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PHOSPHATE ESTER GROUPS IN PROTEOGLYCANS FROM BOVINE NASAL CARTILAGE

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Summary

1. Proteoglycan subunits isolated by standard procedures from bovine nasal cartilage, previously incubated in the presence of [^{32}P]phosphate contain [^{32}]-phosphate ester groups as a regular structural component.

2. Contamination of the proteoglycan subunit with ^{32}P-labelled nucleic acids could be excluded by repeated cesium chloride density gradient centrifugation under associative and dissociative conditions, lanthanum chloride precipitation, gel filtration and by the resistance of the proteoglycan subunit associated ^{32}P to phosphoric diester hydrolases.

3. The [^{32}P]phosphate ester groups are associated to the chondroitin sulfate peptide fraction obtained by proteolytic digestion of the proteoglycan subunit molecule. Degradation of the chondroitin sulfate peptide by chondroitinase ABC resulted in a ^{32}P-labelled oligosaccharide peptide fraction, that contains xylose, galactose, glucuronic acid and inorganic phosphate in a molar ratio 1 : 2 : 1 : 0.12.

4. ^{32}P radioactivity is released as inorganic phosphate by treatment of the ^{32}P-labelled oligosaccharide peptide with acid phosphatase or alkali.

Introduction

Proteoglycan subunits are a major structural component of hyaline cartilage. Proteoglycan-subunit molecules are composed of a central protein core to which a large number of chondroitin sulfate and a few keratan sulfate chains are covalently linked. About one third of the protein core, to which few or no polysaccharide chains are attached, is capable of interaction with hyaluronate [1]. The major portion of the keratan sulfate chains is attached to a keratan-

sulfate-rich region, whereas the bulk of the chondroitin sulfate chains is bound to the chondroitin sulfate rich region (for review, see Ref. 2).

Although the general structure of proteoglycans is well documented, it is not impossible that proteoglycans of connective tissue have additional structural peculiarities. Thus, *N*-glycosyl and *O*-glycosyl linked, partly sialylated oligosaccharides, present in the hyaluronate binding and in the polysaccharide attachment region have been detected in cartilage proteoglycans derived from chick limb bud chondrocytes [3] and from rat chondrosarcoma [4]. A pig skin proteodermatan sulfate was reported to bear ten glucosamini-containing oligosaccharide chains [5] and proteoglycans secreted by cultured skin fibroblasts were shown to possess beside their glycosaminoglycan chains, a carbohydrate structure capable of specific interaction with concanavalin A [6]. Recently, Schwartz and Miller [7] described the phosphorylation of proteoglycans when the cartilage was incubated in vitro in the presence of [γ-^{32}P]ATP or inorganic [^{32}P]phosphate.

In this report it will be shown that proteoglycan subunits of bovine nasal cartilage contain phosphate ester groups that are sensitive to acid phosphatase and are considered to be a regular constituent of the proteoglycan subunit molecule.

Materials

Chemicals were obtained from the following sources. Tris(hydroxymethyl)-aminomethane (Tris) and guanidine hydrochloride from Riedel de Haen AG (Seelze-Hannover, F.R.G.), [^{32}P]ortho phosphate (PBS II, carrier free) from Amersham Buchler (Braunschweig, F.R.G.), EDTA, sodium acetate, lanthanum chloride from Merck (Darmstadt, F.R.G.), bovine serum albumin (Cohn fraction V/BSA) from Serva (Heidelberg, F.R.G.), all Sephadex and Sepharose gels from Pharmacia (Freiburg, F.R.G.). Chondroitinase ABC was obtained from Seikagaku (Japan), Papain from papaya carica (10 U/mg, twice crystallized), deoxyribonuclease and ribonuclease from bovine pancreas (2000 and 100 Kunitz units per mg, respectively) were obtained from Serva (Heidelberg, F.R.G.) and acid phosphatase (potato, 2 U/mg, analytical grade II) from Boehringer (Mannheim, F.R.G.).

Methods

Preparation of [^{32}P]proteoglycan subunits

Batches of 8-g sliced bovine nasal cartilage obtained immediately after slaughtering were incubated in 10 ml phopshate-free Krebs-Ringer buffer under CO_2 and O_2 * for 24 h at 37°C in the presence of 10 mCi [^{32}P]phosphate, then exhaustively washed with 0.15 M NaCl and extracted with 9 vols. 4 M guanidine hydrochloride containing protease inhibitors [8]. The guanidine hydrochloride extract was submitted to repeated CsCl density gradient centrifugation steps under associative (1.69 g CsCl/ml) and dissociative (1.50 g CsCl/ml) conditions according to Hascall and Sajdera [9]. 50 mg of the proteoglycan subunit fraction obtained from the second dissociative gradient ($A_1D_1D_1$) ** were

* 5% CO_2/95% O_2.

** Fractions A and D were obtained from associative and dissociative gradients, respectively.

submitted to a gel filtration on a Sepharose 2B column (2.0 × 93 cm) equilibrated and eluted with 0.5 M acetate buffer, pH 6.8. The fraction retarded by the gel at a K_{av} value of 0.1 was subjected to exhaustive digestion by deoxyribonuclease and ribonuclease and recovered after gel filtration on Sepharose 6B (see below). Alternatively the 4 M guanidine hydrochloride extract was dialysed against distilled water and brought to a final concentration of 0.5 M $LaCl_3$ in 50 mM acetate buffer, pH 5.8. After removing of insoluble material the proteoglycans were precipitated by adjusting the solution to 0.05 M $LaCl_3$ by addition of distilled water [10,11]. The lanthanum salt of the ^{32}P-labelled proteoglycan was dissolved in EDTA, dialysed and lyophilyzed [10]. Further purification was achieved by gel filtration on Sepharose 2B and dissociative density gradient centrifugation (see above).

Degradation of ^{32}P-labelled proteoglycan subunits by DNAase and RNAase

Samples of ^{32}P-labelled proteoglycan subunits (Fraction A_1D_1, $A_1D_1D_1$) and of the fractions A_1D_2—A_1D_5 containing 0.05—1.0 μMol uronic acid were incubated in 75 mM acetate buffer pH 5.0 containing 3.8 mM $MgSO_4$ with 10 μg/ml DNAase I or in 0.2 M Tris-HCl buffer, pH 7.5 containing 10 mM EDTA (disodium salt) with 10 μg/ml RNAase A at 37°C for 12 h. The proteoglycans were recovered by gel chromatography on Sepharose 6B and lyophilization.

Isolation of ^{32}P-labelled oligosaccharide peptide

Procedure I. 50 mg ^{32}P-labelled proteoglycan subunits were digested with 2 units chondroitinase ABC in a final volume of 5 ml 50 mM Tris-HCl buffer, pH 8.0, containing 60 mM sodium acetate and 0.05% bovine serum albumin for 24 h at 37°C, half of the chondroitinase was added at the beginning of each incubation and after 12 h [12]. The digest was applied to a Sepharose 6B column (1.1 × 98 cm) and eluted with 50 mM Tris-HCl, pH 7.5. The material excluded from the gel (2.5 mg proteoglycan subunit/ml) was degraded by papain [13] after adjusting the pH to 6.5 and adding EDTA (85 mM) and cysteine (10 mM). Gel chromatography of the digest was performed as described in Fig. 5.

Procedure II. 10 g bovine nasal cartilage preincubated with [^{32}P]phosphate and then washed with 0.15 M NaCl, were submitted to papain digestion under the conditions described above, for 48 h at 60°C, the same amount of enzyme was added, after 24 h. The digest was centrifuged and to the clear supernatant, cetyl pyridinium chloride was added to a final concentration of 2%. The precipitate formed was washed several times with 0.25 M $MgCl_2$ containing 0.25% cetyl pyridinium chloride and dissolved in 1 M $MgCl_2$. To the filtered solution, 2.5 vols. ethanol and solid potassium acetate 1% (final concn.) were added. The precipitate was washed with ethanol and diether (yield 0.385 g). The ethanol precipitate was redissolved in and exhaustively dialysed against distilled water then subjected to ion exchange chromatography on Dowex 1 × 2 (100 ml bed volume, Cl^--form, 200—400 mesh) equilibrated in 0.1 M NaCl. After stepwise elution of the column with 40 ml each of 0.10, 0.25, 0.5 and 0.75 M NaCl, a linear NaCl gradient was applied, generated from 200 ml 0.75 M NaCl and 200 ml 3 M NaCl [14]. 5.4-ml fractions were collected and assayed for uronic acid and radioactivity. The uronic acid containing material was applied to a

Sepharose 6B column and the material occupying the position of chondroitin sulfate was subjected to chondroitinase ABC degradation and gel filtration on Sephadex G-50, as described above.

Degradation of phosphorylated oligosaccharides by acid phosphatase

1 mg of the lyophilyzed fractions 39—45 (peak III of Fig. 5) was dissolved in 950 μl 0.1 M acetate buffer pH 5.5 and incubated with 50 μl (10 U) acid phosphatase at 25°C for 90 min. The products were applied to a Sephadex G-25 column (0.9 × 55 cm). 1.2-ml fractions eluted with distilled water were monitored for radioactivity and hexosamine.

Analyses

Uronic acid [15], hexosamine [16], hexoses [17], protein [18], inorganic phosphate [19] and galactose (galactose dehydrogenase test combination, Boehringer, Mannheim) were determined according to the specified references, using glucuronic acid, glucosamine, galactose and bovine serum albumin as standards.

Radioactivity was assayed in a Packard Tricarb liquid scintillation counter, 1 ml of aqueous samples being mixed with 2 ml scintillation mixture (Unisolve I, Zinsser/Frankfurt) and counted for 1—5 min. Thin-layer chromatography of glycosaminoglycans was performed according to Humbel and Chamoles [20].

Gas liquid chromatography

400 μg of lyophilyzed material (Fraction III, Fig. 5) was dissolved in 100 μl of 1 M H_2SO_4 hydrolyzed for 3 h at 105°C and the solution neutralized with barium hydroxide. After centrifugation, the supernatant was applied to a Dowex 50W-X12 (200—400 mesh, H^+-form, 0.5 ml bed volume) column, and eluted successively with 2 ml distilled water and 2 ml 1 M HCl. After evaporation of the distilled water eluate to dryness, sugars were analyzed by gas liquid chromatography as trimethylsilyl ethers, essentially according to Chambers and Clamp [21]. The analysis was carried out with a 3 m glass column (4 mm diameter) packed with 3% (w/w) SE 30 on Gas-Chrom Q 125—150 μm (Serva, Heidelberg, F.R.G.) on a Pye Unicam Series 204 chromatography apparatus.

Results

Isolation of ^{32}P-labelled proteoglycan subunits

The ^{32}P-incorporation studies on cartilage are based on the observation that [^{32}P]phosphate is readily incorporated into nucleic acids which are extractable with 4 M guanidine hydrochloride. These nucleic acids copurify with polyanionic polysaccharides and are, hence, potential contaminants of the cartilage proteoglycans.

In vitro incubation of bovine nasal cartilage in the presence of [^{32}P]phosphate, followed by extraction of cartilage with 4 M guanidine hydrochloride and cesium chloride density gradient centrifugation of the guanidine hydrochloride extract under associative and successively under dissociative conditions, results in a distribution of ^{32}P-radioactivity as given in Fig. 1. The fraction of highest density, referred to as A_1D_1 by Hardingham and Muir [1] con-

tains the majority of the uronic acid (and hexosamine) containing material and corresponds to the proteoglycan subunit of largest size, whereas proteoglycan molecules of smaller size and higher protein content occupy the region of lower density.

In contrast, the ^{32}P-radioactivity profile indicates a low specific labelling of the fractions A_1D_1 and A_1D_2. The significant higher specific labelling of the fractions A_1D_3, A_1D_4 and A_1D_5 must be partly attributed to a contamination with ^{32}P-labelled nucleic acids, which sediment with the proteoglycan complex in the associative gradient (not shown) but occupy a density region between 1.371—1.356 under dissociative conditions [22]. The ^{32}P-label of fraction A_1D_1 was shown to be an integral constituent of proteoglycan by 2 independent experiments: (1) The fraction A_1D_1 was collected and recentrifuged under dissociative conditions. The resulting bottom fraction $A_1D_1D_1$ was dialyzed and subjected to digestion by either deoxyribonuclease or ribonuclease. No release of [^{32}P]phosphate was observed. (2) Precipitation of the guanidine hydrochloride-soluble ^{32}P-labelled proteoglycans by lanthanum chloride, successive gel filtration of the redissolved material on Sepharose 2B and cesium chloride density gradient centrifugation of the ^{32}P-labelled macromolecules excluded

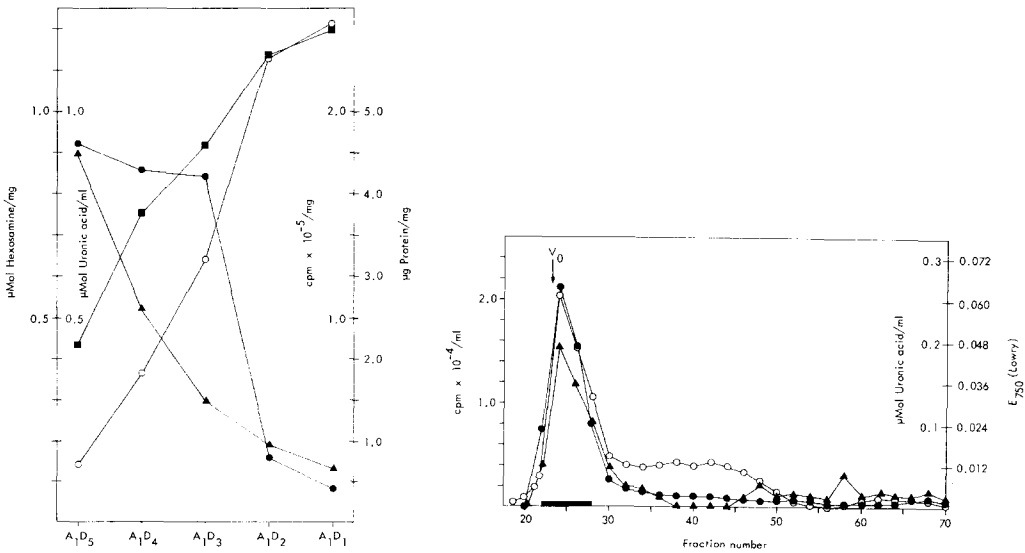


Fig. 1. Dissociative cesium chloride density gradient centrifugation of ^{32}P-labelled proteoglycan complex. ^{32}P-labelled proteoglycan complex was obtained by associative density gradient centrifugation of a 4 M guanidine hydrochloride extract of bovine nasal cartilage which has been incubated in vitro in the presence of [^{32}P]phosphate for 24 h. The bottom fraction A_1 (according to 3) was applied to dissociative density gradient centrifugation. Fractions A_1D_1—A_1D_5 were dialysed and lyophilized ○, uronic acid; ▲, protein; ●, ^{32}P-radioactivity; ■, hexosamine.

Fig. 2. Gel filtration of ^{32}P-labelled proteoglycan subunits on Sepharose 2B. A 4 M guanidine hydrochloride extract of bovine nasal cartilage previously incubated in vitro in the presence of [^{32}P]phosphate was precipitated by lanthanum chloride. 10 mg of the lyophilyzed precipitate was redissolved in 0.5 M acetate buffer, pH 6.8 applied to a Sepharose 2B column (2.0 × 93 cm) and eluted with 0.5 M acetate buffer, pH 6.8. The fractions (4.7 ml) were analyzed for radioactivity (●), uronic acid (○) and protein (▲). Solid bar denotes the fractions used for density gradient centrifugation.

from the gel (Fig. 2), resulted in a clear separation of the ^{32}P-radioactivity into a proteoglycan-subunit-associated, ribonuclease-resistant pool and an uronic-acid-free, but ribonuclease-degradable fraction. This indicates the successful separation of [^{32}P]RNA and ^{32}P-labelled proteoglycan subunits by repeated equilibrium centrifugation under dissociative conditions (Fig. 3).

Oligosaccharide peptide associated ^{32}P-labelled ester phosphate

The proteoglycan subunit fraction $A_1D_1D_1$ was subjected to gel filtration on Sepharose 2B. The material excluded from the gel was exhaustively degraded either by chondroitinase ABC or by papain and applied to a Sepharose 6B column (procedure I).

The elution profile of the chondroitinase ABC treated material (Fig. 4) indicates that the ^{32}P-radioactivity containing material is completely excluded from the gel and is exactly coincident with the galactose profile. It represents the protein core of the proteoglycan subunits with the keratan sulfate side chains and the sugar residues of chondroitin sulfate, which remain subsequent to the action of chondroitinase, still attached. Gel filtration of the papain digest of the proteoglycan fraction $A_1D_1D_1$ produced a single radioactivity peak in the elution position of chondroitin sulfate peptides. The failure to detect labelled low molecular weight material excludes the presence of ^{32}P-labelled peptides or

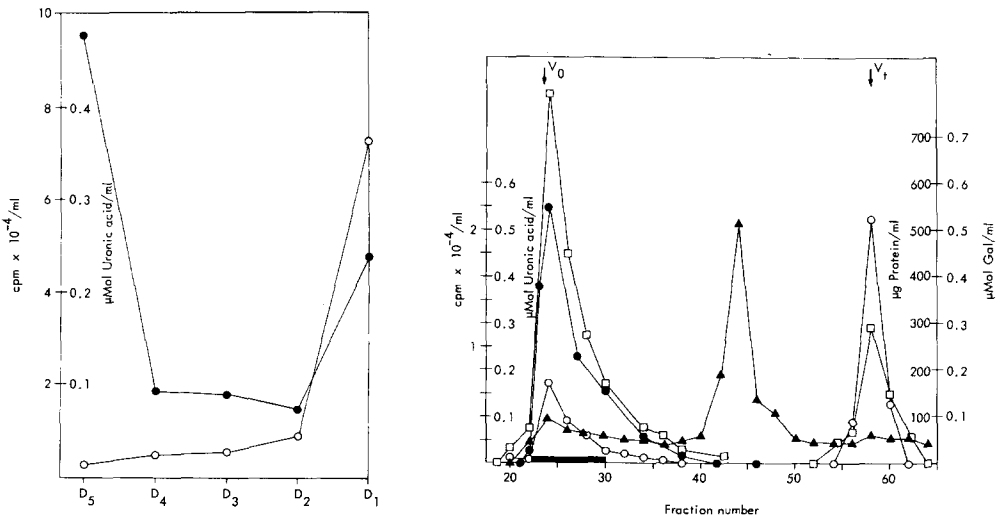


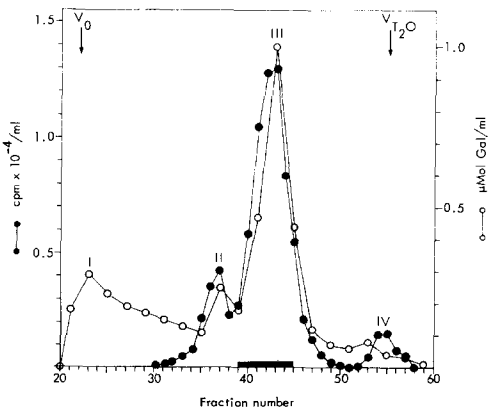
Fig. 3. Dissociative cesium chloride density gradient centrifugation of ^{32}P-labelled proteoglycans purified by lanthanum chloride precipitation and gel filtration on Sepharose 2B. The proteoglycan fractions excluded from the Sepharose gel (see Fig. 2) were collected (4.9 µmol uronic acid) and subjected to a dissociative cesium chloride gradient centrifugation. ○, uronic; ●, ^{32}P radioactivity.

Fig. 4. Gel filtration of chondroitinase ABC degraded ^{32}P-labelled proteoglycan subunits. 80 mg ^{32}P-labelled proteoglycan subunits obtained by repeated dissociative density gradient centrifugation ($A_1D_1D_1$) were exhaustively degraded by chondroitin sulfate lyase ABC and applied to a Sepharose 6B column (1.1 × 98 cm, 115 ml) which was equilibrated and eluted with 50 mM Tris-HCl buffer, pH 7.5. Fractions (1.8 ml) were analyzed for radioactivity (●), uronic acid (○), protein (▲), and galactose (□). Solid bar denotes the fractions used for subsequent papain digestion. Central protein peak represents enzyme added to the incubation mixture.

oligosaccharide peptides in the papain digest. The uronic-acid-containing material derived from chondroitinase ABC action of the chondroitin sulfate chains is eluted with the total volume of the column and is void of ^{32}P radioactivity.

The chondroitinase digested material excluded from the Sepharose 6B gel was digested by papain, and applied to a Sephadex G-50 column. 4 Peaks were obtained (Fig. 5). The 3rd peak (fractions 39—45) contained more than 80% of the label and was identified as an oligosaccharide peptide, containing xylose, galactose, uronic acid, mannose and *N*-acetylhexosamine as carbohydrate constituents. Sialic acid was not detectable. After acid hydrolysis and removal of amino acids, peptides and amino sugars by ion-exchange chromatography on Dowex 50, quantitative gas-liquid chromatography revealed a molecular ratio of xylose : galactose : uronic acid : mannose : inorganic phosphate as 1 : 2 : 1 : 0.15 : 0.12. The nonlabelled galactose containing fractions 20—29 (Peak I of Fig. 5) were identified as keratan sulfate peptide by chemical analysis and by TLC using authentic keratan sulfate as reference. Only a minor portion of ^{32}P was associated with the unidentified fraction II or with low molecular weight degradation products formed by the papain digestion (fraction IV).

The ^{32}P-labelled oligosaccharide peptide was isolated by independent experiments (procedure II) as follows: bovine nasal cartilage preincubated with [^{32}P]-phosphate was digested by papain. From the digest, the chondroitin sulfate peptide fraction was separated from contaminating nucleic acid and nucleotides by cetyl pyridinum precipitation, ion exchange chromatography and gel filtration on Sepharose 6B. Successive degradation of the ^{32}P-labelled chondroitin sulfate peptide by chondroitinase ABC followed by gel filtration on Sephadex



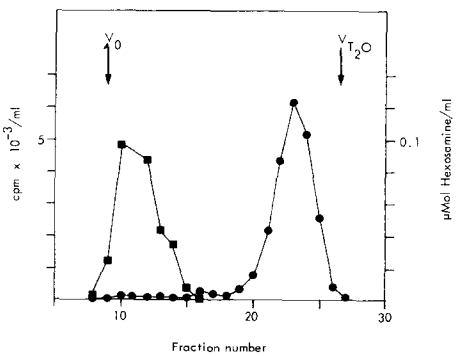


Fig. 5. Gel filtration of ^{32}P-labelled proteoglycan subunits on Sephadex G-50 after degradation by chondroitinase ABC and papain. I, Keratan sulfate peptides; III, oligosaccharide peptides derived from the linkage region of chondroitin sulfate chains. Equilibration and elution of the column (1 × 97 cm, 98 ml) with 50 mM Tris-HCl buffer, pH 7.4 containing 0.5 M NaCl. Fractions 39—45 (fraction volume 1.8 ml) were used for incubation with acid phosphatase.

Fig. 6. Gel filtration of acid phosphatase treated ^{32}P. Fraction III of Fig. 5 (containing 0.53 µmol galactose) was incubated with 10 U acid phosphatase, applied to a Sephadex G-25 column (0.9 × 38, 35 ml) and eluted with distilled water. Fractions (1.2 ml) were analyzed for hexosamine (■) and ^{32}P radioactivity (●).

G-50 resulted in an elution profile resembling that given in Fig. 5, except that keratan sulfate peptides were absent because of prior removal by ion-exchange chromatography. The bulk of ^{32}P radioactivity and the galactose exhibit a parallel distribution pattern (III), distinctly separated from the $\Delta^{4,5}$-uronic-acid-containing disaccharides (IV).

Enzymatic release of [^{32}P]phosphate

When the ^{32}P-labelled oligosaccharide peptide (fractions 39—45 of Fig. 5) was incubated with acid phosphatase, the ^{32}P was effectively removed as inorganic phosphate and separated from the oligosaccharide peptide by gel filtration on Sephadex G-25 (Fig. 6). Likewise, treatment of the ^{32}P-labelled oligosaccharide peptide by 0.1 M NaOH for 3 h caused complete elimination of ^{32}P as inorganic phosphate. No release of ^{32}P-containing, low molecular weight degradation products could be detected after digestion by deoxyribonuclease or ribonuclease and re-chromatography on Sephadex G-50.

Discussion

The present results provide evidence that phosphate ester groups attached to the linkage region of chondroitin sulfate side chains are a structural component of the proteoglycan monomer (proteoglycan subunit) prepared from bovine nasal cartilage. This conclusion is based upon the isolation of ^{32}P-labelled proteoglycan monomers and their degradation to a defined phosphorylated oligosaccharide peptide. The covalent linkage of the phosphate groups by a monoester bond is indicated by their sensitivity for acid phosphatase and their resistance to phosphodiesterases. The purification procedure of the ^{32}P-labelled proteoglycan monomer effectively removes nucleic acid labelled during the pre-incubation of cartilage with [^{32}P]phosphate and coextracted with the proteoglycans. Repeated density gradient centrifugation under associative and dissociative conditions, lanthanum chloride precipitation, gel filtration and treatment with DNAase and RNAase removed contamination with nucleic acids and/or nucleotides.

The chondroitin sulfate linkage region of the proteoglycan molecules was recognized as the site of phosphoester binding, but the precise localization has not been elucidated. From sugar and amino acid analyses, the phosphate-bearing oligosaccharide peptide isolated after enzymatic degradation of ^{32}P-labelled proteoglycan subunits by papain and chondroitinase ABC is assumed to have the following structure:

Peptidyl-Ser-Xyl-Gal-Gal-GlcUA-GalNAc-Δ4,5GlcUA

and additional mannose residues being present in a few oligosaccharide peptides. This result is confirmed by sugar analysis that revealed a molar ratio of Xyl : Gal : GlcUA : Man as 1 : 2 : 1 : 0.15, taking into account that amino acids and amino sugars were removed after hydrolysis by ion-exchange chromatography, and that the unsaturated glucuronic acid residues are not recovered in gas-liquid chromatography. On the basis of a galactose : phosphate ratio of 2 : 0.12, one phosphate ester group could be calculated per 20 chondroitin sulfate chains.

Although amino acid analysis of the phosphorylated oligosaccharide confirmed serine as the predominant amino acid, the attachment of the phosphate ester group to an immediately adjacent peptide region is not completely excluded. Thus, the carbohydrate composition of the phosphate-bearing oligosaccharide does not exclude the possibility that the phosphate group could be linked to oligosaccharides residues bound to the same oligopeptide core of the chondroitin sulfate linkage region. Although the composition of these glucosamine-containing, and partially-sialylated, oligosaccharides was found to be completely different from that of the chondroitin sulfate-linkage region [4,5], it is not certain that those oligosaccharides are removed after proteolysis of the ^{32}P-labelled proteoglycan subunits by gel chromatography on Sepharose 6B. The elution profiles of ^{32}P radioactivity and galactose in peak III (Fig. 5) were not entirely coincident, and imply heterogeneity of the oligosaccharide-peptide fraction.

Phosphorylated mannosyl residues of lysosomal enzymes function as recognition markers and may be responsible for a cycling of lysosomal enzymes via the cell surface in a receptor-bound form [23], but there is no evidence as yet for the function of the phosphoester groups in proteoglycans.

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