, the core protein of HSPG III is tentiatively identified as the K_{av} = 0.63 peak. However, the shift of double-labeled cell layer HSPG III after digestion suggests that this subclass may bear, on the same core, some HS chains, or chain portions, that are relatively heparitinase insensitive and perhaps idoUA rich. Media-derived HSPG III may bear a higher proportion of such chains, in agreement with periodate oxidation results (Figure 5). In addition, the appearance of a labeled sulfate-free apparent core peak at K_{av} = 0.63 after heparitinase digestion of HSPG III may indicate that this population is heterogeneous and may include some cores or core fragments that bear chains which are relatively heparitinase sensitive. The observations that considerable portions of the [^{35}S]sulfate associated with HSPG subclasses II and III were heparitinase insensitive and that all subclasses appear to bear variable amounts of nonsulfated oligosaccharide (Figure 6) suggest that the apparent M_r for the cores of HSPG II (~50 kDa) and HSPG III (~15 kDa) must represent maximal values, with the actual core sizes somewhat smaller.

Differences among HSPG Subclasses Isolated from Wounded and Confluent Cultures. A previous study (Kinsella & Wight, 1986) has shown that proteoglycan synthesis is stimulated and proteoglycan subclasses of lower molecular weight accumulate in wounded aortic endothelial cell cultures. Therefore, HSPG monomers were isolated from wounded cultures and compared with those isolated from confluent cultures to find whether structurally different subclasses are made under differing conditions of growth and migration. The results of this comparison indicate that HSPG subclasses isolated from wounded cultures are similar in most respects to those isolated from confluent cultures. Specifically, four subclasses, which eluted from Sepharose CL-4B with similar K_{av} as those prepared from confluent cultures, were isolated from wounded culture cell layers and media. Papain digests of wounded culture HSPG I and II were indistinguishable from those of confluent cultures (i.e., both indicated proteoglycans with GAG chains of, respectively, 38 and 22 kDa). In contrast to subclasses isolated from confluent cultures, however, HSPG III from wounded cultures shifted position slightly (media) or not at all (cell layer) after papain digestion, and HS chains from both eluted from Sepharose CL-6B with a K_{av} ~ 0.48–0.50 (22 kDa) (Figure 8). This result indicates that wounded culture HSPG III chains are comparable in size to those released from HSPG II and larger than chains released

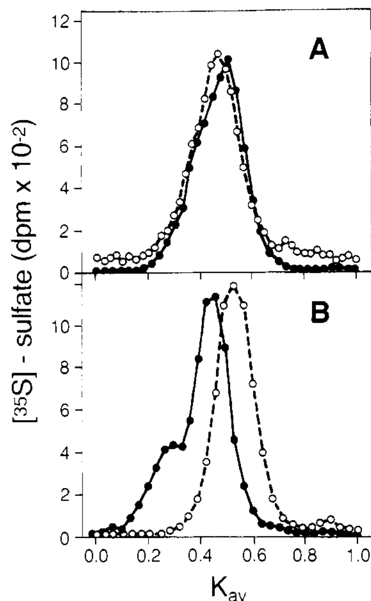

FIGURE 8: Gel filtration profiles of [^{35}S]sulfate-labeled HSPG III isolated from wounded cultures. HSPG III was prepared from wounded culture cell layer (A) and media (B) extracts and chromatographed prior (●) to papain treatment, in 4 M Gdn-HCl with detergent, or after (○) papain digestion (see Materials and Methods) on Sepharose CL-6B in 0.1 M Tris-HCl, pH 7.0, with 0.2 NaCl.

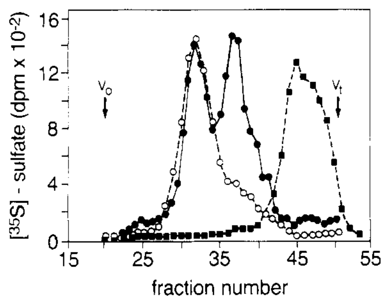

FIGURE 9: Sepharose CL-6B gel filtration chromatograph of HS chains from the media of wounded and confluent cultures after heparitinase digestion. HSPG subclasses were isolated from culture media extracts, and HS chains were prepared by papain treatment and digested with heparitinase as described under Materials and Methods. HS III chains from wounded (●) or confluent cultures (○) showed varying degrees of sensitivity to heparitinase, while HS I chains isolated from confluent cultures (■), and included as a positive control for digestion, showed little remaining high molecular weight label after heparitinase treatment.

from HSPG III (13 kDa) prepared from confluent cultures (see Figure 3). When portions of HSPG III were analyzed on Sepharose CL-6B after heparitinase digestion, 52% of wounded media but only 18% of confluent media HSPG III was found to change elution position, with most of the degradation products eluting within the column included volume (Figure 9). Also, the heparitinase-insensitive portion of HSPG III from wounded culture media eluted at the same column volume as a similar fragment derived from confluent culture media HSPG III (Figure 9). Calculation of the size of the HS chain fragment that remained after digestion indicated a fragment of 10.7 kDa was present in both cases. In contrast, nearly all of the [^{35}S]sulfate asssociated with HSPG I from wounded culture media was digested with heparitinase and eluted near the column total volume. These results were similar to results obtained with confluent culture derived HSPG I (see Figure 7).

Discussion

In this study, the structural characteristics of three subclasses of HSPG isolated from BAEC cultures are compared, and the presence of multiple HSPG species in BAEC cultures is confirmed (Oohira et al., 1983; Buonassissi & Colburn, 1983; Marcum et al., 1986; Keller et al., 1987). HSPG I constituted the major subclass in both cell layer and media extracts. This subclass forms disulfide-bonded aggregates, in agreement with reports of HSPG isolated from various cell types (Cöster et al., 1983; Lowe-Krentz & Keller, 1984; Hassell et al., 1985) including endothelial cells (Hiss et al., 1987). It is unclear, however, whether aggregated HSPG I is a hetero- or homodimer (Cöster et al., 1983; Hiss et al., 1987) or is disulfide bonded to other matrical proteins, such as collagen or laminin as suggested by other workers (Lowe-Krentz & Keller, 1984; Parthasarathy & Spiro, 1984; Hassell et al., 1985). HSPG I is similar in hydrodynamic size (M_r ~350–400 kDa) to the salt-extractable HSPG isolated from the basement membrane-secreting (EHS) tumor (Fujiwara et al., 1984; Hassell et al., 1985), and HSPGs from PYS-2 cells (Oohira et al., 1982), human fibroblasts (Carlstedt et al., 1983; Lories et al., 1986), and the detergent-insoluble matrix of rat embryo fibroblasts (Woods et al., 1985). HSPG I has a relatively large glycoprotein core and HS chains of M_r ~38 kDa. The broad (M_r ~100–300 kDa) elution profile of HSPG I core (Figure 7B) allows only a relative estimate of molecular range, perhaps indicating similarities to preparations from lung fibroblasts that have a heterogenous core size distribution (Lories et al., 1987). Nonreducible HSPG Ia, present only in cell layer samples, may be similar to large (600–800 kDa) matrix-associated HSPG extractable from EHS tumor (Hassell et al., 1985), or human lung fibroblasts (Lories et al., 1986), or the cell surface HSPG of colon carcinoma cells (Iozzo, 1984). HSPG II (M_r ~150–200 kDa) has a smaller core glycoprotein (<50 kDa) and bears HS chains of 22 kDa. HSPG II (Sepharose CL-4B K_{av} ~0.45) is similar in size to a HSPG from glomerular basement membrane (Parthasarathy & Spiro, 1984) and the cell surface HSPGs isolated from a variety of cell types (Oldberg et al., 1979; Kjellén et al., 1980; Rapraeger & Bernfield, 1983; Woods et al., 1985). HSPG III includes a small peptide core (<15 kDa) with short HS chains (13 kDa) and a small amount of nonsulfated oligosaccharide. Interestingly, HSPG III isolated from wounded cultures has a HS chain of 22 kDa and occurs as a single chain with a small amount of peptide (media) or as an apparent free chain (cell layer). Like the smaller HS chain of HS III from confluent cultures, a 10–11-kDa segment of HS III from wounded media is heparitinase insensitive. In other systems, HS-free chains have been isolated from prelysosomal and other intracellular compartments (Yanagishita & Hascall, 1984b; Bienkowski & Conrad, 1984; Kjellén et al., 1985; Fedarko & Conrad, 1986) as well as from presumed extracellular compartments, such as adhesion sites (Lark & Culp, 1982) and culture medium (Bienkowski & Conrad, 1984).

Our data indicate that all HSPG subclasses have certain structural similarities including (1) similar size classes of nonsulfated oligosaccharides and (2) HS chains, which, although varying in average molecular weight (Figure 3), contain similar proportions of *N*- and *O*-sulfate, with the *N*-sulfate distributed in blocks whose proportions appear equivalent among the different subclasses (Figure 4).

Two observations suggest that media-derived HS III from confluent cultures is idoUA rich. Media HS III is relatively insensitive to periodate oxidation, which is specific for glcUA-rich, N-acetylated HS chain regions (Figure 5), while having similar distributions and amounts of N-sulfation (Figure 4). Also, the heparitinase insensitivity of media HS III may indicate idoUA epimerization (Hovingh & Linker,

1970). However, since cell layer derived HSPG II and III chains were found, relative to media HS III, to be more periodate (Figurge 5) and heparitinase (Figure 7) sensitive and since a subclass of HS-free core is generated by heparitinase digestion of HSPG III (Figure 7F,H), it is possible that only a subclass of the HS chains borne by the different HSPG subclasses are idoUA rich. Also, it is not possible at present to exclude the possibility that different subpopulations within the HS subclasses show reduced heparitinase or periodate reactivity, respectively.

Previous studies of the biosynthesis of heparin, using model systems, have demonstrated that mastocytoma-derived uronosyl C-5 epimerase requires the N-sulfated rather than the N-acetylated polysaccharide intermediate for substrate recognition (Jacobsson et al., 1984). If an enzyme with similar substrate specificity is active in the epimerization of glcUA to idoUA in heparan sulfate biosynthesis in endothelial cells, those HS chains bearing increased proportions of idoUA might be expected to bear more N-sulfated residues [however, see Cifonelli and King (1977)]. This was not the case for media HS III (Figure 4). Therefore, it seems likely that the level of N-sulfation ($\sim$50%) of the residues in these short chains is sufficient to provide substrate for conversion to idoUA-containing residues. Lyon and his co-workers (1987) showed that isolated HS chains may bear a series of approximately eight glcNAc-containing residues, adjacent to the protein–polysaccharide linkage region. If present, this region would constitute about one-fourth of the molecular weight of the 13-kDa HS III chains, and lacking $glcNSO_3$, would represent a domain of the chain that may offer no potential sites for epimerization to idoUA. The shift of the portion of HSPG III that is only partially susceptible to heparitinase (Figure 4F and 9) suggests that the putative idoUA-rich region of this class of HS chains is present in an $\sim$11-kDa fragment proximal to the core. Other workers have demonstrated that blocks of *N*-sulfate-substituted residues may be concentrated distally (i.e., at the nonreducing end) on some HS chains (Fransson et al., 1980; Parthasaranthy & Spiro, 1984). Such structure might also suggest that highly sulfated, idoUA-containing (heparin-like) regions might occur distally on some HS chains. In contrast to this expectation, and in agreement with our results, a recent study has suggested that the content of glcUA increases toward the distal (nonreducing) end of some heparin chains (Radoff & Danishefsky, 1985).

In summary, structural characterization indicates that the isolated HSPG subclasses form a series of generally similar molecules bearing progressively smaller HS chains on cores of decreasing size. Interestingly, a portion of the HS chains borne by some HSPG subclasses contain a distinctive region, proximal to the protein core, which was relatively insensitive to heparitinase digestion and/or periodate oxidation and which may be idoUA rich. Structural differences among the subclasses may derive in part from synthesis of unique HSPGs on cores that are distinct products of separate genes (Jalkanen et al., 1985; Stow & Farquhar, 1987). However, the similarities in the structures described do not preclude the possibility that some HSPG subclasses are generated by processing or turnover of precursors (Bienkowski & Conrad, 1984; Yanagishita & Hascall, 1984; Ledbetter et al., 1985). If this is the case, then the accumulation of lower molecular weight HSPGs during the migration of BAEC in wounded cultures (Kinsella & Wight, 1986) may represent enhanced HSPG turnover and suggest that alterations in the activity of HSPG degradative enzymes occurs during migration. In this respect, migrating BAEC may be similar to tumor cells (Kramer et al., 1982; Vlodavsky et al., 1982), macrophages (Savion et al., 1984; 1987), and lymphocytes (Fridman et al., 1987) that appear to secrete heparanase during active cell migration.

References

Bienkowski, M. J., & Conrad, H. E. (1984) *J. Biol. Chem. 259*, 12989–12996.

Buonassisi, V., & Colburn, P. (1983) *Biochim. Biophys. Acta 760*, 1–12.

Carlstedt, I., Cöster, L., Malmström, A., & Fransson, L.-Å, (1983) *J. Biol. Chem. 258*, 11629–11635.

Castellot, J. J., Addonizio, M. J., Rosenberg, R. D., & Karnovsky, M. J. (1981) *J. Cell Biol. 90*, 372–379.

Chang, Y., Yanagishita, M., Hascall, V. C., & Wight, T. N. (1983) *J. Biol. Chem. 258*, 5679–5688.

Cheng, C.-F., Oosta, G. M., Bensadoun, A., & Rosenberg, R. D. (1981) *J. Biol. Chem. 256*, 12893–12898.

Cifonelli, J. A., & King, J. A. (1977) *Biochemistry 16*, 2137–2141.

Cöster, L., Malmström, A., Carlstedt, I., & Fransson, L.-Å. (1983) *Biochem. J. 215*, 417–419.

Fedarko, N. S., & Conrad, H. E. (1986) *J. Cell Biol. 102*, 587–599.

Fransson, L.-Å., Sjöberg, I., & Havsmark, B. (1980) *Eur. J. Biochem. 106*, 59–69.

Fridman, R., Lider, O., Naparstek, Y., Fuks, Z., Vlodavsky, I., & Cohen, I. R. (1987) *J. Cell. Physiol. 130*, 85–92.

Fujiwara, S., Wiedemann, H., Timpl, R., Lustig, A., & Engel, J. (1984) *Eur. J. Biochem. 143*, 145–157.

Hassell, J. R., Leyshon, W. C., Ledbetter, S. R., Tyree, B., Suzuki, S., Kato, M., Kimata, K., & Kleinman, H. K. (1985) *J. Biol. Chem. 260*, 8098–8105.

Hayman, E. G., Oldberg, Å., Martin, G. R., & Ruoslahti, E. (1982) *J. Cell Biol. 94*, 28–35.

Hedman, K., Johansson, S., Vartio, T., Kjellên, L., Vaheri, A., & Höök, M. (1982) *Cell (Cambridge, Mass.) 28*, 663–671.

Hiss, D., Scott-Burden, T., & Gevers, W. (1987) *Eur. J. Biochem. 162*, 89–94.

Hovingh, P. M., & Linker, A. (1970) *J. Biol. Chem. 245*, 6170–6175.

Iozzo, R. V. (1984) *J. Cell Biol. 99*, 403–417.

Jacobsson, I., Lindahl, U., Jensen, J. W., Rodên, L., Prihar, H., & Feingold, D. S. (1984) *J. Biol. Chem. 259*, 1056–1063.

Jalkanen, M., Nguyen, H., Rapraeger, A., Kurn, N., & Bernfield, M. (1985) *J. Cell Biol. 101*, 976–984.

Kanwar, Y. S., & Farquhar, M. G. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 1303–1307.

Keller, R., Silbert, J. E., Furthmayr, H., & Madri, J. A. (1987) *Am. J. Pathol. 128*, 286–298.

Kinsella, M. G., & Wight, T. N. (1986) *J. Cell Biol. 102*, 679–687.

Kjellên, L., Oldberg, Å., & Höök, M. (1980) *J. Biol. Chem. 255*, 10407–10413.

Kjellên, L., Pertoft, H., Oldberg, Å., & Höök, M. (1985) *J. Biol. Chem. 260*, 8416–8422.

Koda, J. E., Rapraeger, A. C., & Bernfield, M. (1985) *J. Biol. Chem. 260*, 8157–8162.

Kramer, R. H., Vogel, K. G., & Nicolson, G. L. (1982) *J. Biol. Chem. 257*, 2678–2686.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Lark, M. W., & Culp, L. A. (1982) *J. Biol. Chem. 257*, 14073–14080.

Laterra, J., Silbert, J. E., & Culp, L. A. (1983) *J. Cell Biol. 96*, 112–123.

Ledbetter, S. R., Tyree, B., Hassell, J. R., & Horigan, E. A. (1985) *J. Biol. Chem. 260*, 8106-8113.
Lindahl, U., Bäckström, G., Jansson, L., & Hallen, A. (1973) *J. Biol. Chem. 248*, 7234-7241.
Lories, V., David, G., Cassiman, J.-J., & Van Den Berghe, H. (1986) *Eur. J. Biochem. 158*, 351-360.
Lories, V., De Boeck, H., David, G., Cassiman, J.-J., & Van Den Berghe, H. (1987) *J. Biol. Chem. 262*, 854-859.
Lowe-Krentz, L. J., & Keller, J. M. (1984) *Biochemistry 23*, 2621-2627.
Lyon, M., Steward, W. P., Hampson, I. N., & Gallagher, J. T. (1987) *Biochem. J. 242*, 493-498.
Marcum, J. A., Atha, D. H., Fritze, L. M. S., Nawroth, P., Stern, D., & Rosenberg, R. D. (1986) *J. Biol. Chem. 261*, 7507-7517.
Oldberg, Å., Kjellên, L., & Höök, M. (1979) *J. Biol. Chem. 254*, 8505-8510.
Oohira, A., Wight, T. N., McPherson, J., & Bornstein, P. (1982) *J. Cell Biol. 92*, 357-367.
Oohira, A., Wight, T. N., & Bornstein, P. (1983) *J. Biol. Chem. 258*, 2014-2021.
Parthasarathy, N., & Spiro, R. G. (1984) *J. Biol. Chem. 259*, 12749-12755.
Radoff, S., & Danishefsky, I. (1985) *J. Biol. Chem. 260*, 15106-15111.
Rapraeger, A. C., & Bernfield, M. R. (1983) *J. Biol. Chem. 258*, 3632-3636.
Saito, H., Yamagata, T., & Suzuki, S. (1968) *J. Biol. Chem. 243*, 1536-1542.
Shimada, K., Gill, P. J., Silbert, J. E., Douglas, W. H. J., & Fanburg, B. L. (1981) *J. Clin. Invest. 68*, 995-1002.
Shively, J. E., & Conrad, H. E. (1976) *Biochemistry 15*, 3932-3942.
Stevens, R. L., & Hascall, V. C. (1981) *J. Biol. Chem. 256*, 2053-2058.
Stow, J. L., & Farquhar, M. G. (1987) *J. Cell Biol. 105*, 529-539.
Wasteson, Å. (1971) *J. Chromatogr. 59*, 87-97.
Wight, T. N., & Hascall, V. C. (1983) *J. Cell Biol. 96*, 167-176.
Woods, A., Höök, M., Kjellên, L., Smith, C. G., & Rees, D. A. (1984) *J. Cell Biol. 99*, 1743-1753.
Yanagishita, M., & Hascall, V. C. (1984a) *J. Biol. Chem. 259*, 10260-10269.
Yanagishita, M., & Hascall, V. C. (1984b) *J. Biol. Chem. 259*, 10270-10283.

Enzymatic Properties of Proteolytic Derivatives of Human α-Thrombin

Jan Hofsteenge,* Paul J. Braun, and Stuart R. Stone
Friedrich Miescher Institut, Basel, Switzerland
Received July 14, 1987; Revised Manuscript Received November 10, 1987

ABSTRACT: The use of derivatives of α-thrombin obtained by limited proteolysis, that have only a single peptide bond cleaved, allowed the unequivocal correlation between the change in covalent structure and alteration of the enzymatic properties. β_T-Thrombin contains a single cleavage in the surface loop corresponding to residues 65-83 of α-chymotrypsin [Birktoft, J. J., & Blow, D. M. (1972) *J. Mol. Biol. 68*, 187-240]. Compared with α-thrombin, this modification had a minor effect on the following: (1) The Michaelis constant (K_m) for two tripeptidyl *p*-nitroanilide substrates increased 2-3-fold, whereas the catalytic constant (k_{cat}) remained unaltered. (2) A 2-3-fold increase in the binding constant (K_I) of a tripeptidyl chloromethane inhibitor was observed, but the inactivation rate constant (k_i) was the same, which indicated that the nucleophilicity of the active-site histidyl residue had not changed. (3) The second-order rate constant for the inhibition by antithrombin III decreased 2-fold. Heparin accelerated the inactivation, and the degree of acceleration was similar to that obtained with α-thrombin. Pronounced effects of the cleavage of this loop were found. (1) The cleavage of fibrinogen was approximately 80-fold slower than that with α-thrombin. This was mainly due to a 40-fold decrease in k_{cat}. In contrast, only a 1.9-fold increase in the Michaelis constant was observed. (2) The affinity for thrombomodulin had decreased 39-fold compared to α-thrombin. ϵ-Thrombin contains a single cleaved peptide bond in the loop corresponding to residues 146-150 in α-chymotrypsin. The kinetic parameters for all of the above-mentioned ligands, except fibrinogen, were not affected by this modification. With fibrinogen, a 40% reduction in k_{cat} was observed, but the K_m was found to be unaltered. The same set of parameters was also obtained with γ_T-thrombin which contained cleavages in both loops. These parameters have been compared with those for β_T- and ϵ-thrombin, and the results indicated that γ_T-thrombin was more similar to β_T-thrombin than to ϵ-thrombin.

Thrombin is a serine protease that interacts with a number of substrates, inhibitors, and receptors that are essential to the process of hemostasis (Fenton, 1981). The specificity of these interactions is determined not only by the active site and its specificity pocket but also by secondary binding sites distinct from the active site (Magnusson, 1972).

One approach to study the structure-function relationship of thrombin has been the use of proteolytically cleaved derivatives (Berliner, 1984). With human thrombin, these studies have used γ-thrombin, which is derived from α-thrombin by the cleavage of at least two peptide bonds. Comparison of the primary structures of human α-thrombin and α-chymotrypsin indicates that these cleavages probably occur in surface loops

* Correspondence should be addressed to this author at the Friedrich Miescher Institut, P.O. Box 2543, CH-4002 Basel, Switzerland.

0006-2960/88/0427-2144$01.50/0 © 1988 American Chemical Society