Purification of Keratan Sulfate Proteoglycan from Monkey Cornea

An explant culture of 15 cynomolgus monkey corneas was incubated with \(^{35}S\)sulfate and \(^{2-3}H\)mannose as labeling precursors. A 4 m guanidine HCl extract of the corneal stromas was prepared and combined with a 4 m guanidine HCl extract of stromas from 300 unlabeled corneas. The keratan sulfate proteoglycans in the combined extracts were purified by a combination of DEAE-cellulose chromatography, chondroitinase ABC digestion to remove chondroitin-dermatan sulfate proteoglycans, and elution from immobilized concanavalin A. The purified keratan sulfate proteoglycan was digested with papain and the digest was eluted on DEAE-Sephacel. The unbound fraction contained 59% of the \(^{3}H\) activity and consisted of intact oligosaccharide-peptides. The bound fraction, consisting of keratan sulfate chains linked to peptides, eluted during a linear 0-0.75 M NaCl gradient as a peak centered at ~0.6 M NaCl and contained 41% of the \(^{3}H\) and all of the \(^{35}S\) activity in the original proteoglycan. The chains were digested with endo-β-galactosidase, and the digest was eluted on DEAE-Sephacel with a linear 0-0.75 M NaCl gradient. Most of the sulfated digestion fragments from the chains eluted as several distinct peaks during the gradient. All the \(^{3}H\) activity eluted in the unbound volume along with a small proportion of \(^{35}S\) activity. This unbound fraction was eluted on Bio-Gel P-10 to give a \(^{3}H\) peak \((K_v = 0.46)\) well resolved from the remaining \(^{35}S\) activity which eluted near the total volume. This \(^{3}H\) peak contained the oligosaccharide-peptides derived from the linkage region between the keratan sulfate chains and the core protein. Structural analyses of the linkage region oligosaccharides and the intact oligosaccharides (Nilsson, B., Nakazawa, K., Hassell, J. R., Newsome, D. A., and Hascall, V. C. (1983) J. Biol. Chem. 258, 6056-6063) in combination with the \(^{3}H\)-labeling data suggest that the intact keratan sulfate proteoglycans contain an average of about one intact oligosaccharide per keratan sulfate linkage site.

Glycosaminoglycan side chains of proteoglycans are joined to the protein core through unique sequences of carbohydrates which constitute linkage regions. The structure of the linkage region in chondroitin (dermatan) sulfate proteoglycans is

\[\text{(uronic acid-Gal-Gal-Xyl-core)} \] (1). Knowing this structure has allowed a more thorough understanding of the biosynthesis of proteoglycans and has suggested possible locations where synthesis could be regulated. For example, \(p\)-nitrophenyl derivatives of xylose have been shown to stimulate the synthesis of chondroitin sulfate chains by substituting for the xylose that is normally linked to a serine in the protein core (2).

The linkage region for keratan sulfate in cartilage proteoglycans is distinctly different from that for chondroitin sulfate. Further, the linkage region for cartilage keratan sulfate is different from that for corneal keratan sulfate. Studies on the cartilage proteoglycan have shown that the keratan sulfate chains are covalently bound to the core protein by \(O\)-glycosidic bonds between \(N\)-acetylgalactosamine and hydroxyl groups of serine and threonine residues in the protein core (3-10). In contrast, several studies have indicated that corneal keratan sulfate chains contain mannose residues (10-15) which are located in the linkage region (13-15). Further, several studies (15-17) have provided evidence that the linkage between the chain and the core protein consists of an \(N\)-glycoside bond between \(N\)-acetylgalactosamine and asparagine.

This was in agreement with Stuhlsatz et al. (15) who initially proposed a linear sequence of \((\text{Man-Man-GlcNAc-N-Asn})\) as a structure for the linkage region. However, the subsequent experiments of Hart and Lemarr (18), which showed that tunicamycin treatment of corneal explants inhibited keratan sulfate synthesis, indicated that dolichol-mediated \(N\)-linked oligosaccharides are directly involved as an intermediate in the biosynthesis of keratan sulfate. This finding suggests that the linkage region should contain at least 2 \(N\)-acetylgalactosamines and perhaps 3 mannose residues. More recently, Keller et al. (19) proposed a linkage region structure that resembles an \(N\)-linked complex-type oligosaccharide.

The cartilage proteoglycan contains both \(O\)- and \(N\)-linked oligosaccharides (7-9), and recent work indicates that the \(O\)-linked oligosaccharides are closely related to the keratan sulfate linkage region structure. In contrast, little is known about the structures of the oligosaccharides on the corneal keratan sulfate proteoglycan, except that they are similar to the linkage region in that they are asparagine linked and contain mannose (20, 21). This suggests that both the linkage region and the oligosaccharides on the corneal keratan sulfate proteoglycan are synthesized through the dolichol pathway and that only some of the \(N\)-linked oligosaccharides provide linkage regions for keratan sulfate chains. Consequently, in order to gain more insight into the biosynthesis of keratan sulfate on \(N\)-linked oligosaccharides, we have isolated and, in the accompanying paper (22) characterized, both the linkage region and the oligosaccharides on the corneal keratan sulfate proteoglycan.

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Keratan Sulfate Proteoglycan Purification

EXPERIMENTAL PROCEDURES

Materials—Chondroitinase ABC (Proteus vulgaris) and endo-β-
galactosidase (κeratanase, Pseudomonas sp.) were purchased from Miles Laboratories; papain (twice crystallized) was from Sigma Chemicals; [2-3H]mannose (17.3 Ci/mmol) and [35S]sulfate (100 Ci/mmol) were from New England Nuclear; DEAE-cellulose (DE52) was from Whatman; DEAE-Sephacel and ConA-Sepharose were from Pharmacia, and Bio-Gel P-10 was from Bio-Rad. Aqueous counting scintillants (ACS and Aquasol) were purchased from Amersham Corp.

Corneas—Three hundred corneas were obtained from freshly enucleated eyes of cynomolgus monkeys which were being killed for other purposes. The corneas were immediately rapidly frozen and stored at -70°C until extraction.

Labeling of Corneas with [2-3H]Mannose and [35S]Sulfate—Fifteen corneas were excised with intact scleral collars and were incubated in Coon's modified Ham's F-12 medium at the end of the incubation period and rinsed briefly in phosphate-buffered saline. They were then trimmed to remove the scleral rims, rapidly frozen, and stored at -70°C until extraction.

Extraction and Isolation of Keratan Sulfate Proteoglycan—Corneas were individually thawed on ice, rapidly scraped free of epithelium and endothelium, and placed in 4 ml guanidine HCl containing 0.01 M sodium EDTA, 0.01 M sodium acetate, 0.1 M 6-amino-n-heptanoic acid, and 0.005 M benzamidine HCl, pH 5.8 (25). The 300 unlabelled corneas were extracted with 200 ml of the 4 M guanidine HCl solution for 20 h at 4°C. The extract was deanted, and the residual tissue was re-extracted in 100 ml of 4 M guanidine HCl containing the same protease inhibitors for an additional 20 h at 4°C. The two extracts were combined and concentrated to about 130 ml by ultrafiltration with a PM-10 membrane (Amicon) followed by dialysis against 8 M urea in 0.05 M sodium chloride, pH 6.8 (26).

The reaction mixture after 17 h dialysis, for hexose and for hexosamine were pooled as described in Fig. 5 below and lyophilized.

RESULTS

Isolation of Keratan Sulfate Proteoglycan—The 4 M guanidine HCl extracts of corneal stromas were dialyzed into 8 M urea, and applied to a column of DEAE-cellulose (2.5 x 10 cm) equilibrated with 8 M urea, 0.05 M Tris-HCl, pH 6.8. The column was washed with 285 ml of 8 M urea, 0.05 M Tris-HCl, pH 6.8. The bound material was then eluted from the column with a linear gradient of 0-0.75 M NaCl (total 200 ml) in the same buffer, and then with 80 ml of 3.0 M NaCl in the urea solution. Fractions of 8.5 ml were collected. The main peak containing both 3H and 35S activity was pooled as described in Fig. 2 below, concentrated by ultrafiltration with an Amicon PM-10 membrane, and dialyzed against 1 M NaCl, 0.05 M Tris-HCl, pH 7.0.

The dialyzed fraction was applied to a concanavalin A-Sepharose column (2.5 x 10 cm) equilibrated with 1 M NaCl, 0.05 M Tris-HCl, pH 7.0. The column was washed with 300 ml of 1 M NaCl, 0.05 M Tris-HCl, pH 7.0, and the bound material was eluted with 300 ml of 1 M methylmannoside, 1 M methylmannoside, 1 M Tris-HCl, pH 7.0. Fractions of 16 ml were collected. The ConA-bound fraction, containing 3H and 35S activity, was pooled as described in Fig. 3 below, dialyzed against distilled water, and lyophilized (yield, ~50 mg). This ConA-bound fraction contained purified keratan sulfate proteoglycans.

Isolation of the Keratan Sulfate Linkage Region Oligosaccharide and of Mannose-containing Oligosaccharide—All of the purified keratan sulfate proteoglycan fraction was dissolved in 3 ml of 1 M sodium acetate, pH 6.5, containing 5 mM sodium EDTA and 5 mM cysteine, and digested with 3 mg (70 units) of papain at 55°C for 7 h. The reaction was terminated with the addition of 6 mg of iodoacetamide. The digest was dialyzed 10-fold with water and applied to a column of DEAE-Sephacel (1.6 x 6.5 cm) equilibrated with 0.1 M sodium acetate, pH 7.0. The column was washed with 100 ml of 0.1 M sodium acetate, pH 7.0. The bound material was eluted with a linear gradient of 0-0.1 M sodium chloride, pH 7.0. The bound material was washed with 40 ml of 3.0 M NaCl in 0.1 M sodium acetate, pH 7.0. Fractions of 4 ml were collected. The unbound fractions containing 3H activity and the bound fractions, containing both 3H and 35S activity were pooled, respectively, as described in Fig. 4 below. The unbound fraction, which contained intact glycopeptides, was directly lyophilized and subsequently treated as described below. The bound fraction, containing purified keratan sulfate chains, was lyophilized after dialysis against distilled water.

The keratan sulfate chain fraction (85.6 mmol as galactose) was digested with 2.0 ml of 1.0 M 0.1 M sodium phosphate, pH 7.5, 0.01 M sodium acetate, pH 7.0, and the resulting end sugar moieties produced by the enzymatic hydrolysis were assayed (24). The concentration of reducing groups reached a plateau at 15.4 mmol of hexosamine. The reaction mixture after 42 h, the digestion mixture was applied directly to a column (1.6 x 6.0 cm) of DEAE-Sephacel equilibrated with 0.05 M Tris-HCl, pH 6.8. The column was washed with 80 ml of 0.05 M Tris-HCl, pH 6.8. The unbound fractions containing 3H activity and 35S activity were pooled as described in Fig. 6 below and lyophilized.

The lyophilized fraction from the DEAE-Sephacel column was then dissolved in 2 ml of 0.4 M ammonium acetate, pH 5.5, and applied to a column (1.0 x 140 cm) of Bio-Gel P-10 (<400 mesh) equilibrated with 0.4 M ammonium acetate, pH 5.5. The column was eluted with the same buffer, and fractions of 1.0 ml were collected. Aliquots of each fraction were assayed for 3H and 35S activity and for hexose. The fractions containing both 3H activity and hexose were pooled as described in Fig. 6 below and lyophilized (yield, 2.4 mg).

The intact mannose-containing glycopeptides in the unbound fraction from DEAE-Sephacel chromatography of the papain-digested proteoglycan (described above) were dissolved in 4 ml of distilled water. The solution was then applied to and eluted on the Bio-Gel P-10 column. Aliquots of each fraction were assayed for 3H activity and hexose. Two peaks were pooled as described in Fig. 7 below and lyophilized. The lyophilized samples were analyzed for sugars. The yields of the two samples were 1.0 mg (peak I) and 6.5 mg (peak II).

The abbreviation used is: ConA, concanavalin A.
Keratan Sulfate Proteoglycan Purification

FIG. 1. DEAE-cellulose chromatography of the combined extracts in 8 M urea.

FIG. 2. DEAE-cellulose chromatography of the chondroitinase ABC digest of the proteoglycan fraction isolated from the DEAE-cellulose column as indicated in Fig. 1. KSPG, keratan sulfate proteoglycan.

1). Additional peaks with \(^{2}\text{H}\) activity eluted near fraction 30 at the beginning of the salt gradient and between fractions 40 and 60 where the majority of \(^{35}\text{S}\) activity also eluted. The final 30 M NaCl wash eluted only a small peak, indicating that the salt gradient eluted the majority of the proteoglycans. The \(^{35}\text{S}\) and \(^{3}\text{H}\)-labeled peak in fractions 40-60 was pooled as shown by the bar in Fig. 1 to give the proteoglycan fraction. About 30% of the total \(^{3}\text{H}\) activity was recovered in the proteoglycan fraction while the rest was presumably in glycoproteins which did not bind to the column or which bound only weakly.

The DEAE-purified proteoglycan fraction was then incubated with chondroitinase ABC in the presence of protease inhibitors (27) to degrade the chondroitin-dermatan sulfate, dialyzed into urea, and applied to a column of DEAE-cellulose. The core protein molecules of the dermatan sulfate proteoglycans eluted in the unbound fractions as indicated by the \(^{3}\text{H}\) activity and absorbance at 280 nm in fractions 5-25 (Fig. 2). The intact keratan sulfate proteoglycans were eluted by the salt gradient between fractions 50 and 90 as shown by the peak of \(^{3}\text{H}\) and \(^{35}\text{S}\) activity and by the absorbance at 280 nm in this fraction. About 52% of the \(^{3}\text{H}\) activity was present in the keratan sulfate proteoglycan peak. Previously (20), it was shown that the macromolecular \(^{3}\text{H}\) activity incorporated with \([2-{3}\text{H}]\text{mannose as a precursor remains almost entirely in mannose and fucose.}\)

The keratan sulfate proteoglycan fraction was recovered and eluted on a column of ConA-Sepharose (Fig. 3). About all of the \(^{3}\text{H}\) activity and 80% of the \(^{35}\text{S}\) activity bound to the column, eluting as a single peak in the presence of \(\alpha\)-methylmannoside. The column was then washed with another aliquot of 1 M \(\alpha\)-methylmannoside after 18 h (Fig. 3, arrow at fraction 40) to check for complete elution of the homoglycan (29). Heparan sulfate proteoglycans, which eluted with the keratan sulfate proteoglycans on DEAE-cellulose, eluted in the unbound fractions on the concanavalin A column along with unbound keratan sulfate proteoglycan as described elsewhere (30). The ConA-bound material between fractions 22 and 35 in Fig. 3 was pooled, dialyzed against distilled water, and lyophilized, yielding ~50 mg of a highly purified keratan sulfate proteoglycan.

**Isolation of the Linkage Region Oligosaccharides and the Mannose-containing Oligosaccharides**—The purified keratan sulfate proteoglycan fraction was digested with papain and applied to DEAE-Sepharose. A mannose-containing oligosaccharide-peptide fraction eluted in the unbound fraction as shown by \(^{3}\text{H}\) activity between fractions 2 and 20 (Fig. 4). The keratan sulfate peptide fraction eluted in the latter half of the NaCl gradient as shown by both the \(^{35}\text{S}\) and \(^{3}\text{H}\) activity after fraction 50. The presence of a significant level of \(^{35}\text{S}\) activity at the end of the salt gradient (fraction 75) indicated that some keratan sulfate peptides had not eluted with 0.7 M NaCl, and therefore the column was washed further with 3.0

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\(^{3}\text{H}\) activity assessed by polyacrylamide gel electrophoresis before and after digestion with chondroitinase ABC or endo-\(\beta\)-galactosidase. Coomassie blue staining of the gels indicated that the proteoglycans were the only components detected in this fraction (K. Nakazawa, unpublished data).

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When DEAE-cellulose was used in a preliminary experiment using a smaller number of corneas, subsequent analyses of the linkage region and mannose-containing oligosaccharide fractions were contaminated with glucose-containing material, presumably derived from the DEAE-cellulose. Therefore, DEAE-Sephacel was used for this step of the isolation procedure described above. The DEAE-Sephacel gave rise to little or no glucose contamination of the linkage region and the mannose-containing oligosaccharide fractions (22).
Keratan Sulfate Proteoglycan Purification

Fig. 4. DEAE-Sephacel chromatography of the papain digest of the ConA-bound keratan sulfate proteoglycan fraction isolated from the ConA-Sepharose column as indicated in Fig. 3.

Fig. 5. DEAE-Sephacel chromatography of the endo-β-galactosidase digest of the keratan sulfate peptide fraction isolated from the DEAE-Sephacel column as indicated in Fig. 4.

Fig. 6. Bio-Gel P-10 chromatography of the linkage region glycopeptide fraction isolated from the DEAE-Sephacel column as indicated in Fig. 5.

Fig. 7. Bio-Gel P-10 chromatography of the oligosaccharide-peptide fraction isolated from the DEAE-Sephacel column as indicated in Fig. 4.

M NaCl. Fractions with 3H and 35S activity that eluted with both the salt gradient and the 3.0 M NaCl wash were pooled as shown in Fig. 4 to give the keratan sulfate peptide fraction. About 39% of the 3H activity in the original purified keratan sulfate proteoglycan was recovered in the glycopeptide fractions, with the remainder present in the keratan sulfate peptide fraction.

The keratan sulfate peptide fraction was digested with endo-β-galactosidase, and the digest was applied to a column of DEAE-Sephacel. All of the 3H activity eluted in the unbound fractions along with a slightly retarded 35S-labeled peak (Fig. 5). Most of the 35S activity eluted as several peaks during the salt gradient and in the 3.0 M NaCl wash. These are keratan sulfate fragments of different sizes and charge densities (28).

The 3H peak in the unbound fractions along with the associated 35S peak was pooled as shown in Fig. 5 and lyophilized. The sample was then dissolved in a small volume of 0.4 M ammonium acetate, pH 5.5, and applied to a column of Bio-Gel P-10 (Fig. 6). The 35S activity eluted with a hexose-positive peak at the total column volume. This peak probably contains monosulfated disaccharides (28). A broad, 3H-labeled peak eluted with a hexose-positive peak in the middle of the column. This peak was pooled as shown in Fig. 6 to give the keratan sulfate linkage region glycopeptides. This fraction was lyophilized, yielding 2.4 mg. Its structure was determined as indicated in the accompanying paper (22).

The oligosaccharide-peptide fraction Fig. 4) was isolated and applied to the Bio-Gel P-10 column (Fig. 7). Two broad peaks of 3H activity eluted within the excluded and partially included column volume. The middle-eluting peak II (between fractions 55 and 69) was accompanied by a substantial amount of hexose, and its elution position was almost the same as that of the keratan sulfate linkage region glycopeptides in Fig. 6. The large hexose peak eluting after fraction 70 is probably derived from α-methylmannoside, which was used to elute the keratan sulfate proteoglycan from the ConA-Sepharose column and which was incompletely dialyzed. The two 3H-labeled peaks were pooled as shown in Fig. 7 and lyophilized. The second (peak II) contained 6.5 mg. The structures of the oligosaccharides in this fraction were determined as described in the accompanying paper (22).

DISCUSSION

[2-3H]Mannose was used as a labeling precursor along with [35S]sulfate to label the keratan sulfate proteoglycan in explant cultures of monkey cornea (20). These labeled macromolecules were mixed with a large quantity of unlabeled macromolecules, and, by following the incorporated radioactivity, about 50 mg of highly purified keratan sulfate proteoglycan sample was isolated from 315 corneas by a series of steps including: first, DEAE chromatography, to isolate proteogly-
use of [2-3H]mannose and [35S]sulfate as labeling precursors for structural determination (22) while utilizing minimal facilitate the purification of the intact glycopeptides and the amounts ride content and in numbers galactosidase, an enzyme from linkage site in the intact proteoglycans.

The keratan sulfate peptides were treated with endo-\(\beta\)-galactosidase, an enzyme from *Pseudomonas* which selectively degrades keratan sulfate (27, 28). After digestion, the linkage region oligosaccharide-peptide fraction, which contained all of the \(^3\)H activity but no \(^35\)S activity, was purified. The absence of \(^35\)S activity indicates the effectiveness of the endo-\(\beta\)-galactosidase for removing all of the backbone structure of the chains from the linkage region. Clearly, then, the use of [2-\(^3\)H]mannose and [\(^3\)S]sulfate as labeling precursors facilitated the purification of the intact glycopeptides and the linkage region oligosaccharide-peptides in sufficient quantities for structural determination (22) while utilizing minimal amounts of material.

In separate studies (30), procedures similar to those described above were used to show that both the dermatan sulfate proteoglycans and the keratan sulfate proteoglycans contain subpopulations based upon differences in oligosaccharide content and in numbers of glycosaminoglycan chains. In these studies, it was found that the concanavalin A step was essential for removing a small amount of heparan sulfate from the keratan sulfate proteoglycan fraction. However, a proportion (20–30%) of the keratan sulfate proteoglycan does not bind to the column, and appears to have fewer intact oligosaccharide-peptides (30).

After papain digestion of the keratan sulfate proteoglycan, glycopeptides, which contained 59% of the \(^3\)H activity, were separated from keratan sulfate peptides, which contained 41% of the \(^3\)H activity and all of the \(^35\)S activity. The ratio of \(^3\)H activity in these two fractions in combination with their structures (22) suggests that there is an average of approximately one intact oligosaccharide for each keratan sulfate linkage site in the intact proteoglycans.

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